Role of Shear Stress in Endothelial Cell Morphology and Expression of Cyclooxygenase Isoforms

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Role of Shear Stress in Endothelial Cell Morphology and Expression of Cyclo-oxygenase Isoforms


Objective—The goal of this study was to examine the effect of chronic heterogeneous shear stress, applied using an orbital shaker, on endothelial cell morphology and the expression of cyclo-oxygenases 1 and 2.

Methods and Results—Porcine aortic endothelial cells were plated on fibronectin-coated Transwell plates. Cells were cultured for up to 7 days either under static conditions or on an orbital shaker that generated a wave of medium inducing shear stress over the cells. Cells were fixed and stained for the endothelial surface marker CD31 or cyclo-oxygenases 1 and 2. En face confocal microscopy and scanning ion conductance microscopy were used to show that endothelial cells were randomly oriented at the center of the well, aligned with shear stress nearer the periphery, and expressed cyclo-oxygenase-1 under all conditions. Lipopolysaccharide induced cyclo-oxygenase-2 and the production of 6-keto-prostaglandin F1α in all cells.

Conclusion—Cyclo-oxygenase-1 is expressed in endothelial cells cultured under chronic shear stress of high or low directionality. (Arterioscler Thromb Vasc Biol. 2011;31:384-391.)

Key Words: eicosanoids ■ endothelium ■ prostacyclin ■ cyclo-oxygenase ■ shear stress

Endothelial cells line the luminal surface of blood vessels and are continuously exposed to hemodynamic shear stress. The level of shear stress that cells experience varies from region to region within the vasculature. In areas of high laminar shear stress, endothelial cells are elongated, aligned, and protected from inflammation. In areas of low, oscillatory shear stress, endothelial cells are randomly orientated and susceptible to inflammation. Areas of low shear stress are thought to be atheroprone, whereas areas of high shear stress are thought to be atheroprotected.1-3

Cultured endothelial cells are routinely studied under static conditions, where they appear nonaligned, with a cobblestone morphology.4,5 It is increasingly recognized that endothelial cells grown under static conditions may not be representative of endothelial cells in the body.6,7 In addition, evidence suggests that endothelial endocrine function and expression of key enzymes, including cyclo-oxygenase (COX), is also regulated by shear stress.8

COX is present in 2 isoforms: COX-1 and COX-2. Generally, COX-1 is expressed constitutively, whereas COX-2 is induced at sites of inflammation.9 In endothelial cells, COX-1 activity results predominantly in the production of the antithrombotic hormone prostacyclin.10 COX-1 and COX-2 are the targets for nonsteroidal anti-inflammatory drugs (NSAIDs). They have attracted much media attention since the association of COX-2-selective NSAIDs with adverse cardiovascular events,11,12 although the mechanism behind this association remains unclear. One leading hypothesis is that COX-2-selective drugs reduce the production of the cardioprotective hormone prostacyclin,13,14 which, in susceptible individuals, increases the risk of arterial thrombosis. Prostacyclin is formed mainly by endothelial cells, which express high levels of COX. It has previously been shown that COX-1 predominates over COX-2 in endothelial cells cultured under static conditions,15-17 which raises the question of how COX-2-selective drugs might be affecting prostacyclin in the body. One possibility is that prostacyclin is formed in the kidney, where COX-2 is expressed and where COX-inhibition is thought to cause the hypertension seen in patients taking NSAIDs, including COX-2-selective drugs. On the other hand, our own research has shown that COX-2-selective drugs are effective at inhibiting COX-1 in endothelial cells because of relatively low cellular lipid peroxide levels.15 A third possibility is that the ratio of COX-1 to COX-2 expression in endothelial cells cultured under static conditions is not representative of expression patterns under
physiological conditions. It is therefore important to examine COX expression in endothelial cells cultured under shear stress. Others have used flow chambers to investigate how shear affects COX expression in endothelial cells, but these studies are generally conducted over relatively short periods of time, and the results have not been consistent; some studies have shown upregulation of COX-2 mRNA\textsuperscript{18,19} and protein\textsuperscript{19} by shear stress, but contradictory findings have also been reported.\textsuperscript{20}

In this communication, we report a study that used an orbital shaker to apply shear stress for up to 7 days to investigate effects on the expression of COX-1 versus COX-2 in porcine aortic endothelial cells (PAEC). This technique creates different temporal patterns of shear at different radial locations in the well of a Transwell plate, although the time-averaged shear is approximately uniform.\textsuperscript{21} In the present study, further postprocessing of computational flow simulations was performed that additionally characterized the magnitude and temporal pattern of the radial and tangential components of the shear at various radial positions in the well during 1 orbital period. Confocal microscopy and scanning ion conductance microscopy (SICM) were used to image the effects of the shear stress on endothelial cell morphology and COX expression. Other parameters, including cell number, CD31 expression, and cell volume were also measured.

**Materials and Methods**

**Cell Isolation and Subculture**

PAEC were isolated from descending thoracic aortas of 2-year-old white Landrace cross pigs, obtained from an abattoir (Fresh Tissue Supplies), using the method of Bogle et al.\textsuperscript{22} Primary cultures were assumed to be >99% pure based on the observation of CD31 staining throughout. Cells were subcultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% fetal calf serum (Biosera).

**Application of Shear Stress**

PAEC at passage 2 were plated on fibronectin (Sigma; 50 μg/mL)–coated 6-well Transwell filter inserts (Corning; Supplemental Information I, available online at http://atvb.ahajournals.org) at a density of 400,000 cells per well. Experimental conditions were applied 24 hours after seeding. To assess the effect of shear stress, a PS-300 orbital shaker (Grant Instruments) was used as previously described\textsuperscript{6}. As COX-2 is known to be upregulated in response to inflammatory stimuli, some wells were incubated with either 0.01 or 0.1 μg of lipopolysaccharide (LPS) (0111:B4 Escherichia coli, Sigma) as positive controls. Cells were incubated under either static conditions (no shear) or shear stress for up to 7 days. Medium was replaced every 2 days, with fresh LPS added where appropriate.

**Modeling Shear Stress**

For our previous study (which used identical filter inserts and the same orbital shaker and settings), the movement of medium was modeled by solving the 3-dimensional Navier-Stokes equations with commercial computational fluid dynamics software (Fluent 6.2; Figure 1).\textsuperscript{6} Shear stress at the base of the well was derived from the computed fluid motion. In the present study, further postprocessing of this solution also identified the temporal pattern of the radial and tangential components of the shear at various radial positions in the well during 1 orbital period (Figure 1).

**Western Blot Analysis**

To verify COX-1 (Cayman 160108) and COX-2 (Cayman 160126) antibody specificity, Western blots were carried out on cells grown under static conditions. Before Western blotting, protein concentration for loading was estimated by Bradford assay. Samples were loaded on 7.5% (w/v) acrylamide gels (reagents from Sigma and National Diagnostics) and transferred onto polyvinylidene difluoride membranes (Amersham) after approximately 1 hour. Visualization was by autoradiography (Supplemental Information II). Anti-β-actin antibodies (AbCam) were used as loading controls.

**Radioimmunooassay**

Prostacyclin was measured by the accumulation of its breakdown product 6-keto-prostaglandin (PG) F\textsubscript{1α} by radioimmunooassay as described previously.\textsuperscript{23}

**Immunohistochemistry and Confocal Microscopy**

All immunohistochemistry procedures were carried out in the Transwells. Cells were permeabilized by treatment with 0.2% Triton X-100 (Sigma) for 15 minutes before incubation with primary rabbit polyclonal antibodies for either COX-1 (Cayman 160108) or COX-2 (Cayman 160126) for 2 hours. Subsequently cells were incubated with the goat secondary anti-rabbit antibody Alexa Fluor 568 (Invitrogen) for 1 hour. Both incubations were carried out at room temperature. A negative control, with the primary antibody omitted, was used to quantify nonspecific background fluorescence. All wells were incubated with an Alexa Fluor 488–conjugated mouse anti-porcine CD31 antibody (MCA1746F, AbD Serotec) overnight at 4°C to confirm that the cells were endothelial in origin, and the nuclear and chromosome counterstain 4′,6′-diamidino-2-phenylindole (DAPI) (DH106 Invitrogen) was used to stain the endothelial cell nuclei. Details of the mounting procedure can be found in the Supplemental Information. Confocal imaging was carried out using a Leica SP5 inverted confocal microscope, with a ×40 1.25 oil objective and 405 diode, argon, and HeNe 543 lasers. LAS AF software was used for quantification. Images were taken at 9.3 μm from the center (denoted edge) and at the center of the Transwell. Further detail of the methodology is provided in the Supplemental Information I.

**SICM**

The SICM method has been described previously.\textsuperscript{24} Briefly, the SICM probe consists of a glass nanopipette filled with electrolyte. An Ag/AgCl electrode plugged into it is connected to a current amplifier that measures the ion current passing through the pipette tip. The probe, mounted on a 3-axis piezo translation stage, vibrates vertically (amplitude, 100 nm; frequency, 200 Hz) when close to the sample surface. The modulated current is amplified and fed into a lock-in amplifier tuned to the modulation frequency. The output is connected to a DSP card to generate a feedback signal to maintain the probe-sample separation distance. The control/data acquisition electronics record both the lateral and vertical positions of the probe and generate the topographical image. We used hopping probe ion conductance microscopy without continuous feedback.\textsuperscript{25} The images generated were used to estimate the volume of live endothelial cells as described previously.\textsuperscript{26}

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism version 5.0. Where n values were 8 or more, the D’Agostino and Pearson omnibus normality test showed that the data were normally distributed. One-way ANOVAs followed by the Bonferroni multiple comparison post test, paired 2-tailed t tests, or 2-way ANOVA followed by Bonferroni post tests were performed as appropriate. Data were considered statistically significant as indicated in the figures (*P<0.05). In all figures, results are displayed as mean±SEM.

**Results**

**Pattern of Shear Stress Applied to Endothelial Cells**

The computational fluid dynamics solution showed that a wave of culture medium swirls around the Transwell as a...
result of the motion of the shaker platform, which generates shear stress across cell surfaces. Figure 1A maps the pattern of shear stress acting on the cells at one instant in time. The flow is periodic so the pattern remains the same as the map rotates synchronously with the motion of the well. To illustrate the spatial and temporal variations, Figure 1A also shows resultant shear stress magnitudes at radii of 0, 2.3, 5.9, and 9.4 mm during one complete orbit. The amplitude of oscillation is essentially zero at \( r = \text{constant} \) (i.e., the center of the well) and increases with increasing radius, reaching a peak near the periphery. The radial increase is reversed in a narrow band close to the side wall, where the viscous drag on the side wall has an influence. The time-averaged mean level of shear is \( \approx 2 \text{ dyne/cm}^2 \) and approximately constant across the well.

Figure 1B also shows that although the average magnitude of the shear is approximately constant across the well, its direction is not. This has been illustrated by resolving shear stress vectors into radial and circumferential components; the radial component is plotted against the circumferential component for each of the 4 radii during one orbit. If data were confined to a single point on this plot, that would indicate constant magnitude and direction of shear during the orbit. A vertical line at \( x = 0 \) would indicate pure tangential flow of fluctuating magnitude, and a horizontal line at \( y = 0 \) would indicate pure radial flow of fluctuating magnitude. A circle centered on the origin would indicate flow that oscillates equally between both directions. The plot for the center of the well \((r=0)\) is indicative of the latter—there is no preferred direction. Points on the lines indicate intervals of 25 milliseconds; their regular distribution for \( r=0 \) shows that the change in direction is smooth and even. At increasing radii, however, the center of the plot shifts to the left, the excursion of the tangential shear exceeds that of the radial shear, and the spacing of the 25-millisecond markers becomes uneven, indicating a preferred direction of shear.

**Effect of Shear Stress on Morphology of Endothelial Cells**

Endothelial cells cultured under static conditions expressed the endothelial cell specific marker CD31 (Figure 2) and displayed typical cobblestone/polygonal morphology (Figure 3). Morphology was largely unchanged when cells were incubated with LPS for either 24 hours or 7 days (see Supplemental Information). It was found that cell number was higher (per unit area) at the center of the well in both static and sheared conditions (Figure 2). In static conditions, cell number was reduced at the center and edge of wells by LPS, whereas CD31 was reduced at the center only. When
endothelial cells were cultured under an applied shear stress on an orbital shaker, the effect of LPS on cell number was prevented (in regions of directional and pulsatile flow; edge) or lessened (in regions of nondirectional and steady flow; center) (Figure 2). Cellular alignment was evident by day 2 in some cases, but consistent alignment was not seen until day 3 (Figure 3A). By day 7, cellular alignment was seen in all images taken toward the edge of the well (Figure 3B) and in some cases at the center of the well (Figure 3D). Blinded visual scoring of cellular alignment confirmed statistically significant responses toward the edge of the wells after 7 days (Supplemental Information II). In occasional frames taken at the center of the well, cells displayed directionality, with the flow producing an image consistent with a swirl confirmation (Figure 3D). Using SICM, it was found that aligned endothelial cells grown in edge regions had a somewhat lower cellular volume than those showing no directional alignment at the center or those grown under static conditions (Figure 3).

Expression of COX-1 and COX-2 in Endothelial Cells Grown Under Static and Shear Stress Conditions

Endothelial cell medium contained relatively low levels of the prostacyclin metabolite 6-keto-PGF\(_{1\alpha}\) when cells were grown under static or sheared conditions for up to 7 days (Table). The levels of 6-keto-PGF\(_{1\alpha}\) increased when cells were incubated with 0.01 (data not shown) or 0.1 \(\mu\)g LPS (Table). Although results at some time points showed trends, no significant difference was seen in 6-keto-PGF\(_{1\alpha}\) levels in medium from cells cultured under static versus sheared conditions (Table). Primary antibodies to COX-1 or COX-2 used in this study were validated for specificity using standard Western blotting technique (Supplemental Information I). Antibodies to COX-1 recognized a protein band of approximately 70 kDa in endothelial cells. Antibodies to COX-2 did not detect proteins in control endothelial cell extracts but recognized a 70-kDa band in homogenates of cells treated with LPS for 24 hours (Supplemental Information II). PAEC cultured under static or shear stress conditions expressed readily detectable COX-1 with low levels of COX-2 staining in all regions of the well (Figures 4 and 5). Shear stress did not affect the relative expression of COX-1 and COX-2 immunoreactivity in cells at either 24 hours (Supplemental Information III) or 7 days (Figure 4). COX-1 immunofluorescence appeared localized throughout the cytosol, with lower levels in the nucleus (Figure 4). LPS did not increase COX-1 expression in cells cultured under any conditions studied (Supplemental Information III). However, as expected, 24 hours of exposure to LPS increased COX-2 immunoreactivity expression in endothelial cells (Figure 5). Concentrated COX-2 immunoreactivity appeared as a distinctive bright ring around the nucleus, with lower levels in the remainder of the cytoplasm. Where visible in untreated cells, COX-2 appeared as granular staining around the nucleus. There was no significant difference in levels of LPS-induced COX-2 expression in endothelial cells cultured under static conditions or shear stress (Supplemental Information III). When cells were incubated with LPS for 7 days, COX-2 expression declined and was no longer found to be increased above levels seen in control cells (Supplemental Information III).

Discussion

Endothelial cells are exposed to shear stress caused by the passage of blood over the luminal surface of blood vessels in their normal physiological state. However, when endothelial cells are studied in vitro, they are often cultured under static conditions. The transfer of endothelial cells from blood...
vessels into static culture affects morphology and the expression of genes regulating important vasoactive enzymes within the cells. In addition, within blood vessels, the level and waveform of shear varies with the architecture of the vessel. Areas of endothelium exposed to low and nonunidirectional shear stress, such as at vessel bifurcations or in the lesser curvature of the aortic arch, are predisposed to inflammation and the subsequent development of atherosclerosis. It is therefore important when considering the expression of vasoactive enzymes in the endothelium to compare levels in cells exposed to different patterns of shear stress. This is particularly relevant when considering COX-1 and COX-2; it is necessary to know relative expression of COX-1 and COX-2 in the endothelium to compare levels in particular regions of our wells and to better understand the cardiovascular side effects of NSAIDs, including COX-2-selective drugs.

Other studies have investigated how COX-1 and COX-2 levels change in endothelial cells exposed to shear stress during culture, but they have generally been short-term experiments, which may give an incomplete picture. Our approach differs from these studies: we have investigated effects of shear over longer periods, and we have measured levels of COX isoforms within particular regions of our wells where our computational fluid dynamics results show different shear waveforms and directionality and where our imaging results show different cell morphology. This method may be more physiologically relevant.

Our first consideration was to ensure that our model of shear was robust and, as far as possible, represented the effects of shear in vivo. Cells at the outer region of the well, where shear has high pulsatility and a preferred direction, were elongated and aligned. Cells at the center of the well, an area experiencing the same mean shear but with no pulsatility (and hence lower maximum shear) and no preferred direction, appeared nonaligned and resembled the classic cobblestone appearance of cultured endothelial cells. SICM revealed that the aligned cells from the edge of the well had a lower volume than the nonaligned endothelial cells imaged at the center of the well and also a lower volume than cells grown under static conditions. These morphologies parallel observations at regions of the mouse aorta with high probability of developing atherosclerosis (nonaligned, cobblestone cells) or low probability of developing atherosclerosis (elongated and aligned cells). The high-probability region appears to experience low wall shear stress but greater changes in the direction of shear stress during the cardiac cycle.

Using the model, we demonstrated that COX-1 is readily detectable in endothelial cells, whereas COX-2 immunoreactivity remains relatively low in endothelial cells grown under both shear stress and static conditions. Levels of COX-1 or COX-2 did not vary significantly in aligned versus nonaligned endothelial cells. This is an important observation, because we might expect COX-2 levels to be higher in regions at the center of the wells where peak shear stress is
low and mean shear stress lacks directionality, consistent with an atheroprone region of the vessel. As with any protocols reliant on immunoreactivity we cannot make direct comparisons between COX immunoreactivity and specific activity; however, we have used well-validated antibodies and are able to conclude on any change in expression of individual isoforms within our model. Indeed, if shear had increased COX-2 expression, we would have detected it, because LPS treatment for 24 hours increased COX-2 significantly. In line with this observation, endothelial cells released increased prostacyclin after the addition of LPS, although it should be noted that this result may equally relate to increased expression or activation of phospholipase A2 (a prerequisite to arachidonic acid liberation and subsequent prostaglandin production). The effect of LPS on COX-2 and prostacyclin release had declined by 7 days of continuous exposure. Similarly, 7 days of LPS treatment was associated with reduced endothelial cell number and CD31 expression. Our findings are consistent with the notion that endothelial cells predominantly express COX-1 under static culture conditions and, importantly, that this is also the case after acute (24 hours) or chronic (7 days) periods of shear stress. Our observations at the 7-day time point are particularly important, as this allows for cells to reacclimatize to any acute shear insult. It is noteworthy that COX-1 expression tended to be lower in cells after 7 days of culture than after 24 hours. This tendency was not affected by shear and may well represent the limitation in continued culture of confluent monolayers of endothelial cells.
It is important to point out that other groups have shown that shear stress increases COX-2 gene and protein expression. However, previous studies often focus on changes after relatively acute shear (often <24 hours), which is likely perceived by cells as an inflammatory stimulus. A recent study that used human umbilical vein endothelial cells expressing COX-1 but not COX-2 under static conditions demonstrated robust COX-2 expression after acute (6-hour) shear and showed that resultant prostacyclin release was only partially inhibited by a COX-2 inhibitor, suggesting that a component of prostacyclin release was COX-1 driven. Although we did not find alterations in COX expression by long-term shear in endothelial cells, short-lived changes in shear at specific, highly localized sites of the vasculature may well regulate COX-1 and COX-2 transiently; the importance of which remains the subject of investigation.

Although we did not observe dramatic effects of shear on COX expression, we did find that shear stress protected cells against the effects of LPS on cell number. Clearly, more experiments are required before we fully understand how shear protects the endothelium; however, this observation may well be meaningful because systemic LPS (associated with bacterial sepsis) is linked to endothelial cell death and loss of function. Interestingly, others have shown that nuclear factor κB responses in endothelial cells of mice exposed to LPS in vivo are less in areas of high laminar shear than in areas of interrupted shear stress (the atheroprotected and atheroprotected regions respectively). In summary, we have used a simple and effective model of shear stress and demonstrated that endothelial cells align according to shear directionality. Our data show that in the center of the well, the shear stress generated has a low peak level and lacks directionality, and at the edge of the well, it has a high peak level and more directionality, even though the mean level is approximately constant. We observe that endothelial cells at the center of the well appear similar in morphology to those seen at atheroprotected regions of the aortic arch, whereas those at the edge of the well appear like endothelium in atheroprotected regions. Endothelial cells predominantly express COX-1 immunoreactivity under static or shear stress conditions, supporting the idea that COX-1 is the predominant isofrom in the healthy vasculature. In order for us to better understand why COX-2 selective drugs reduce arterial prostacyclin metabolites and are associated (as are other NSAIDs) with increased cardiovascular risk, it remains important for us to continue to consider potential off-target effects of COX-2 selective inhibitors on prostacyclin formation and to consider that COX-2 in specialized cardiovascular regions, such as the kidney, may play a major role in protecting cardiovascular health. Finally, our findings show that shear stress protects the endothelium from the toxic effects of LPS.

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Disclosures

None.

References


Supplement Material:
Role of shear stress in endothelial cell morphology and expression of cyclooxygenase isoforms

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1Cardiothoracic Pharmacology, Pharmacology and Cardiovascular Sciences, National Heart and Lung Institute and 2Department of Bioengineering, Imperial College London, UK. 3William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, London, UK. 4Department of Chemical Engineering, University of Louisville, USA
Data gathering, quantification and analysis of Immunohistochemistry imaging data.

For each protocol, n=6 images using cells from 3 separate pigs were gathered for analysis. The size of each image was 380µm². Images were taken in 4 regions of each filter, two from the centre and one each from opposite sides at the edge. Images at the edge of the well were taken 7 fields of view from each edge along the centre line (approximately 9.3mm from the centre). Images at the centre of the well were taken one each side of a reference mark made at the centre point of the filter. These areas correspond to those thought to experience pulsatile and non-laminar flow respectively in our shear stress model. Pilot studies were carried out to optimise staining and visualisation conditions and once established microscope settings were rigorously adhered to.

Detection wavelength settings were 493–544nm for CD31 and 585–680nm for COX-1 and COX-2 imaging protocols. Images were analysed using Leica LAS AF software. Fluorescence intensity units (FIU) were calculated by the multiplication of average pixel intensity by total number of pixels per frame (385 µ).

Non-specific FIU background values were obtained from separate, but parallel wells where secondary antibody only was added (secondary antibody controls). Background values from cells cultured under corresponding conditions were subtracted prior to further data analysis. FIU data points were analysed using GraphPad software using appropriate statistical analysis.

Image reproduction

Images shown were selected as representative of the group. Where linear adjustment of contrast and brightness were made they were applied equally to all parts of an entire image and to all other images presented in the paper.
Transwell filters are cut out and mounted on cover slips with Vectashield aqueous mounting medium (H-100, Vector, UK). Images are taken in regions consistent with areas predicted by our model to experience directional, pulsatile flow and non-directional flow.
Western Blots indicating the specificity of our COX-1 and COX-2 antibody.

1=PAEC cultured under static conditions for 24 hours; 2=PAEC cultured under static conditions for 24 hours in the presence of 0.1µg/ml LPS; 3=Murine macrophage cell line J774 cultured for 24 hours under static conditions; 4=Murine macrophage cell line J774 cultured for 24 hours under static conditions in the presence of 0.1µg/ml LPS.

COX-1 Blot
1=J774 + LPS
2= J774 Control
3= EA.hy + LPS
4= EA.hy Control
5= PAEC + LPS
6= PAEC Control

COX-2 Blot
1= PAEC Control
2= PAEC + LPS
3= J774 Control
4= J774 + LPS
SIII

Pooled data for COX-1, COX-2 and CD31 immunoreactivity in all experimental protocols.

Blind scoring results for endothelial alignment scored by 3 assessors on a scale between 0 and 3 where 0 = no alignment, cells appear randomly orientated and 3 = all cells are in unidirectional alignment.

Data is mean±S.E.M for n=6–12.

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N/D = Not determined