Increased Mitogenic and Decreased Contractile P2 Receptors in Smooth Muscle Cells by Shear Stress in Human Vessels With Intact Endothelium

Lingwei Wang, Maria Andersson, Lena Karlsson, Marie-Ange Watson, Diane J. Cousens, Sverker Jern, David Erlinge

Objective—We investigated the role of shear stress in regulating P2 receptors in human umbilical vein.

Methods and Results—Using a novel, computerized, biomechanical perfusion model, parallel vessel segments were randomized to simultaneous perfusion under high (25 dyn/cm²) or low (<4 dyn/cm²) shear stress at identical mean perfusion pressure (20 mm Hg) for 6 hours. In the endothelium, no significant P2 receptor mRNA differences were found. In smooth muscle cells (SMCs), high shear stress decreased P2X₁ receptors, whereas P2Y₂ and P2Y₆ receptors were upregulated. These findings were consistent at the mRNA level (real-time reverse transcription–polymerase chain reaction), protein level (Western blot), and morphologically (immunohistochemistry). The changes were more pronounced in the subintimal layer of the media.

Conclusions—Our findings suggest that shear stress might regulate gene expression in SMCs more than in the endothelium in intact vessels. Decreased expression of the contractile P2X₁ receptor could lead to reduced vascular tonus and increased blood flow. Because P2Y₂ and P2Y₆ receptors stimulate growth and migration of SMCs, increased expression of these receptors could promote vascular remodeling induced by shear stress. The pattern of upregulation of mitogenic P2Y receptors and downregulation of contractile P2X₁ receptor is similar to changes seen in the phenotypic shift from contractile to synthetic SMCs. (Arterioscler Thromb Vasc Biol. 2003;23:1111–1119.)

Key Words: smooth muscle cells ■ shear stress ■ purinoceptors ■ gene expression ■ vascular remodeling

Shear stress, compressive force, and tensile force are the 3 major types of biomechanical forces acting on the vessel wall.¹,² Fluid shear stress is a frictional force caused by the blood flow, which acts parallel to the longitudinal axis of the vessel wall. This frictional force, which is determined by blood flow, vessel geometry, and fluid viscosity in the layer of the wall, acts on the endothelium. However, recent studies have shown that shear stress might also modulate smooth muscle cells (SMCs) either indirectly by way of endothelium-mediated changes in vascular tone or indirectly by transmural pressure gradients causing interstitial flow.³,⁴ Indeed, SMCs might experience even higher shear stress levels than do endothelial cells (ECs) exposed to normal blood flow.³ Shear stress can regulate SMC migration,⁵–⁷ proliferation,⁸,⁹ differentiation,¹⁰,¹¹ and endothelial function.¹²,¹³ Shear stress is an important trigger for vascular remodeling in the adaptation of the vascular tree. For example, increased blood flow is required in growing organs, tumors, or ischemic areas in need of collaterals, and this causes an increased flow through existing blood vessels. An unaltered diameter in the vessels will lead to increased shear forces. The initial response will be an endothelium-dependent dilatation. However, if the flow is continuously increased over a longer time, vascular remodeling and enlargement occur. The mechanisms of this phenomenon are poorly understood.

Vascular SMC proliferation is a key event in vascular remodeling and growth, in the development of atherosclerosis, angioplasty-induced restenosis, pulmonary hypertension, venous bypass graft atherosclerosis, and possibly hypertension.¹⁴,¹⁵ Many factors are believed to be responsible for SMC growth or hypertrophy in the vessel wall. It has been demonstrated recently that extracellular nucleotides can act as growth factors for vascular SMC by activation of several P2Y receptors.¹⁶,¹⁷ A large number of shear stress studies have been performed in vitro on cultured cells, especially ECs. Often, the cells have been of animal origin. However, the intact blood vessel is more relevant, where both ECs and SMCs can be studied in their natural environments. We have investigated the role of shear stress by using a novel, computerized,
biomechanical perfusion system. In this model, intact vessels are perfused under well-defined, fluid mechanical forces, while the essential hemodynamic variables, such as intraluminal pressure, flow, and shear stress, are continuously measured to permit studies of differential effects of each variable. Shear stress was kept constant for 6 hours in human umbilical cord veins, after which ECs and SMCs were analyzed for changes in P2 receptor expression.

We have recently devised methods to quantify the expression of P2 receptors in humans at the mRNA level with real-time polymerase chain reaction (PCR) and at the protein level with Western blotting and to study their morphological expression with immunohistochemistry. Using these methods, we examined how shear stress regulates P2 receptor expression in human umbilical vein SMCs and ECs.

Methods
The studies were approved by the local Ethics Committees of Lund University and Göteborg University and were conducted according to the principles of the Declaration of Helsinki.

Preparation Procedure
Human umbilical cords were collected immediately after single, full-term, vaginal deliveries (Department of Obstetrics, Sahlgrenska University Hospital/Ostra, Göteborg, Sweden) and divided into two 20-cm segments for parallel perfusion. After an initial 10-minute noncircuiting washout period, the vessel pairs were equilibrated for another 20 minutes under constant mean intraluminal pressure and a flow of 20 mm Hg and 10 ml/min, respectively. The vessel segments were thereafter randomized to perfusion under high (25 dyn/cm²) or low shear stress (<4 dyn/cm²) at an identical mean perfusion pressure of 20 mm Hg for 6 hours (n=8). The perfusion medium consisted of Tyrode’s saline solution and a standard antibiotic mixture (penicillin and streptomycin [PES]).

Vascular Perfusion Model
The perfusion system has recently been described in detail. In brief, fresh human umbilical veins are perfused unequally in 2 parallel, gravity-fed circuits. The medium is continuously pumped into an upper reservoir whose height is regulated with a computer-controlled proportioning solenoid valve. The 37.0°C medium is bubbled with a computer-controlled amount of gas mixture of 90% N₂/5% O₂/5% CO₂ to maintain target pH (7.4), PO₂ and PCO₂. Pressure is monitored through upstream and downstream pressure catheters. Shear stress calculation was based on the following formula:

\[ \tau = \frac{1}{2} \left( \frac{\Delta P}{L} \right) \times \left( \frac{8 \eta Q}{\pi} \right) \]

where \( \tau \) is wall shear stress, \( \Delta P \) is the pressure drop over the vessel, \( L \) is the vessel length, \( \eta \) is viscosity of the fluid, and \( Q \) is the flow through the vessel. Reynolds’ number was monitored continuously to ensure a laminar flow profile.

RNA and Protein Extraction
After perfusion, ECs were removed by incubating the vessel with 0.1% collagenase. The cell suspension was centrifuged for 10 minutes at 260g. The media (smooth muscle layers) was obtained by dissecting them free of adhering tissue under sterile conditions. Smooth muscle layers and the EC pellet were snap-frozen in LN₃ and then placed in a –70°C freezer for RNA and protein extraction. The identity of ECs and SMCs was checked by using the specific markers α-actin (for SMCs) and von Willebrand factor (for ECs). Total cellular RNA and protein were extracted with reagent (TRizol, Gibco BRL, Life Technology, Invitrogen Corp) according to the supplier’s instructions. The resulting RNA pellets were cleaned by using a commercially available kit (RNeasy mini cleanup protocol, Qiagen). The protein pellet was finally resolved in 1% sodium dodecyl sulfate (SDS) solution. DC protein assay was used to detect the protein concentration.

Quantitative Real-Time RT-PCR Assay
A commercially available kit (TaqMan reverse transcription [RT] reagent kit) was used to transcribe mRNA into cDNA. Real-time PCR was performed with use of a sequence detector (Prism 7700), as described previously. Oligonucleotide primers and TaqMan probes were designed by using Primer Express, based on sequences from the GenBank database. Constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and rRNA were selected as external, endogenous control genes to correct for potential variation in RNA loading, cDNA synthesis, or efficiency of the amplification reaction.

Methodological Validation
The quantity of total cellular RNA extracted from each vessel segment was similar in vessels exposed to high or low shear stress. Transcript levels of the endogenous control GAPDH and rRNA were independent of shear stimulation.

The amount of target and endogenous reference was determined from the corresponding standard curve. The input amount was calculated according to the formula \[ \frac{y}{x} = \frac{b}{m} + \frac{a}{m} \], where \( b = y \) is the intercept of the standard curve and \( m \) is the slope of the standard curve. The target amount was divided by the endogenous reference amount to obtain a normalized target value. A normalized target gene under low shear stress was arbitrarily chosen to be the calibrator in a comparative analysis. To further verify the specificity of PCR assays, TaqMan PCR was performed with non-RT total cellular RNA and samples lacking the DNA template. No significant amplifications were obtained in any of these samples (data not shown).

SDS-PAGE and Western Blotting
SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting were performed as described previously. In brief, protein electrophoresis was performed on 10% Tris-HCl polyacrylamide ready gels and electrophoretically transferred to nitrocellulose membranes (Hybond-C extra). Protein loading was 10 μg for each well. Membranes were incubated with anti-P2Y₁ (diluted 1:400), anti-P2Y₂ (diluted 1:200), anti-P2Y₆ (diluted 1:250), and anti-P2X₁ (diluted 1:200) antibodies and a negative control (peptide antigen preincubated with the same amount of antibody). Then the membranes were incubated with a peroxidase-conjugated secondary antibody (anti-rabbit immunoglobulin G, diluted 1:1500). Membranes were then exposed to x-ray film after chemiluminescence detection. Membranes were reprobed with the anti-GAPDH antibody (diluted 1:20 000) as a control. The blots were visualized as described previously. The immunoreactive band densities were quantified with a scanner and appropriate software (Quantity One). With this software, volume background subtraction was used, which was the volume of the band minus the volume of the same area of background. Thus, higher band values reflect darker and/or larger bands. The optimized band value was calculated from the value of peptide receptor divided by the value of GAPDH, which were obtained from the same blot membrane.

Immunocytochemistry
After perfusion, 10-mm vascular segments were cut from the middle part of each umbilical cord, fixed in 4% formalin, and then embedded in paraffin. Five-micron paraffin sections were cut and mounted on glass slides (Superfrost Plusglass, Merck Ltd). Each matched vessel pair was placed on the same slide and stained at the same time and under the same conditions. The avidin-biotin-peroxidase complex method was used. After incubation with normal...
serum, the sections were incubated with anti-P2Y1 (diluted 1:200), anti-P2Y2 (diluted 1:100), anti-P2Y6 (diluted 1:100), and anti-P2X1 (diluted 1:100) antibodies and a negative control (peptide antigen preincubated with antibody). Bound primary antibody was detected by using a standard kit (Vectastain Elite ABC) and developed with a diaminobenzidine substrate kit for peroxidase. After being counterstained with Vector hematoxylin Q5 nuclear counterstain (modified Mayer’s formula), the slides were examined by light microscopy.

**Drugs and Antibodies**

Unless otherwise stated, all reagents and drugs were purchased from Sigma. PCR consumables were purchased from Perkin-Elmer Applied Biosystems. Some Western blot reagents were from Amersham Pharmacia Biotech or Bio-Rad Laboratories. Immunohistochemistry reagents were from Vector Laboratories.

Anti-P2X1, anti-P2Y1, and anti-P2Y2 antibodies were purchased from Alomone Labs, which were affinity-purified and raised against highly purified peptides (identity confirmed by mass spectrometry and amino acid analysis), corresponding to specific epitopes not present in any other known protein (see product certificate). The anti-P2Y1 antibody was tested in transfected astrocytoma cells (hP2Y1-1321N1), demonstrating a strong 180-kDa and a weak 50-kDa band, indicating possible oligomerization of the P2Y1 receptor (data not shown). Both bands were absent or very weak in 2 control cells, indicating the specificity of the antibody. Our previous studies of tissues known to express P2Y1 receptors (platelets and ECs) have also shown 180-kDa bands. Anti-P2Y6 antibody was produced by GlaxoSmithKline Research & Development (Stevenage, UK). Anti-GAPDH antibody was purchased from Chemicon International, Inc.

**Statistical Methods**

Data analysis was performed on the computer using Microsoft Excel and Prism 3.0 software. Data are expressed as mean and SEM, unless otherwise stated. n indicates the number of experimental vessel pairs. For comparison of gene expression, parametric methods (ANOVA and Student’s *t* test) were used after logarithmic transformation of the normalized target value. In the present study, GAPDH and rRNA were chosen as endogenous control genes. Similar statistical significance was obtained by using a target gene normalized to either GAPDH or rRNA. Only results normalized to GAPDH are shown, because they were similar when normalized to rRNA. For comparison of protein expression, paired Student’s *t* test was used. Significant differences were considered at *P*<0.05 (2-tailed test).

**Hemodynamics**

The average shear stress levels in the 6-hour series were 25.0±0.5 and 2.3±0.2 dyn/cm² in high- and low-shear circuits, respectively (*P*<0.0001). The mean perfusion pressure was maintained at identical levels in both systems (20.0±0.0 mm Hg, *P*<0.05). The average flow rate throughout the experiment was 114.67±13.37 and 17.75±2.38 mL/min in the high-and low-shear stress systems, respectively (*P*<0.0001).

**mRNA Quantification of P2 Receptors**

The relative P2 receptor expression in SMCs under low shear stress is shown in Figure 1B. P2X1 was the most highly expressed P2 receptor. A normalized target gene under low shear stress was chosen to be the calibrator, which means that gene expression under low shear stress was set to 1 and under high shear stress was expressed in relation to this level. In SMCs, the expression of P2X1 under high shear stress was lower than under low shear stress (*P*<0.05), whereas P2Y1 and P2Y4 under high shear stress were higher than under low shear stress (*P*<0.05). The expression of P2Y1 (Figure 1A) was similar under low and high shear stress, and the difference was not statistically significant (*P*>0.05). P2Y4 (1.65±1.62; *n* = 5, *P*<0.05), P2Y11 (1.91±0.72; *n* = 5, *P*<0.05), P2X1 (1.72±0.55; *n* = 4, *P*<0.05), and P2X2 (1.31±0.17; *n* = 4, *P*<0.05) were little changed under high shear stress compared with low shear stress.

P2Y2, P2Y3, P2Y4, P2Y6, P2Y11, P2X2, and P2X4 in the ECs were also analyzed. Again, a normalized target gene under low shear stress was chosen to be the calibrator, ie, arbitrarily set to 1. The comparative values of P2 receptors under high shear stress were 1.12±0.23 (P2Y2; *n* = 6, *P*<0.05), 2.19±1.48 (P2Y4; *n* = 6, *P*<0.05), 0.78±0.10 (P2Y6; *n* = 4, *P*<0.05), 2.60±1.65 (P2Y11; *n* = 6, *P*<0.05), 0.97±0.27 (P2X1; *n* = 6, *P*<0.05), 0.86±0.19 (P2X2; *n* = 4, *P*<0.05), and 1.18±0.11 (P2X4; *n* = 4, *P*<0.05).

**Western Blots for P2 Receptors in SMCs**

A 35-kDa band was found in the parallel detection of GAPDH to verify the amount of protein loaded and to calibrate integration of the protein bands. The distribution of P2X1 receptors under high shear stress and low shear stress is shown in Figure 2. There were bands of ≈55-kDa on the P2X1 membrane and no band in the peptide control membrane. The band intensity under high shear stress was lower than under low shear stress (*P*<0.05).

The P2Y1 receptor was found with bands of ≈180 kDa under both high and low shear stress (Figure 3). There was no significant difference in intensity between high and low shear stress (*P*>0.05). The P2Y1 receptor (Figure 4) showed a stronger band of 33 kDa under high shear stress than under low shear stress (*P*<0.05). For P2Y4 (Figure 5), a 45-kDa band was found. The band under high shear stress was stronger than under low shear stress (*P*<0.05).

**Immunohistochemistry**

Positive immunoreactivity (brown) was seen for P2X1, P2Y1, P2Y2, and P2Y6, and the bands disappeared when control peptide was added or in controls without the primary antibodies (Figure 6). P2X1 staining was only found in the SMC layer. P2Y1, P2Y2, and P2Y6 staining was found in both EC and SMC layers. SMCs had more pronounced staining right beneath the internal elastic lamina than at a distance from it. In high- compared with low-shear stress sections, the staining intensity in the SMCs was decreased for the P2X1 receptor, whereas the staining intensity in the ECs was increased for the P2Y1 and P2Y2 receptors, and similar for the P2Y6 receptor, whereas the staining patterns were similar in all vessel pairs.

**Discussion**

The main finding of the study was that in an intact blood vessel, shear stress induced more alterations of expression patterns in SMCs than in ECs. The P2X1 receptor that mediates contraction was downregulated, whereas the P2Y1 and P2Y6 receptors that mediate mitogenesis, migration, and contraction were upregulated in SMCs.
Somewhat surprisingly, alterations in receptor expression during shear stress were far more pronounced in SMCs than in ECs. In fact, we did not find any significant change in the expression of P2 receptors in ECs. Most previous studies have focused on the effects on the endothelium, and although the effects of shear stress on cultured ECs are well documented, very few studies have addressed the effects of shear stress on intact blood vessels. The endothelium is believed to be the sensor of shear stress, responding by generating biochemical signals that affect SMCs. However, there is

Figure 1. Relative P2 gene expression in SMCs after 6-hour perfusion. A, Bar graph shows relative P2X<sub>1</sub>, P2Y<sub>1</sub>, P2Y<sub>2</sub>, and P2Y<sub>6</sub> receptor gene expression normalized to GAPDH in high (HSS) compared with low (LSS) shear stress system in SMCs after 6-hour perfusion. Ratio of target to GAPDH expression under low shear stress was arbitrarily set to 1. Results are presented as mean±SEM, n=6 per group. *P<0.05, ΔP<0.05 vs LSS, by Student’s t test. B, Bar graph shows relative P2 receptor gene expression under low shear stress system in SMCs after 6-hour perfusion. Results are presented as mean±SEM, n=8 per group.

Figure 2. Western blots demonstrating effect of P2X<sub>1</sub> under different shear stress conditions in SMCs. A, Band of ~55 kDa appeared under high (HSS) and low (LSS) shear stress. B, Parallel detection of GAPDH (35 kDa) is shown. C, Optimized band values (P2X<sub>1</sub>/GAPDH) are shown. Results are presented as mean±SEM, n=5 per group. *P<0.05 vs LSS, by Student’s t test.
evidence that SMCs could be influenced by wall shear stress derived from transmural pressure gradients, even in the presence of an intact endothelium. Furthermore, fluid dynamics models of intact blood vessels suggest that SMCs also experience shear stress as a result of interstitial flow through fenestral pores in the internal elastic lamina. The average shear stress for SMCs in the immediate vicinity of the fenestral pore could be as much as 10 to 100 times greater than for SMCs in the outer layers of the media. The proximal SMCs might experience shear stress levels that are even higher than those to which ECs exposed during normal blood flow. In the present study, alterations in P2 receptor expression were more pronounced in SMCs in the layer immediately below the internal elastic lamina.

Downregulation of the P2X<sub>1</sub> Receptor in SMCs
P2X<sub>1</sub> is the most highly expressed receptor in SMCs and is the primary receptor coupled to vasoconstriction. During stimulation by shear stress, ECs are able to release endogenous nucleotides. After activation with higher doses of ATP, P2X<sub>1</sub> receptors are rapidly desensitized. Thus, P2X<sub>1</sub> receptor function might be reduced by continued ATP release from ECs. The present study demonstrates that high shear stress decreases P2X<sub>1</sub> expression. This might further down-regulate the contractile capacity of SMCs, causing reduced tonus. These mechanisms might contribute to vascular wall relaxation in response to prolonged shear stress that increases the blood supply before vascular wall remodeling comes into effect.

Figure 3. Western blots demonstrating effect of P2Y<sub>1</sub> under different shear stress conditions in SMCs. A, Band of ∼180 kDa appeared under high (HSS) and low (LSS) shear stress. B, Parallel detection of GAPDH (35 kDa) is shown. C, Optimized band values (P2Y<sub>1</sub>/GAPDH) are shown. Results are presented as mean±SEM, n=5 per group. ∆P<0.05 vs LSS, by Student’s t test. There were no bands on the peptide control membrane.

Figure 4. Western blots demonstrating effect of P2Y<sub>2</sub> under different shear stress conditions in SMCs. A, Band of ∼33 kDa appeared under high (HSS) and low (LSS) shear stress. B, Parallel detection of GAPDH (35 kDa) is shown. C, Optimized band values (P2Y<sub>2</sub>/GAPDH) are shown. Results are presented as mean±SEM, n=5 per group. *P<0.05 vs LSS, by Student’s t test. In the peptide control membrane, no band was found.
Upregulation of P2Y<sub>2</sub> and P2Y<sub>6</sub> Receptors in SMCs
Both P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors were upregulated in SMCs during shear stress, both at the mRNA and protein levels. Even if these receptors are contractile, they might play a more important role in SMC growth, migration, and differentiation. Extracellular nucleotides (ATP, ADP, UTP, and UDP) are mitogens for SMCs and stimulate DNA synthesis, protein synthesis, increased cell number, immediate-early gene expression, cell-cycle progression, and tyrosine phosphorylation. The effect is mediated by activation of the G<sub>q</sub> protein–coupled P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors. Furthermore, UTP stimulates SMC migration by an osteopontin-dependent mechanism. Nucleotides also act synergistically with other growth factors. UTP and ATP are equally effective at the P2Y<sub>2</sub> receptor, whereas UDP is the agonist of P2Y<sub>6</sub> receptors. Both UTP and UDP can be released from the endothelium in response to mechanical stimulation. In the present study, P2Y<sub>2</sub> and P2Y<sub>6</sub> were upregulated by high shear stress, which, in combination with increased release of ATP, UTP and UDP from ECs, could lead to proliferation and migration of SMCs.

Regulatory Mechanisms for P2 Receptor Expression
The pattern of upregulation of mitogenic P2Y receptors combined with downregulation of contractile P2X<sub>1</sub> receptors is also seen in the shift from a contractile to a synthetic SMC phenotype. This phenotypic shift is a central mechanism for SMC participation in the development of vascular disease. The cells lose contractile proteins, start to produce growth factors and matrix, become less differentiated and more fibroblast-like, migrate, and proliferate. Thus, our findings indicate that shear stress induces changes in SMCs that are similar to a shift toward a more synthetic phenotype.

A large number of mechanisms are probably involved in shear stress–induced alterations of P2 receptor expression. P2Y<sub>2</sub> receptor mRNA is increased by fetal calf serum and other growth factors in a mitogen-activated protein kinase kinase–dependent manner. Shear stress influences the release of growth factors, such as fibroblast growth factor-2 and platelet-derived growth factor, from SMCs and ECs. These growth factors might upregulate P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors, which might be a mechanism for their increased levels under high shear stress. As mentioned earlier, ATP is released from ECs in response to shear stress. Interestingly, ATP upregulates the expression of both P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors in SMCs. Thus, ATP itself can be involved in the P2 receptor changes seen during shear stress.

In conclusion, our findings suggest that shear stress might regulate gene expression in SMCs more than in the endothelium in intact vessels. Decreased expression of the contractile P2X<sub>1</sub> receptor could lead to reduced vascular tonus and increased blood flow. Because P2Y<sub>2</sub> and P2Y<sub>6</sub> receptors stimulate growth and migration of SMCs, increased expression of these receptors could promote vascular remodeling induced by shear stress. This might be 1 reason why high flow results in an increased passive lumen diameter and hypertrophy of the vessel wall.
Figure 6. P2 receptor expression as revealed by immunohistochemistry. P2 receptor expression was shown in human umbilical vein treated with high (H) and low (L) shear stress. Brown (diaminobenzidine substrate) was the positive staining color. Controls with antigenic peptides (AP) of each receptor and controls without primary antibodies (control) are shown; n=6 per group.


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Arterioscler Thromb Vasc Biol. published online June 5, 2003;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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