Role of shear stress in endothelial cell morphology and expression of cyclo-oxygenase isoforms

Claire M.F. Potter[1], Martina H. Lundberg[1], Louise S. Harrington[1], Christina M. Warboys[2], Timothy D. Warner[3], R. Eric Berson[4], Alexey V. Moshkov[1], Julia Gorelik[1], Peter D. Weinberg[2] and Jane A. Mitchell[1]

[1]Cardiothoracic Pharmacology, National Heart and Lung Institute and [2]Department of Bioengineering, Imperial College London, UK. [3]William Harvey Research Institute, Bart's and the London School of Medicine and Dentistry, London, UK. [4]Department of Chemical Engineering, University of Louisville, USA

Correspondence to Jane A Mitchell:

Cardiothoracic Pharmacology, National Heart and Lung Institute, Imperial College London, Dovehouse Street, London SW3 6LY.

j.a.mitchell@imperial.ac.uk

CMFP and MHL contributed equally to this study and share first authorship.

Total Word count:

Abstract Word Count:

Keywords: endothelium, cyclo-oxygenase, shear stress, orbital shaker

Abstract:

Objectives: To examine the effect of chronic heterogeneous shear stress as 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 and cultured for up to 7 days either under static conditions or on an orbital shaker which generated a wave of shear stress over the cells. Cells were fixed and stained for the endothelial surface marker CD31 or cyclo-oxygenase 1 and 2. *En face* confocal microscopy or scanning ion microscopy were used to analyse cell morphology and protein expression. Endothelial cells aligned with shear stress and expressed predominately cyclo-oxygenase-1 under all conditions studied. shear stress protected cells from death under some conditions.

Conclusion: Cyclo-oxygenase-1 is the dominant cyclo-oxygenase isoform in endothelial cells under static conditions or cultured under chronic shear stress.

Introduction:

Endothelial cells line the luminal surface of blood vessels and are continuously exposed to haemodynamic shear stress. The level of shear stress 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 and development of atherosclerosis. At areas of low, oscillatory shear stress endothelial cells are randomly orientated and susceptible to inflammation and atherosclerosis development [1-3].

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

COX is present in two isoforms: COX-1 and COX-2. Generally, COX-1 is expressed constitutively whilst COX-2 is induced at sites of inflammation [9]. In endothelial cells COX-1 activity results predominately in the production of the anti-thrombotic hormone prostacyclin [10]. COX-1 and 2 are the targets for non-steroidal anti-inflammatory drugs (NSAIDs). They have gathered much media attention since the association of COX-2 selective NSAIDs with adverse cardiovascular events [11-12] though the mechanism behind this association remains unclear. It has previously been shown that COX-1 predominates over COX-2 in endothelial cells cultured under static conditions [13]. However, the relative effect of shear stress, particularly long term shear, is less well understood. Others have used flow chambers to investigate how shear affects COX expression in endothelial cells. Some studies have shown the upregulation of levels of COX-2 mRNA by shear stress [14-15] but other contradictory findings have also been reported [16].

In this communication we have utilised an orbital shaker to apply shear stress for up to 7 days and investigated effects on the expression of COX-1 versus COX-2 in porcine aortic endothelial cells (PAEC). This technique creates a gradient of shear across a well of endothelial cells [10]. Confocal microscopy and scanning ion conductance microscopy (SICM) were used to image the resultant effects of 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 abattoir (Fresh tissue supplies, UK) using the method of Bogle et al [17]. Primary cultures were assumed to be 99.99% pure, based on results from studies into internalisation of DiI-labelled acetyl LDL and CD31 staining throughout. Cells were subcultured in DMEM (Sigma, UK) containing 10% FCS (Biosera, UK)

Modelling Shear Stress

PAEC at passage 2 were plated on fibronectin (Sigma, UK) (50µg/ml) coated 6 well Transwell™ plates (Corning, UK) at a density of 400,000 cells per well. Experimental conditions were applied 24 hours after seeding. In order to assess the effect of shear stress a PS-300 orbital shaker (Grant Instruments, UK) was used under conditions described in Warboys et al [18]. As COX-2 is known to be upregulated in response to inflammatory stimuli, some wells were incubated with either 0.01 or 0.1µg LPS (0111:B4 *E.coli*, Sigma, UK) as positive controls. Cells were incubated under either static conditions (no shear) or shear stress conditions for up to 7 days. A complete media change was carried out every 2 days with fresh LPS added where appropriate.

Western blot analysis

To verify COX-1 (Cayman 160108, US) and COX-2 (Cayman 160126, US) antibody specificity, western blots were carried out. Prior to western blotting, protein concentration for loading was estimated by Bradford Assay. Protein Samples were loaded on 7.5% (v/v) acrylamide gels (reagents from Sigma and National Diagnostics, UK) and transferred onto PVDF membranes (Amersham, UK) over 1.15 hours. Visualisation was by autoradiography. Loading concentrations were confirmed using either anti-GAPDH (AbCam, UK) or anti-β-actin antibodies (AbCam, UK).

Radioimmunoassay

Prostacyclin was measured by the accumulation of its break down product 6-keto $PGF_{1\alpha}$ by radioimmunoassay as described previously [19].

Immunohistochemistry and Confocal Microscopy

All immunohistochemistry procedures were carried out in the Transwells™. Cells were incubated with primary rabbit polyclonal antibodies for either COX-1 (Cayman 160108, US) or COX-2 (Cayman 160126, US) for 2 hours and with secondary antibody goat anti rabbit Alexafluor 568 (Invitrogen, UK) for 1 hour, both incubations were carried out at room temperature. A negative control where no primary antibody was added (only secondary antibody was added) was also prepared and used for non-specific background fluorescence quantification purposes. All wells were incubated with an Alexa Fluor 488 conjugated anti-CD31 antibody (MCA1746F, AbD Serotec,UK) overnight at 4^{o}C to provide validation that the cells were endothelial in origin and the nuclear and chromosome counter stain 4',6'-diamidino-2-phenylindole (DAPI) (DI106 Invitrogen, UK) was used to stain the endothelial cell nuclei. Details of mounting procedure can be found in the supplementary information (see Figure S1). Confocal imaging was carried out using a Leica SP5 inverted confocal microscope, with a 40x1.25 oil objective with 405 Diode, 15% Argon and HeNe543 lasers. LAS AF software was used for quantification. Images were taken at 2.7mm from the edge and the centre of the transwell. Further detail of the methodology is provided in the supplementary literature.

Scanning ion microscopy (SICM)

(JULIA to ADD IN PLEASE)

Statistical Analysis

All statistical analysis was performed using GraphPad Prism version 5.0. ANOVAs were performed with the Bonferroni Multiple Comparison post test. In all figures results are displayed as mean +/- standard error of the mean (SEM). Statistical significance was taken as p=<0.05 and is indicated by *.

Results:

Effect of shear stress on morphology of endothelial cells

Endothelial cells cultured under static conditions expressed the endothelial cell specific marker CD31 (Figure 1) and displayed typical cobblestone/polygonal morphology (Figure 2). Morphology was largely unchanged when cells were stimulated with LPS for either 24 hours or 7 days. It was found that cell number was higher (per unit area) at the centre of the well in both static and sheared conditions (Figure 1). In static conditions cell number was reduced by LPS treatment in the centre and edge of static wells and CD31 was reduced at the centre of the wells. When endothelial cells were cultured under conditions of shear stress on an orbital shaker the effect of LPS on cell number was prevented (in high shear regions; edge) or lessened (in low shear regions; centre) in cells of sheared wells (Figure 1). Cellular alignment was evident by day 2 in some cases, but consistent alignment was not seen until day 3 (Figure 2). By day 7, cellular alignment was seen in all images taken at regions of high shear stress (Figure 2B) and in some cases at regions of low shear stress (Figure 2D). Blinded visual scoring of cellular alignment confirmed statistically significant responses in the high shear regions of the wells after 7 days (Table 1). In some frames taken at the low shear centre of the well, cells displayed directionality with the flow producing an image consistent with a 'swirl' confirmation (Figure 2D). Using SICM it was found that aligned endothelial cells grown in regions of high shear had a somewhat lower cellular volume than those showing no directional alignment at the low shear regions or cell grown under static conditions (Figure 2; Table 1).

Expression of COX-1 and COX-2 in endothelial cells grown under static and shear stress conditions

Endothelial cells released low or undetectable levels of the prostacyclin metabolite 6-keto$PGF_{1\alpha}$ when grown under static or sheared conditions for up to 7 days (Table2). Endothelial cells released increased levels of 6-keto $PGF_{1\alpha}$ when stimulated with 0.01 or 1µg LPS. No notable difference was seen in LPS-induced release of 6-keto $PGF_{1\alpha}$ in cells grown under static or sheared conditions (Table 2). Primary antibodies to COX-1 or COX-2 used in this study were validated for specificity using standard western blotting technique (Figure S2). Antibodies to COX-1 recognized a protein band of approximately 70KDa in endothelial cells (Figure S2). Antibodies to COX-2 did not detect proteins in control endothelial cell extracts but recognised a 70KDa band in homogenates of cells treated with LPS for 24 hours (Figure S2). PAEC cultured under static or shear stress conditions expressed predominately COX-1 with low levels of COX-2 in all regions of the well studied (Figure 3). Shear stress did not affect the relative expression of COX-1 and COX-2 in cells when studied at either 24

hours (not shown) or 7 days (Figure 3). COX-1 immunofluoresence appeared localized throughout the cytosol with lower levels in the nucleus (Figure 3).

LPS had no effect on COX-1 expression in cells cultured under any conditions studied (data not shown). However, as expected 24 hour exposure to LPS increased COX-2 expression in endothelial cells grown under static (Figure 4) and shear stress (data not shown) conditions. Concentrated COX-2 immunoreactivity appeared as a distinctive bright ring around the nucleus with lower levels in the cytoplasm. Where visible in untreated cells COX-2 appeared as a granular staining around the nucleus. There was no significant difference in levels of LPS-induced COX-2 expression in endothelial cells cultured under static, high shear or low shear stress conditions (data not shown). It should be noted that when cells were stimulated with LPS for 7 days, COX-2 expression had declined and was no longer found to be increased above levels seen in control cells (data not shown).

Discussion:

Endothelial cells are exposed to shear stress caused by the passage of blood over the luminal surface of blood vessels as part of their normal physiological state. However, when endothelial cells are studied *in vitro* they are often cultured in standard static conditions. The transfer of endothelial cells from blood vessels (out of shear conditions) into static culture affects morphology and potentially the expression of important vasoactive genes within the cells. In addition, within blood vessels the level and the type of shear vary with the architecture of the vessel. Areas of endothelium exposed to low and interrupted shear stress, that occurs at vessel bifurcations or in the inner curvature of the aortic arch, are predisposed to inflammation and the subsequent development of atherosclosis [1]. It is therefore important when considering the expression of vasoactive enzymes in the endothelium to compare levels in cells exposed to different types of shear stress. This is particularly relevant when considering COX-1 and COX-2.

In this study we show that endothelial cells grown on an orbital shaker subjected to a gradient of shear stress caused by motion of the media wave [20] are present in two shear-associated phenotypes. Cells at the outer region of the well where shear is relatively high (~4dyn/cm^2) and laminar, were elongated and aligned (with the direction of the wave). However, cells at the centre of the well in areas of relatively low and interrupted flow appeared non-aligned and resembled the classic cobble-stone appearance of cultured endothelial cells. SICM revealed that the aligned cells from the edge of the well had a lower volume than the non aligned endothelial cells imaged at the centre of the well or than the cells grown in static conditions. Interestingly, these observations are remarkably consistent with endothelial cell morphology at regions of blood vessels experiencing high laminar shear stress (elongated and aligned) versus low and interrupted shear stress (non aligned, cobblestone) typified by the outer and inner curvature of the aortic arch respectively [21]. These observations validate this model and show, for the first time, how it can be used to study typical endothelial cells (in terms of morphology) from different regions of blood vessels.

Using this model we demonstrate that COX-1 dominates over COX-2 immunoreactivity 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 non aligned endothelial cells. This is an important observation since we might expect that COX-2 levels be higher in regions of low shear stress consistent with the notion that this isoform is associated with inflammation. COX-2 immunoreactivy was however significantly increased in endothelial cells stimulated with LPS for 24 hours. In line with this observation, endothelial cells released detectable levels of prostacyclin which were increased by the

addition of LPS. Prostacyclin levels were not significantly altered by shear stress in this system. It was interesting to note however that 7 days treatment with LPS reduced endothelial cell number with a corresponding drop in CD31 expression. The effect of LPS on cell number was reduced in cells grown under low shear stress and completely prevented for cells grown under high shear stress.

Figure legends

Figure 1

Numbers of PAEC grown on transwell filters subjected to shear stress were counted in two regions of the well which had experienced different levels of shear, Edge (HS; ~4dyn/cm^2), Centre (LS; ~2 dyn/cm^2). Cells were also counted in corresponding regions of static wells. LPS was present in the medium in half of the wells throughout the 7 days of the experiment. Levels of CD31 (an endothelial cell marker) were quantified in the same areas. Data was analysed by One Way ANOVA with Bonferroni's multiple comparison post-test. **A.** LPS significantly reduced the number of cells at the edge of the static wells ($P<0.05$ compared to static no LPS, shear no LPS and static LPS) but had no effect on cells that experienced high shear **B.** LPS significantly reduced the number of cells in the centre of static wells ($P<0.05$ compared to static no LPS, shear no LPS, shear LPS). To a lesser extent, LPS reduced the level of cells in the centre of the sheared wells (#$P<0.05$ compared to sheared no LPS, static no LPS) but the number of cells in this region remained greater than at the centre of static wells without LPS. **C.** No significant difference was seen in levels of CD31 expressed in cells grown at the edge of static or sheared wells with and without LPS treatment. **D.** In static cells a significant decrease in levels of CD31 ($P<0.05$) was seen between cells that had been treated with LPS and all other studied conditions

Figure 2

A distinct morphology differences can be seen between PAEC cultured under static conditions and those cultured under conditions of shear stress. Images on the left were acquired using Scanning Ion Conductance Microscopy (SICM) and images on the right shows immunohistochemically stained PAEC cells imaged by confocal microscopy. Blue=Nuclei stained with 4',6-diamidino-2-phenylindole (DAPI). Green=Alexa Fluor 488 conjugated anti-porcine CD31. **A & B.** Cells cultured under the greatest level of shear stress appeared elongated and aligned with the direction of flow. **C & D.** Cells that experience a low level of shear are of mixed phenotype. Some, though not all cells, appeared elongated. **E & F.** Cells grown under static conditions displayed typical polygonal/'cobblestone' conformation.

Figure 3

Representative images of cells cultured under static or shear stress conditions for 7 days.lue=Nuclei stained with 4',6-diamidino-2-phenylindole (DAPI). Green=Alexa Fluor 488 conjugated anti-porcine CD31. Red=COX-1 or COX-2 as indicated. COX-1 can clearly be seen as the dominant isoform present, in all conditions studied.

Figure 4

Representative images of cells cultured under static conditions for 24 hours presence of 0.1ug LPS. Blue=Nuclei stained with 4',6-diamidino-2-phenylindole (DAPI). Green=Alexa Fluor 488conjugated anti-porcine CD31. Red=COX-1 or COX-2 as indicated. COX-1 can be seen as the dominant isoform under all conditions studied though an increase in COX-2 expression is seen following LPS exposure.

Figure 5

After immunostaining cells were imaged in the regions of interest using confocal microscopy and the fluoresence was quantified using LAS AF software. **A.** Fluorescent Intensity Units of COX-1 staining of cells n=6 from 3 wells. Data was analysed by One Way ANOVA with Bonferroni's multiple comparison post-test. No significant difference between groups was detected. **B.** Fluorescent Intensity Units of COX-2 staining of cells n=6 from 3 wells Data was analysed by One Way ANOVA with Bonferroni's multiple comparison post-test. No significant difference between groups was detected.

Supplementary Figures

Figure S1

PAEC were grown on insert wells and placed on an orbital shaker. The wave of media produced causes a graded pattern of shear over the well (B). Mathmatical modelling allows us to predict the distance from the edge of the well where each force is likely to occur (B). Inserts are cut out of the wells and mounted on cover slips. Estimates of distance from the edge of the well (C) consistent with predicted shear force are made and images taken in those regions.

Figure S2

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

1. Caro, C.G., J.M. Fitz-Gerald, and R.C. Schroter, *Atheroma and Arterial Wall Shear Observation, Correlation and Proposal of a Shear Dependent Mass Transfer Mechanism for Atherogenesis.* Proceedings of the Royal Society of London. Series B. Biological Sciences, 1971. **177**(1046): p. 109-133.
2. Cheng, C., et al., *Atherosclerotic Lesion Size and Vulnerability Are Determined by Patterns of Fluid Shear Stress.* Circulation, 2006. **113**(23): p. 2744-2753.
3. Hajra, L., et al., *The NF-kappa B signal transduction pathway in aortic endothelial cells is primed for activation in regions predisposed to atherosclerotic lesion formation.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(16): p. 9052-9057.
4. Folkman, J., C.C. Haudenschild, and B.R. Zetter, *Long-term culture of capillary endothelial cells.* Proceedings of the National Academy of Sciences of the United States of America, 1979. **76**(10): p. 5217-5221.
5. Schor, A., S. Schor, and T. Allen, *Effects of culture conditions on the proliferation, morphology and migration of bovine aortic endothelial cells.* J Cell Sci, 1983. **62**(1): p. 267-285.
6. Warboys, C.M., et al., *Acute and chronic exposure to shear stress have opposite effects on endothelial permeability to macromolecules.* Am J Physiol Heart Circ Physiol.
7. Caro, C.G., *Discovery of the role of wall shear in atherosclerosis.* Arterioscler Thromb Vasc Biol, 2009. **29**(2): p. 158-61.
8. Topper, J.N., et al., *Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress.* Proc Natl Acad Sci U S A, 1996. **93**(19): p. 10417-22.
9. Seibert, K., et al., *PHARMACOLOGICAL AND BIOCHEMICAL DEMONSTRATION OF THE ROLE OF CYCLOOXYGENASE-2 IN INFLAMMATION AND PAIN.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(25): p. 12013-12017.
10. Moncada, S., et al., *An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation.* Nature, 1976. **263**(5579): p. 663-5.
11. Bombardier, C., et al., *Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis.* New England Journal of Medicine, 2000. **343**(21): p. 1520-1528.
12. Bresalier, R.S., et al., *Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial.* New England Journal of Medicine, 2005. **352**(11): p. 1092-1102.
13. Caughey, G.E., et al., *Roles of cyclooxygenase (COX)-1 and COX-2 in prostanoid production by human endothelial cells: Selective up-regulation of prostacyclin synthesis by COX-2.* Journal of Immunology, 2001. **167**(5): p. 2831-2838.
14. Di Francesco, L., et al., *Induction of Prostacyclin by Steady Laminar Shear Stress Suppresses Tumor Necrosis Factor-alpha Biosynthesis via Heme Oxygenase-1 in Human Endothelial Cells.* Circulation Research, 2009. **104**(4): p. 506-513.
15. Okahara, K., B. Sun, and J. Kambayashi, *Upregulation of prostacyclin synthesis-related gene expression by shear stress in vascular endothelial cells.* Arteriosclerosis Thrombosis and Vascular Biology, 1998. **18**(12): p. 1922-1926.
16. Dancu, M.B., et al., *Asynchronous shear stress and circumferential strain reduces endothelial NO synthase and cyclooxygenase-2 but induces endothelin-1 gene expression in endothelial cells.* Arteriosclerosis Thrombosis and Vascular Biology, 2004. **24**(11): p. 2088-2094.
17. Bogle, R.G., et al., *Regulation of L-arginine transport and nitric oxide release in superfused porcine aortic endothelial cells.* The Journal of Physiology, 1996. **490**(Pt 1): p. 229-241.

18. Warboys, C.M., et al., *Acute and chronic exposure to shear stress have opposite effects on endothelial permeability to macromolecules.* Am J Physiol Heart Circ Physiol, 2010: p. ajpheart.00114.2010.
19. Bishop-Bailey, D., et al., *Characterization of the induction of nitric oxide synthase and cyclo-oxygenase in rat aorta in organ culture.* Br J Pharmacol, 1997. **121**(1): p. 125-33.
20. Berson RE, P.M., Sharp MK. and 189–198., *Computationally determined shear on cells grown in orbiting culture dishes.* Adv Exp Med Biol 2008.(614): p. 189–198.
21. Iiyama, K., et al., *Patterns of Vascular Cell Adhesion Molecule-1 and Intercellular Adhesion Molecule-1 Expression in Rabbit and Mouse Atherosclerotic Lesions and at Sites Predisposed to Lesion Formation.* Circ Res, 1999. **85**(2): p. 199-207.

Table 1

Cell Culture Conditions	Mean Cell Count	Mean Cell Volume	Mean Alignment Score (0=no alignment, 3=full, unidirectional alignment)
Static Edge	44±3	3777±781μm^3	0.18±0.09
Static Centre	55±4		0.18±0.05
Shear Edge (HS)	49±3	2736±262μm^3	1.46±0.21
Shear Centre (LS)	38±4	3600±302μm^3	0.69±0.15

Table 1: Cell volume and morphology characteristics of endothelial cells grown under different conditions.

Table 2

Time (hours)	Control (6-ketoPGF$_{1\alpha}$ ng/ml)		0.1µg LPS (6-ketoPGF$_{1\alpha}$ ng/ml)	
	Static	Sheared	Static	Sheared
1	1.82±0.69	1.88±0.70	0.89±0.39	0.95±0.54
3	1.27±0.48	7.40±6.31	1.40±0.57	1.37±0.72
6	0.99±0.45	2.97±2.21	42.25±14.06	30.41±12.24
24	2.88±1.10	2.81±1.13	54.52±18.20	47.70±15.63
48	4.69±2.03	1.03±0.37	67.83±26.72	59.60±27.80
72	3.61±1.4	11.52±3.44	49.71±12.23	63.72±20.89
96	5.44±3.02	8.55±2.21	67.77±21.70	57.61±14.93
120	6.98±1.76	12.02±3.79	34.33±16.79	33.06±6.92
144	8.12±2.19	5.16±0.67	37.99±17.52	22.67±9.35
168	24.02±10.48	11.02±1.88	46.50±24.36	16.31±6.16

Table 2: release of prostacyclin (measured as 6-ketoPGF1α) by endothelial cells cultured under different conditions.