Shear stress modulates endothelial KLF2 through activation of P2X4

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Received: 5 November 2014 / Accepted: 16 December 2014 / Published online: 8 January 2015
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Abstract Vascular endothelial cells that are in direct contact with blood flow are exposed to fluid shear stress and regulate vascular homeostasis. Studies report endothelial cells to release ATP in response to shear stress that in turn modulates cellular functions via P2 receptors with P2X4 mediating shear stress-induced calcium signaling and vasodilation. A recent study shows that a loss-of-function polymorphism in the human P2X4 resulting in a Tyr315>Cys variant is associated with increased pulse pressure and impaired endothelial vasodilation. Although the importance of shear stress-induced Krüppel-like factor 2 (KLF2) expression in atheroprotection is well studied, whether ATP regulates KLF2 remains unanswered and is the objective of this study. Using an in vitro model, we show that in human umbilical vein endothelial cells (HUVECs), apyrase decreased shear stress-induced *KLF2*, *KLF4*, and *NOS3* expression but not that of *NFE2L2*. Exposure of HUVECs either to shear stress or ATPγS under static conditions increased KLF2 in a P2X4-dependent manner as was evident with both the receptor antagonist and siRNA knockdown. Furthermore, transient

transfection of static cultures of human endothelial cells with the Tyr315>Cys mutant P2X4 construct blocked ATP-induced KLF2 expression. Also, P2X4 mediated the shear stress-induced phosphorylation of extracellular regulated kinase-5, a known regulator of KLF2. This study demonstrates a major physiological finding that the shear-induced effects on endothelial KLF2 axis are in part dependent on ATP release and P2X4, a previously unidentified mechanism.

Keywords Shear stress · Purinergic receptors · Krüppel-like factor 2 · ATP

Abbreviations

HUVECs	Human umbilical vein endothelial cells
HAECs	Human aortic endothelial cells
IVF	In vitro fertilization
qRT-PCR	Quantitative real-time polymerase chain reaction
HKG	Housekeeping genes
KLF2	Krüppel-like factor 2
KLF4	Krüppel-like factor 4
NFE2L2	Nuclear factor (erythroid derived-2) like-2
NOS3	Nitric oxide synthase 3
THBD	Thrombomodulin
SNP	Single nucleotide polymorphism
Y315C	Tyr315>Cys
WT	Wild type
ERK5	Extracellular regulated kinase-5

Electronic supplementary material The online version of this article (doi:10.1007/s11302-014-9442-3) contains supplementary material, which is available to authorized users.

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Introduction

The endothelium is a dynamic structure, and its dysfunction is critical to the pathophysiology of cardiovascular disease states

such as atherosclerosis and thrombosis. Endothelial cells in the intimal layer of the vessel wall are constantly exposed to shear stress, the mechanical force generated by laminar blood flow [1]. Experimental observations support the role of endothelial cells in mediating shear-induced mechanotransduction thus enabling the physiological regulation of vascular tone, permeability, blood coagulation and arteriogenesis [2–5]. This regulation is mediated by the mechanically stimulated release of potent endothelial-derived vasoactive factors [6–11]. Extracellular ATP released from endothelial cells during blood flow is an important factor that modulates cellular functions including vasodilation and maintenance of vascular tone via activation of P2X and P2Y receptors [8, 12, 13]. The mammalian P2X receptor family comprises of seven subtypes (P2X1 to P2X7) that function as ATP-gated ion channels [14, 15]. The P2X4 receptor is the most highly expressed subtype in endothelial cells and is involved in flow as well as ATP-induced calcium influx [9, 16, 17]. In fact, P2RX4^{-/-} mice lack normal vascular endothelial function, with aberrant calcium fluxes and nitric oxide levels [18]. In addition, cardiac-specific transgenic overexpression of P2X4 receptors in mice resulted in enhanced cardiac function and in rescue of ischemic heart failure [19]. Furthermore, the inheritance of a single nucleotide polymorphism (SNP), rs28360472, creates a Tyr315>Cys (Y315C) variant in the human P2X4 receptor that results in disruption of the agonist-binding site and loss of receptor function. The presence of this SNP correlated with increased pulse pressure as well as with impaired endothelium-dependent vasodilation [20]. These findings suggest the importance of the P2X4 receptors in the physiological functions of the vascular endothelium.

Depending on the structure of the blood vessel, the blood flow patterns vary from uniform laminar atheroprotective flow in unbranched arteries to disturbed turbulent, atheroprone flow patterns at bifurcations of branched arteries [21]. The *in vitro* model of cultured endothelial cells allows the use of defined flow patterns (atheroprotective vs. atheroprone), simulating those present *in vivo*, and has helped in identifying biomechanically responsive transcriptional activators and repressors [22–24]. Well-documented evidence demonstrates enhanced expression of members of the Krüppel-like factor (KLF) family, particularly KLF2 and KLF4, in vascular endothelial cells exposed to laminar shear stress [25–31]. These KLF transcription factors are key regulators of endothelial function promoting vascular homeostasis and conferring an atheroprotective phenotype both *in vitro* and *in vivo* [28, 31]. Reports have shown shear-induced KLF2 and KLF4 expression in endothelial cells, with distinct but overlapping roles in the vasculature including the induction of endothelial nitric oxide synthase (NOS3) and thrombomodulin (THBD), and attenuation of vascular cell adhesion molecule-1 expression [4, 25–27]. While

KLF2-null mice are embryonically lethal characterized by embryonic hemorrhaging [32], mice with endothelial-specific deficiency of KLF4 were susceptible to atherothrombosis [31]. However, understanding the different shear-induced mechanisms regulating their expression remains to be explored.

Vascular homeostasis is governed by a fine interplay between the endothelial cells and shear stress. Although flow-induced KLF2 and KLF4 expression is well studied, whether extracellular ATP is involved in the regulation of these transcription factors by laminar shear stress in endothelial cells remains unknown. To address this, we studied the effects of steady laminar shear stress and ATP focusing on endothelial KLF2 using an *in vitro* orbital shaker model of human umbilical vein endothelial cells (HUVECs).

Materials and methods

Reagents

Basal Medium 200 was supplemented with 10 % fetal bovine serum, 3 ng/ml basic fibroblast growth factor, 10 µg/ml heparin, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, gentamycin, and amphotericin (Life Technologies, USA). High capacity complementary DNA (cDNA) reverse transcription kit, TaqMan[®] assays and master mixes, Lipofectamine[®] RNAiMAX transfection reagent, OPTI-MEM, ProLong[®] gold antifade mounting medium, and Silencer Select siRNA for P2X4 and P2Y₂ were all obtained from Life Technologies, USA. QIAzol lysis reagent, and miRNeasy[®] mini kit were from Qiagen, USA. 2MeSADP, UTP, ATPγS, ivermectin, and apyrase were obtained from Sigma, USA. Prof. Christa Müller (University of Bonn, Germany) provided P2X4 and P2Y₂ receptor antagonists.

Cell culture

Pooled HUVECs from multiple donors and human aortic endothelial cells (HAECs), both from Life Technologies, USA, were used between passages 1–4. These cells were cultured in Medium 200 supplemented with 10 % fetal bovine serum, 3 ng/ml basic fibroblast growth factor, 10 µg/ml heparin, 1 µg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, gentamycin, and amphotericin on dishes coated with attachment factor (0.1 % gelatin). The static (“no shear stress”) cultures of both HUVECs and HAECs were exposed to 200 µmol/L ATPγS for 3 h. In addition, static HUVEC cultures were exposed to 10 µmol/L 2MeSADP or 100 µmol/L UTP for 3 h. For shear stress experiments, HUVECs (300–700 cells/mm²) were cultured in the peripheral area of the *in vitro* fertilization (IVF) dishes (BD Biosciences).

Shear stress model

Uniform shear stress was achieved using the orbital shaker model and the IVF dish with HUVECs restricted to the peripheral area similar to previous studies [23, 24]. Cultures were synchronized for 24 h in 0.5 % FBS media and subsequently switched to 10 % FBS media prior to the shear stress experiments. A shear stress of 0.1 Pa (1 dyne/cm²) or 1 Pa (10 dynes/cm²) was calculated according to the following formula: $\tau_{\max} = \alpha \sqrt{\eta \rho (2\pi f)^3}$ wherein, $\tau_{\max}$ (Pascal or dynes/cm²) is the shear stress, a is the radius of rotation (9.5 mm), ρ is the density of the cell culture medium (1000 kg/m³), η is the viscosity of the cell culture medium (1.01 × 10⁻³ Pascal-second), and f is the frequency of rotation (1.93 Hz for 0.1 Pa and 3.55 Hz for 1 Pa of shear stress). HUVECs from the same passage cultured under static conditions were used as control. The pre-calculated maximal wall shear stress of 1 Pa applied to the cells is within the range of physiological arterial blood flow (~0.6–4 Pa).

In this study, we used the P2X4 receptor-specific antagonist, PSB-12253. The determination of its inhibitory activity at P2X receptor subtypes was performed in calcium influx assays as previously described [33]. Cells were exposed to either shear stress or to ATPγS (200 μmol/L) under static conditions in the presence or absence of PSB-12253 (1 μmol/L), which was added 60 min prior to the addition of the agonist and/or shear stress experiments. In addition, cells were subjected to shear stress in the presence or absence of apyrase (2 U/ml) for the indicated time points of 6, 24, or 48 h. In experiments with static cultures, cells were exposed to 3 μmol/L ivermectin, an allosteric modulator of the P2X4 receptor, for 20 min prior to the addition of ATPγS.

Flow cytometric analysis

A total of 1 × 10⁶ HUVECs were fixed with 2 % paraformaldehyde for 15 min at 4 °C, washed with cold PBS, and stained with anti-P2X4 antibody that recognizes an extracellular epitope (Alomone Labs, Israel) at 1:100 dilution for 1 h on ice. The cells were then incubated with Alexa 488-conjugated donkey anti-rabbit IgG (Life Technologies, USA) at 1:500 dilution for 1 h on ice. Isotype IgG (rabbit IgG; Cell Signaling Technology, USA) was used as a negative control, and for antibody specificity, the antibody was blocked with the corresponding control antigen for 1 h prior to addition to the cells. FACS was performed using Accuri C6 (BD Biosciences), and a total of 30,000 events were collected in the gated population that excluded debris and aggregates.

RNA isolation and reverse transcription-PCR analysis

Cells were harvested and total RNA was isolated using QIAzol either after 3 h (for the agonist-treated static cultures)

or after 6, 24, and 48 h for cultures subjected to shear stress. The total RNA was purified using the miRNeasy® mini kit, and first-strand cDNA was synthesized from 1 μg of RNA using a high capacity cDNA reverse transcription kit. Negative controls with no template and no reverse transcriptase enzyme were also included. Real-time PCR was performed using the ABI StepOnePlus instrument as per manufacturer's instructions. TaqMan® assays were used to measure mRNA expression of *P2X4*, *P2Y₂*, *KLF2*, *KLF4*, nuclear factor (erythroid-derived 2) like-2 (*NFE2L2*), and *NOS3*, which were normalized to the housekeeping genes *PPIA* and *18S*.

siRNA-mediated knockdown

The HUVECs were seeded to achieve a confluency of 70–80 % on the day of transfection. The siRNA at a final concentration of 10 nM/L was incubated with Lipofectamine® RNAiMAX reagent in OPTI-MEM at room temperature for 20 min to form complexes, and then added to the cells cultured in media containing 3 % FBS without antibiotics. At 24 h post-transfection, the culture media was replenished with fresh media (3 % FBS). The cells were either subjected to shear stress for 6 h or exposed to ATPγS for 3 h and harvested for RNA and protein at 48 and 72 h post-transfection, respectively.

Transient transfection

HUVECs and HAECs at 70–80 % confluency were used for transfections. The wild-type (WT) and Y315C mutant P2X4 constructs [20] (1 μg) were incubated with Transfex reagent (ATCC, USA) in OPTI-MEM at room temperature for 15 min to form complexes, and then added to the cells for 24 h before replacing with regular growth media without antibiotics. At 48 h post-transfection, the cells were exposed to ATPγS for 3 h and harvested for RNA analysis.

Immunoblot analysis

For immunoblotting, protein was extracted from cells using ice-cold Nonidet P-40 lysis buffer (50 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium-β-glycerophosphate, 50 mmol/L sodium fluoride, 5 mmol/L sodium pyrophosphate, 1 mmol/L dithiothreitol (DTT), 1 % (w/v) Nonidet P-40, and 270 mmol/L sucrose) with complete protease and phosphatase inhibitor (Roche, USA) mixture. Lysates were centrifuged at 14,000×g for 15 min at 4 °C, and the protein concentration was determined using Micro-BCA protein assay kit (Thermo Scientific, USA). The total cell lysates were heated at 95 °C for 5 min in lauryl dodecyl sulfate sample buffer. Total protein (10–15 μg) was resolved on precast NOVEX 4–12 % Bis-Tris gels (Bio-Rad Laboratories, USA)

and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in 50 mmol/L Tris–HCl (pH 7.6), 137 mmol/L NaCl, and 0.2 % (w/v) Tween 20 (TBS-T) containing either 5 % (w/v) nonfat dried milk or 3 % (w/v) protease-free BSA followed by overnight incubation at 4 °C with the following antibodies in TBS-T containing 5 % (w/v) protease-free BSA—anti-KLF2 (Millipore, USA), anti-P2X4 antibody that recognizes an intracellular epitope (Alomone Labs, Israel), anti-phospho-extracellular regulated-kinase (ERK5) (Thr218/Tyr 220), total ERK5, and anti- β -tubulin (all from Cell Signaling Technology, USA), anti-eNOS and anti-phospho-eNOS (S1177) (BD Biosciences, USA). To test the specificity of P2X4, the anti-P2X4 antibody was pre-adsorbed using the corresponding control antigen (Alomone Labs, Israel). The bands were visualized by enhanced chemiluminescence using horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, USA). Images were acquired with LI-COR Odyssey[®] Fc dual-mode imaging system, and the band intensities were quantified using the Image Studio[™] software. While the phosphorylated protein was normalized to the respective total protein levels, the rest were normalized to β -tubulin. Changes in protein levels are depicted as relative to the control condition.

Immunocytochemistry

HUVEC cultures after exposure to 3 h ATP γ S and 6 h shear stress were washed once with 1 $\times$ phosphate buffered saline (PBS) and fixed with ice-cold 4 % paraformaldehyde for 20 min at room temperature. They were then washed once and permeabilized with 0.25 % Triton-X 100 in PBS for 15 min at room temperature. Subsequent to washes, they were incubated in anti-KLF2 primary antibody (R&D Systems, USA) overnight at 4 °C in a humidified chamber. The following day, the cells were washed three times in PBS and incubated in the Northern Lights[™] anti-mouse-NL567 secondary antibody (R&D Systems, USA) for 2 h at room temperature in the dark. Finally, the cells were stained with ActinGreen[™] 488 and NucBlue[®] Ready Probes (Molecular Probes, USA) as per the manufacturer's protocol. The cells on coverslips were mounted with ProLong[®] gold antifade (Life Technologies, USA) and left to cure overnight. Images were visualized in an Olympus BX60 (Olympus, Tokyo, Japan) epifluorescence microscope and acquired using a Nikon DS-2Mv camera (Nikon, Tokyo, Japan).

Statistical analysis

All data are expressed as mean $\pm$ standard error (s.e.m.). Statistical analysis (Graphpad Prism software) was performed using unpaired Student's *t* test when comparing two situations, or one-way analysis of variance (ANOVA) with Bonferroni

correction for multiple comparisons with *p* values ≤ 0.05 regarded as statistically significant.

Results

Validation of the in vitro orbital shaker model of shear stress

Shear stress resulting from blood flow regulates vascular endothelial phenotype, homeostasis, and gene expression [23, 34, 35]. Vascular endothelial cells are known to express P2 receptors with P2X4 being the most abundantly expressed subtype [9, 16, 17, 36]. HUVECs have been extensively used as an in vitro model to study endothelial cell function and atheroprotection [37–40]. We first evaluated the protein expression of the P2X4 receptor in these cells using FACS and immunoblotting. FACS analysis of HUVECs stained with the anti-P2X4 antibody recognizing an extracellular epitope showed P2X4 protein expression (Fig. 1a) and the pre-adsorption with the corresponding control antigen showed the antibody specificity (Fig. 1b). Additionally, immunoblotting of HUVEC total cell lysates with a P2X4 antibody that recognizes an intracellular epitope confirmed this result (Fig. 1c). The human monocytic cell line, THP-1 known to express P2X4 [41–43] served as a positive control (Fig. 1c). Next, to test our hypothesis that shear stress and ATP regulates KLF2 via P2X4 receptors, we used the orbital shaker model of laminar shear stress, wherein the HUVECs were cultured in an IVF dish and subjected to a pre-calculated shear stress of 0.1 or 1 Pa. This model creates an orbital rotation of the culture medium over the HUVEC monolayer in the outer ring or peripheral area of the IVF dish, thus ensuring a uniform shear stress on the cells. Throughout the study, we refer to “no shear stress condition” as “static”. When HUVECs were subjected to a shear stress of 0.1 Pa for 48 h, we did not observe any changes in cell alignment (Fig. 2a) or in transcript levels of *P2X4* (Fig. 2b) or *KLF2* (Fig. 2c). However, similar to previous reports [23, 24], the HUVECs in the IVF dish subjected to shear stress of 1 Pa were elongated and aligned in the direction of flow as early as 3 h as opposed to the cobblestone morphology observed under static conditions (Fig. 2d).

Since we were interested in studying the atheroprotective effects, the shear stress of 1 Pa was used for the subsequent experiments. Time course experiments showed shear stress to decrease *P2X4* (Fig. 2e; 2.6 ± 0.08 -fold decrease; $p<0.0001$) and increase *KLF2* transcript levels (Fig. 2f; 14.1 ± 1.1 -fold, $p<0.0001$) as early as 6 h. We also observed shear stress to decrease P2X4 protein (Fig. 2g; 21 ± 1.8 %; $p=0.008$) and increase KLF2 protein at 6 h (Fig. 2h; 6.5 ± 0.8 -fold; $p=0.0006$) with the latter being stabilized at 24 h as evident by the nuclear staining (Fig. 2i). Furthermore, 6 h of shear stress increased *P2Y₂* (Supplementary Fig. 1A; 3.5 ± 0.4 -fold

Fig. 1 HUVECs express P2X4 receptors. Flow cytometric analysis of HUVECs using an anti-P2X4 antibody recognizing an extracellular epitope shows these cells to express P2X4 receptors (**a**; in red) and pre-adsorption of the antibody with the corresponding antigen shows antibody specificity (**b**; in blue). Unstained population (in black) and isotype control (in green) are also shown in both the histograms. A representative immunoblot (**c**) probed with an anti-P2X4 antibody recognizing an intracellular epitope shows P2X4 receptor at approximately 60 kDa. The lane assignments are as follows: lane 1 THP-1 cell lysate probed with P2X4 antibody pre-adsorbed with P2X4 peptide, lane 2 THP-1 cell lysate, lane 3 marker lane showing bands at 50 and 75 kDa, and lane 4 HUVEC cell lysate

increase; $p=0.002$), *KLF4* (Supplementary Fig. 1B; 58.3 ± 8.5 -fold; $p=0.003$), *THBD* (Supplementary Fig. 1C; 13 ± 1.5 -fold; $p=0.008$), *NOS3* (Supplementary Fig. 1D; 8.5 ± 1.4 -fold; $p<0.0001$), and *NFE2L2* (Supplementary Fig. 1E; 1.8 ± 0.5 -fold; $p=0.003$) transcript levels. Shear stress also resulted in increased phosphorylated eNOS (S1177) (Supplementary Fig. 1F). These findings are consistent with prior studies.

Extracellular ATP modulates KLF2 in HUVECs

It is well documented that shear stress is implicated in the release of ATP from endothelial cells [8, 9] that in turn exerts its effects through ligand-gated P2X and G protein-coupled P2Y receptors. To investigate whether the extracellular ATP modulates shear stress-induced KLF2 in the HUVECs, we used apyrase (2 U/ml), which is shown to hydrolyze extracellular ATP and inhibit flow-induced calcium responses in a P2X4-dependent manner [9]. Shear stress time course experiments, in the presence or absence of apyrase, showed a decrease of *KLF2* transcript levels (Fig. 3a) at 6 h (38.7 ± 13.5 %; $p=0.0003$), and 48 h (73.4 ± 2.4 %; $p=0.006$) with a trend towards a decrease at 24 h (51.9 ± 4.7 %; $p=0.08$). Similar to *KLF2*, *KLF4* (Fig. 3b; 42.5 ± 9.6 %; $p=0.022$) and *NOS3* (Fig. 3c; 83.7 ± 5.2 %; $p=0.0008$) were significantly reduced by apyrase at 48 h of shear stress. The effect of apyrase was not common to all shear stress-induced genes that we tested as it had no significant effect on *NFE2L2* although there was a trend towards a decrease at 48 h (Fig. 3d; 18.6 ± 4.2 %; $p=0.08$).

Studies have shown HUVECs to express several different P2 receptors [9, 16, 17, 44] with P2X4 in HUVECs [18] and P2Y₂ in different cell types [16, 45] being implicated in mechanotransduction. We, therefore, exposed the cells to the purinergic agonists (200 μ mol/L ATP γ S, 10 μ mol/L 2MeSADP, and 100 μ mol/L UTP) to examine their role in regulating KLF2. The non-hydrolyzable analog of ATP, ATP γ S, increased *KLF2* transcript levels at 3 h (Fig. 4a; 5.1 ± 0.4 -fold; $p<0.0001$). In order to validate this finding in an endothelial cell of arterial origin, we repeated this experiment in HAECs and observed a significant increase (1.6 ± 0.03 -fold; $p=0.004$; Supplementary Fig. 2) in ATP γ S-induced KLF2.

P2X4-specific antagonist and siRNA inhibit ATP γ S-induced KLF2 in HUVECs

We used a P2X4 receptor-specific antagonist (Supplementary Fig. 3), PSB-12253 (1 μ mol/L), in static HUVEC cultures, in the presence or absence of ATP γ S, and observed an inhibition of KLF2 (Fig. 4b; 62.3 ± 9.7 %; $p=0.017$ and Fig. 4c) expression. To confirm the role of P2X4 in the regulation of KLF2 expression, we performed siRNA knockdown of the receptor in the HUVECs. As a control, HUVECs were transfected with scrambled siRNA (negative control siRNA). *P2X4* mRNA was significantly decreased in both static cultures (86 ± 3 %; $p=0.002$; Fig. 5a) and under shear stress conditions (79 ± 2.5 %; $p<0.0001$; Fig. 5b) when transfected with P2X4-specific siRNA. Similarly, the P2X4 siRNA decreased protein expression in static cultures (62.3 ± 3.4 %; $p<0.0001$; Fig. 5c)

Fig. 2 Validation of the in vitro orbital shaker model of shear stress. Representative bright-field images ($\times 10$ objective) show morphology (**a**) of HUVECs exposed to either static (ST) (*left panel*) or cells cultured in the peripheral area of the IVF dish using an orbital shaker to shear stress (SS) conditions for 48 h (0.1 Pa or 1 dyne/cm²; *middle panel* and 1 Pa or 10 dynes/cm²; *right panel*). qRT-PCR analysis reveals altered *P2X4* (**b**) and *KLF2* (**c**) at 1 Pa but not 0.1 Pa shear stress. Time course of cells cultured under shear stress conditions (1 Pa) for 3, 6, 12, 24, and 48 h show cell alignment as early as 3 h (**d**) and qRT-PCR analysis show time-dependent decrease of *P2X4* (**e**) as well as an increase in *KLF2* (**f**) transcript levels normalized to the geometric mean of housekeeping

genes (HKG). Quantitation of Westerns along with representative immunoblots show shear stress to decrease *P2X4* (**g**) and increase *KLF2* (**h**) protein as normalized to the loading control (β -tubulin). Representative epifluorescent images ($\times 20$ objective) (**i**) of HUVECs subjected to 6 h (**a** and **b**) and 24 h (**c** and **d**) static and shear stress conditions (1 Pa) show an increase in *KLF2* (Northern LightsTM-567). Actin cytoskeleton and nucleus were stained with ActinGreenTM-488 and NucBlue[®], respectively. White arrows in **a**, **d**, and **i** indicate the direction of flow. $N=3-6$ experiments each in replicates; $*p \leq 0.05$. Scale bar, 20 μ M

as well as in cells subjected to 6 h of shear stress ($43.4 \pm 9\%$; $p=0.0007$; Fig. 5d). Furthermore, the knockdown of *P2X4* receptor in static cultures inhibited ATP γ S-induced *KLF2* expression (Fig. 6a; $34 \pm 8.7\%$; $p=0.03$ and Fig. 6b) thus confirming our observations with the receptor antagonist.

P2X4 receptor mediates shear stress-induced *KLF2*

We next examined if shear stress-induced *KLF2* is dependent on the *P2X4* receptor. Indeed, PSB-12253 reduced shear stress-induced *KLF2* (Fig. 7a; $31.1 \pm 6.1\%$; $p=0.002$) with *NOS3* mRNA showing a trend towards a decrease at 6 h (data not

shown). To confirm that this effect is dependent on *P2X4*, HUVECs were first transfected with *P2X4* receptor-specific siRNA and then subjected to shear stress for 6 h. Knocking down *P2X4* resulted in a decrease in shear stress-induced *KLF2* mRNA when compared to the negative control siRNA (Fig. 7b and c; $44 \pm 6\%$; $p < 0.0001$).

To further confirm the role of *P2X4* in shear stress-induced *KLF2*, the transfected HUVECs were additionally exposed to PSB-12253 or apyrase under shear stress conditions. Both PSB-12253 (Fig. 7b; $25.2 \pm 3.7\%$; $p=0.003$) and apyrase (Fig. 7c; $43.4 \pm 8.7\%$; $p=0.0002$) independently reduced *KLF2* mRNA in cells transfected with the negative control siRNA similar to that

Fig. 3 Apyrase decreases shear stress-induced *KLF2*, *NOS3*, and *KLF4*, but not *NFE2L2* expression. HUVECs were cultured for 6, 24, and 48 h (a) under static (no shear stress) or shear stress conditions (1 Pa) in the presence or absence of 2 U/ml of apyrase, an ATP hydrolyzing enzyme

with the line graph depicting fold change in *KLF2* mRNA (a). Fold change in *KLF4* (b), *NOS3* (c), and *NFE2L2* (d) mRNA at 48 h with all transcripts normalized to the geometric mean of the housekeeping genes (HKG). $N=3-4$ experiments in replicates; $*p\leq 0.05$

seen in non-transfected HUVECs. However, we did not observe any additive effect when the same experiment was repeated in the presence of P2X4 siRNA (PSB-12253; Fig. 7b; $24.1\pm 9.6\%$; $p=0.17$; apyrase: Fig. 7c; $30.8\pm 3.5\%$; $p=0.33$). In addition, transfection of HUVECs with P2X4 siRNA decreased shear stress-induced *KLF2* protein (Fig. 7d; $21\pm 4.7\%$; $p=0.007$). Furthermore, while the knock down of P2X4 resulted in a significant decrease in *KLF4* (Supplementary Fig. 4A; $43.5\pm 5.3\%$; $p=0.03$) and *THBD* mRNA (Supplementary Fig. 4B; $27.7\pm 5.8\%$; $p=0.004$), we only observed a trend towards a decrease in *NOS3* (Supplementary Fig. 4C; $18.9\pm 7.5\%$; $p=0.25$). The fact that ATP also activates P2Y₂ receptors, which is well in line with our observations that shear stress increases *P2Y₂* mRNA in the HUVECs (Supplementary Fig. 1A), prompted us to examine if P2Y₂ receptor mediates shear stress-induced *KLF2*. However, the P2Y₂-specific antagonist, AR-C118925 (10 $\mu\text{mol/L}$) [46, 47] (Supplementary Fig. 5A; $17.7\pm 18.1\%$; $p=0.56$) and the P2Y₂-specific siRNA (Supplementary Fig. 5B; $17.8\pm 7\%$; $p=0.06$) had no significant effect on shear stress-induced *KLF2*.

In a previous study, Kessler et al. demonstrated that shear stress mimics the effects of ivermectin, which is supported by other studies reporting ivermectin to allosterically induce sustained signaling resulting in prolonged and repetitive ATP stimulation [48–51]. We therefore tested if ivermectin modulates P2X4 receptor and thereby ATP-induced *KLF2* expression in static cultures. As earlier shown in Fig. 4, ATP γ S increased *KLF2* in static cultures as early as 3 h, but this effect was not evident at 6 h (Fig. 8a; $p=0.12$). However, static cultures exposed to ivermectin (3 $\mu\text{mol/L}$), and subsequent application of ATP γ S for 6 h, induced *KLF2* (Fig. 8a; 2 ± 0.13 -fold; $p=0.04$). Furthermore, this effect was not apparent in the presence of P2X4 siRNA thus confirming the role of P2X4 in ATP-induced *KLF2* expression (Fig. 8b).

Y315C polymorphism in human P2X4 receptor inhibits ATP γ S-induced *KLF2* and its targets

Having observed the effects of ATP and shear stress on P2X4-mediated atheroprotective gene expression, we further tested

Fig. 4 ATP γ S induces KLF2 in a P2X4-dependent manner.

HUVECs were cultured for 3 h under static conditions in the presence or absence of 200 μ mol/L ATP γ S, 100 μ mol/L UTP, and 10 μ mol/L 2MeSADP with the *bar graph* (a) depicting fold change in *KLF2* mRNA normalized to the geometric mean of the housekeeping genes (*HKG*). Static HUVEC cultures were exposed to 200 μ mol/L ATP γ S for 3 h in the presence or absence of 1 μ mol/L PSB-12253. qRT-PCR analysis (b) shows the effect of PSB-12253 on *KLF2* mRNA. Representative epifluorescent images ($\times 40$ objective) (c) of KLF2 immunostaining (Northern LightsTM-567), actin cytoskeleton (ActinGreenTM-488), and nucleus (NucBlue[®]) in cultures exposed to PSB-12253. Panels a–d represent KLF2 and (a'–d') merged images of KLF2, actin, and DAPI staining. $N=2-3$ experiments in replicates; $*p \leq 0.05$. Scale bar, 20 μ m

our hypothesis using a recently identified SNP, rs28360472, in the human P2X4 receptor. Transient transfections of static HUVEC cultures with WT and Y315C P2X4 constructs were performed. At 48 h post-transfection, in the presence of ATP γ S, the cells transfected with the Y315C construct showed an inhibition of *KLF2* (Fig. 9a; 52.5 ± 11.7 %; $p = 0.003$), *NOS3* (Fig. 9b; 40.6 ± 15.2 %; $p = 0.04$), *THBD* (Fig. 9c; 34 ± 12.5 %; $p = 0.024$), and *KLF4* (Fig. 9d; 66.7 ± 20.3 %; $p = 0.015$) expression when compared to cells transfected with the WT P2X4 construct. However, we did not observe a significant change in *NFE2L2* (Fig. 9e; 26.5 ± 18.2 %; $p = 0.62$) in the HUVECs expressing the Y315C mutant construct. Furthermore, we show transient transfection of the Y315C construct in HAECs to reduce ATP γ S-induced *KLF2* mRNA (Supplementary Fig. 6; 24.2 ± 2 %; $p = 0.003$).

P2X4 regulates phosphorylation of ERK5 in HUVECs

It is well known that both KLF2 and KLF4 are regulated by ERK5 that is activated by atheroprotective shear stress [30, 52–55], and hence, we investigated the role of P2X4 in shear stress-induced ERK5 phosphorylation. siRNA knockdown of P2X4 in HUVECs resulted in a significant reduction of shear stress-induced phosphorylation of ERK5 (Fig. 10; 43.5 ± 7.6 %; $p = 0.007$) suggesting that P2X4 plays an important

role in the activation of ERK5 and regulation of KLF2 and KLF4.

Discussion

Shear stress, an important regulator of endothelial function, is created by mechanical forces acting on the surface of the endothelial cell. These forces are transmitted through mechanotransductive complexes that subsequently transduce the force-generated signals into the cellular signaling network where they will give rise to a flow-specific gene expression pattern. Earlier observations, from our group and others, have shown the P2X4 receptor to be highly expressed in the endothelial cells [16, 17, 36]. It has been previously reported that vascular endothelial cells exposed to laminar shear stress release ATP that through activation of P2X4 receptors specifically mediate flow-induced calcium influx and control of vascular tone [9, 18]. The objective of this study was to test the hypothesis that endothelial P2X4 is involved in shear stress regulation of KLF2.

We first confirmed expression of P2X4 receptors and validated our in vitro orbital shaker model of laminar shear stress, wherein we observed a decrease in *P2X4* and an increase in *P2Y₂*, *KLF2*, *KLF4*, *NFE2L2*, *NOS3*, and *THBD*. We then

Fig. 5 Validation of siRNA knockdown of P2X4 receptors. Validation of the knockdown of P2X4 48 h post-transfection in static (a and b) and shear stress conditions (c and d) by qRT-PCR (a and c) and Western (b and d) with representative immunoblots for P2X4 (60 kDa) and the loading control, β -tubulin (50 kDa). $N=4-6$ experiments each in replicates; $*p\leq 0.05$; *HKG* housekeeping genes

tested the involvement of purinergic mechanisms in this event and found that apyrase inhibited shear stress-induced *KLF2*, *KLF4*, and *NOS3* transcript levels. Exposure of static cultures to ATP γ S increased *KLF2* in a clearly P2X4-dependent manner as demonstrated by intervention with the P2X4 antagonist PSB-12253 and with siRNA. Furthermore, both PSB-12253 and P2X4 knockdown partially inhibited the shear stress-induced *KLF2*. Transfection of HUVECs and HAECs with the previously described P2X4 SNP (Tyr315>Cys) resulted in the inhibition of ATP-mediated *KLF2* expression. Finally, P2X4 decreased the phosphorylation of ERK5 under shear stress conditions suggesting a role for P2X4 in the well-established ERK5 pathway for the regulation of shear stress-induced *KLF2*. Our findings thus suggest that ATP contributes to the shear stress-induced *KLF2* expression, a key modulator of atheroprotective phenotype in endothelial cells, in part through the P2X4 receptor.

Although the shear stress observed in vivo sometimes is higher compared to the in vitro orbital shaker model, the shear stress of 1 Pa used in this study is within the range of physiological arterial blood flow. In fact, consistent with previous studies [23, 56], our findings demonstrate (i) shear stress to

decrease P2X4 mRNA by approximately 60 %, (ii) a time-dependent cell alignment in the direction of flow, and (iii) an upregulation of *KLF2*. The initial results showed that within 6 h, the level of P2X4 transcript was decreased and the *KLF2* mRNA increased. While the increase in *KLF2* protein levels reflected the increase of *KLF2* transcript, the decrease in P2X4 protein was only 20 % with the remaining functional pool of receptors mediating the ATP-induced effects. The shear stress-mediated down regulation of P2X4 was first described by Korenaga et al. who mapped the stress sensitive part of the P2X4 promoter to be close to the translational start [56]. While Korenaga suggested Sp1 as the major negative regulator, Gu et al. mapped the transcription start to just beside the Sp1 consensus motif and also indicated a GATA-2 site to be the major positive regulator of P2X4 transcription [57]. It is in this context interesting to note that GATA-2 has been reported to directly repress the *KLF2* gene [58]. This indicates the possible existence of a stress-mediated mutual, but bifunctional, control of *KLF2* and P2X4 expression. Another possibility is that interaction of *KLF2* with the Sp1 consensus site represses P2X4 transcription. This mechanism is supported by the fact that Sp1 and *KLF2* belong to the Sp1/*KLF2* family of

Fig. 6 siRNA knockdown of P2X4 inhibits ATP γ S-induced KLF2. Static HUVEC cultures transfected with negative control and P2X4 siRNA for 48–72 h were exposed to 200 μ mol/L ATP γ S for 3 h. qRT-PCR analysis (a) shows the effect of siRNA knockdown (48 h) of P2X4 on *KLF2* mRNA normalized to the geometric mean of the housekeeping genes (*HKG*). Representative epifluorescent images ($\times 40$ objective) (b) of KLF2 immunostaining (Northern LightsTM-567), actin cytoskeleton (ActinGreenTM-488), and nucleus (NucBlue[®]) in cultures with siRNA knockdown (72 h) of P2X4. Panels (a–d) represent KLF2 and (a'–d') merged images of KLF2, actin, and DAPI staining. $N=3$ experiments in replicates; $*p\leq 0.05$. Scale bar, 20 μ M

transcription factors and thus share homology and possibly binding motifs.

ATP release, in response to mechanical stimulation by shear stress, promotes P2 receptor-mediated calcium influx and vasodilation in endothelial cells [8, 10, 11]. In this context, we show a significant reduction of shear stress-induced *KLF2*, *KLF4*, and *NOS3* transcript levels in the presence of apyrase, an enzyme that rapidly hydrolyzes extracellular ATP, thus suggesting the importance of ATP in regulating these genes under shear stress conditions. While it is interesting to note that extracellular ATP regulated some of the atheroprotective genes, we did not observe a significant effect of apyrase on shear stress-stimulated *NFE2L2* transcription. Since the signaling pathway responsible for shear stress-mediated regulation of *NFE2L2* is similar to the one of *KLF2* [59], the most likely explanation is that the level of *NFE2L2* upregulation in this system is too small to allow statistical significance.

To further delineate the role of extracellular ATP and P2X4 in regulating shear stress-induced KLF2, we added ATP γ S to static cultures that increased *KLF2* mRNA at 3 h, which was reversed in the presence of the P2X4 receptor antagonist or after P2X4 knockdown. Furthermore, neither UTP nor 2MeSADP had any effect indicating that P2Y₂ and P2Y₁ are unlikely to participate in this process. This points to the

contribution of ATP to downstream signaling events via the P2X4 receptor. In parallel, we observed shear stress-induced *KLF2* transcripts to be reduced both with the antagonist and with siRNA specific for P2X4, which conforms to our observations using apyrase and demonstrates that shear stress-induced KLF2 is regulated in part by extracellular ATP activating P2X4 receptors. As the combined treatment with siRNA and antagonist was unable to further reduce KLF2 expression, we conclude that the remaining fraction is due to P2X4 independent mechanisms. One such mechanism suggested by Huddleson et al. is through shear stress-mediated chromatin remodeling leading to recruitment of the transcriptional coactivator p300/CBP (PCAF) [60]. This study in HUVECs has shown a direct link between flow-induced phosphoinositide-3-kinase activation, histone acetylation, KLF2, and eNOS transcription. Another possible mechanism is regulation of KLF2 by shear stress-induced microRNAs [61, 62]. Interestingly, we observed an effect similar to that of KLF2 in *KLF4* expression with the P2X4 antagonist and siRNA suggesting that this gene is also regulated by P2X4. The fact that neither the P2Y₂ antagonist nor the siRNA inhibited shear stress-induced KLF2 expression, in spite of P2Y₂ being upregulated, further confirms the importance of P2X4 in this atheroprotective mechanism. The transient increase in *KLF2* mRNA at 3 h in response to acute high levels

Fig. 7 Selective antagonist and siRNA knockdown of P2X4 receptors reduce shear stress-induced KLF2. HUVECs were cultured for 6 h under static or shear stress conditions (1 Pa) in the presence or absence of the P2X4 receptor antagonist, PSB-12253 (1 μ mol/L) (**a** and **b**) or transiently transfected with siRNA (**b–d**). Graphs depict fold change in transcript levels of *KLF2* (**a**) in response to PSB-12253. Knocking down of P2X4 results in decreased levels of shear stress-induced *KLF2* mRNA (**b** and **c**) with no additive effect of the antagonist (**b**) or apyrase (**c**) after siRNA knockdown. Transcript levels were normalized to the geometric mean of the housekeeping genes (*HKG*). In addition, P2X4 siRNA inhibits KLF2 protein (**d**) with a representative immunoblot showing levels of KLF2 and the loading control β -tubulin after siRNA knockdown of P2X4. Fold changes are relative to cells transfected with the negative control siRNA. $N=3$ –6 experiments in replicates; $*p\leq 0.05$

of the non-hydrolyzable exogenous ATP is suggestive of agonist-induced receptor desensitization, leading to alterations in calcium-dependent intracellular signaling. Reports show ivermectin to allosterically modulate P2X4 receptors and

Fig. 8 Ivermectin increases ATP γ S-induced KLF2 at 6 h. Static HUVEC cultures were exposed to 200 μ mol/L of ATP γ S in the presence or absence of 3 μ mol/L ivermectin for 6 h. qRT-PCR analysis shows a significant increase in ATP γ S-induced *KLF2* transcript levels only in the presence of ivermectin (**a**) with no synergistic effect after siRNA knockdown (**b**) of P2X4. $N=3$ experiments in replicates; $*p\leq 0.05$; *HKG* housekeeping genes

increase both the potency as well as the peak amplitude of ATP generated currents [49–51]. Furthermore, Kessler et al. reported that laminar shear stress modulates P2X4 receptor in a way similar to the effect caused by ivermectin. The authors interpret these observations to be due to stabilization of the open state of P2X4 channel by reducing the desensitization and slowing the receptor deactivation. Interestingly, this is in line with our study wherein ATP γ S increased *KLF2* mRNA at 6 h only in the presence of ivermectin and that this effect was not evident after treatment with P2X4 siRNA. These observations further strengthen the idea that ATP-induced KLF2 expression is dependent on P2X4. We therefore speculate that shear stress modulates P2X4 receptor activity in response to extracellular ATP and that this plays a role in sensing mechanical forces.

Endothelial dysfunction is characterized by decreased arterial compliance and increased shear stress [63] resulting in hypertension and elevated cardiovascular risk. Endothelial NOS, one of the primary mediators of vascular tone, is regulated by blood flow and by mechanosensitive channels including the P2X4 receptors. This is well demonstrated in the P2X4 $^{-/-}$ mice, which exhibit increased blood pressure and

Fig. 9 Y315C mutant P2X4 inhibits ATP γ S-induced gene expression. Static HUVEC cultures transiently transfected for 48 h with WT and Y315C mutant P2X4 constructs were exposed to 200 μ mol/L ATP γ S for

3 h. qRT-PCR analysis shows the effect of the SNP on *KLF2* (a), *NOS3* (b), *THBD* (c), *KLF4* (d), and *NFE2L2* (e) mRNA. $N=2$ experiments in replicates; $*p\leq 0.05$

decreased nitric oxide production. Notably, four genetic polymorphisms in the P2X4 receptor gene were recently identified in a large cohort where one of the polymorphisms, the Y315C

variant (rs28360472), was found only in the heterozygous dosage. This variant displayed distinct effects on ATP-induced P2X4 receptor response [20]. The report shows that the ATP-induced currents were reduced by 40 % in macrophages from heterozygous subjects, which was confirmed in HEK-293 cotransfection experiments that mimicked the heterozygous state. Furthermore, Stokes et al. show that the inheritance of the Y315C variant correlated with increased pulse pressure suggestive of reduced large arterial compliance and dysfunctional vasodilation. In our study, HUVECs transfected with the Y315C mutant P2X4 construct inhibited ATP-induced *KLF2* and *KLF4* as well as the target genes that regulate vascular tone (*NOS3*) and thrombosis (*THBD*). Similarly, aortic endothelial cells (HAECs) transfected with the Y315C mutant also inhibited ATP-induced *KLF2*. Taken together, these results indicate the importance of P2X4 receptors not only in regulating shear stress-mediated mechanotransduction but also in atheroprotective gene expression.

Fig. 10 Shear stress induced ERK5 phosphorylation in a P2X4-dependent receptor. HUVECs transiently transfected for 48 h with P2X4 siRNA were cultured for 6 h under static or shear stress (1 Pa) conditions. Graph depict fold difference in shear stress-induced phosphorylated ERK5 (Thr218/Tyr220) normalized to total ERK5, which is dependent on P2X4 with a representative immunoblot showing levels of phosphorylated ERK5 and total ERK5. Fold difference is relative to cells transfected with the negative control siRNA. $N=6$ experiments in replicates; $*p\leq 0.05$

Using our in vitro model of laminar shear stress, we show that P2X4 in part regulates ERK5. These results are well in line with previous reports that demonstrate the stress-induced regulation of *KLF2* and *KLF4* to be primarily channeled through the MEKK2/3/MEK5/ERK5/MEF2 pathway [30, 52–55, 59]. Activation of MEKK2/3 is, besides shear stress, dependent on calcium influx [64], which is one of the primary

intracellular responses to P2X4 activation, and hence, it is probably the most likely entry point for P2X4 signaling. In conclusion, we provide, to our knowledge, the first evidence for ATP-regulated expression of both KLF2 and KLF4 via P2X4 receptors. Our findings contribute to the knowledge of extracellular nucleotides and P2 receptors in mediating mechanosensitive signaling in endothelial cells that govern vascular homeostasis. For instance, it is well established that shear stress is primary to exercise-induced vascular adaptation and is critical not only in ameliorating endothelial dysfunction and cardiovascular disease but also in enhancing arteriogenesis [65–67]. Taken together, our results indicate a role for P2X4 in mediating ATP- and shear stress-induced atheroprotective gene expression thereby regulating endothelial function.

Acknowledgments We thank Siv Svensson, Hanna Gustafsson, and Joanna Daszkiewicz-Nilsson for their technical assistance and Dr. Aliaa Abdelrahman for performing calcium assays for PSB-12253 at P2X receptor subtypes. The Swedish Heart and Lung Foundation, Swedish Scientific Research Council, ALF, and Skåne University Hospital funds supported this work.

Conflict of interest The authors declare that they have no conflict of interest.

References

- Gimbrone MA Jr, Garcia-Cardena G (2013) Vascular endothelium, hemodynamics, and the pathobiology of atherosclerosis. *Cardiovasc Pathol* 22:9–15
- Davies PF (2009) Hemodynamic shear stress and the endothelium in cardiovascular pathophysiology. *Nature clinical practice. Cardiovasc Med* 6:16–26
- Gimbrone MA Jr, Topper JN, Nagel T, Anderson KR, Garcia-Cardena G (2000) Endothelial dysfunction, hemodynamic forces, and atherogenesis. *Ann NY Acad Sci* 902:230–239, discussion 239–240
- Lin Z, Natesan V, Shi H, Dong F, Kawanami D, Mahabeleshwar GH, Atkins GB, Nayak L, Cui Y, Finigan JH, Jain MK (2010) Kruppel-like factor 2 regulates endothelial barrier function. *Arterioscler Thromb Vasc Biol* 30:1952–1959
- Takada Y, Shinkai F, Kondo S, Yamamoto S, Tsuboi H, Korenaga R, Ando J (1994) Fluid shear stress increases the expression of thrombomodulin by cultured human endothelial cells. *Biochem Biophys Res Commun* 205:1345–1352
- Burnstock G (1999) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. *J Anat* 194(Pt 3):335–342
- Corson MA, James NL, Latta SE, Nerem RM, Berk BC, Harrison DG (1996) Phosphorylation of endothelial nitric oxide synthase in response to fluid shear stress. *Circ Res* 79:984–991
- Bodin P, Bailey D, Burnstock G (1991) Increased flow-induced ATP release from isolated vascular endothelial cells but not smooth muscle cells. *Br J Pharmacol* 103:1203–1205
- Yamamoto K, Sokabe T, Ohura N, Nakatsuka H, Kamiya A, Ando J (2003) Endogenously released ATP mediates shear stress-induced Ca²⁺ influx into pulmonary artery endothelial cells. *Am J Physiol Heart Circ Physiol* 285:H793–803
- Milner P, Bodin P, Loesch A, Burnstock G (1990) Rapid release of endothelin and ATP from isolated aortic endothelial cells exposed to increased flow. *Biochem Biophys Res Commun* 170:649–656
- Milner P, Kirkpatrick KA, Ralevic V, Toothill V, Pearson J, Burnstock G (1990) Endothelial cells cultured from human umbilical vein release ATP, substance P and acetylcholine in response to increased flow. *Proc R Soc B* 241:245–248
- Yamamoto K, Furuya K, Nakamura M, Kobatake E, Sokabe M, Ando J (2011) Visualization of flow-induced ATP release and triggering of Ca²⁺ waves at caveolae in vascular endothelial cells. *J Cell Sci* 124:3477–3483
- Corriden R, and Insel PA (2010) Basal release of ATP: an autocrine-paracrine mechanism for cell regulation. *Science Signal* 3, re1
- Burnstock G (2007) Purine and pyrimidine receptors. *Cell Mol Life Sci* 64:1471–1483
- Burnstock G, Kennedy C (1985) Is there a basis for distinguishing two types of P2-purinoceptor? *Gen Pharmacol* 16:433–440
- Wang L, Karlsson L, Moses S, Hultgardh-Nilsson A, Andersson M, Borna C, Gudbjartsson T, Jern S, Erlinge D (2002) P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. *J Cardiovasc Pharmacol* 40:841–853
- Yamamoto K, Korenaga R, Kamiya A, Ando J (2000) Fluid shear stress activates Ca(2+) influx into human endothelial cells via P2X4 purinoceptors. *Circ Res* 87:385–391
- Yamamoto K, Sokabe T, Matsumoto T, Yoshimura K, Shibata M, Ohura N, Fukuda T, Sato T, Sekine K, Kato S, Isshiki M, Fujita T, Kobayashi M, Kawamura K, Masuda H, Kamiya A, Ando J (2006) Impaired flow-dependent control of vascular tone and remodeling in P2X4-deficient mice. *Nat Med* 12:133–137
- Sonin D, Zhou SY, Cronin C, Sonina T, Wu J, Jacobson KA, Pappano A, Liang BT (2008) Role of P2X purinergic receptors in the rescue of ischemic heart failure. *Am J Physiol Heart Circ Physiol* 295:H1191–H1197
- Stokes L, Scurrah K, Ellis JA, Cromer BA, Skarratt KK, Gu BJ, Harrap SB, Wiley JS (2011) A loss-of-function polymorphism in the human P2X4 receptor is associated with increased pulse pressure. *Hypertension* 58:1086–1092
- Chiu JJ, Chien S (2011) Effects of disturbed flow on vascular endothelium: pathophysiological basis and clinical perspectives. *Physiol Rev* 91:327–387
- Topper JN, Gimbrone MA Jr (1999) Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype. *Mol Med Today* 5:40–46
- Dardik A, Chen L, Frattini J, Asada H, Aziz F, Kudo FA, Sumpio BE (2005) Differential effects of orbital and laminar shear stress on endothelial cells. *J Vasc Surg* 41:869–880
- dela Paz NG, Walshe TE, Leach LL, Saint-Geniez M, D'Amore PA (2011) Role of shear-stress-induced VEGF expression in endothelial cell survival. *J Cell Sci* 125:1–13
- Atkins GB, Jain MK (2007) Role of Kruppel-like transcription factors in endothelial biology. *Circ Res* 100:1686–1695
- Dekker RJ, van Thienen JV, Rohlena J, de Jager SC, Elderkamp YW, Seppen J, de Vries CJ, Biessen EA, van Berkel TJ, Pannekoek H, Horrevoets AJ (2005) Endothelial KLF2 links local arterial shear stress levels to the expression of vascular tone-regulating genes. *Am J Pathol* 167:609–618
- Hamik A, Lin Z, Kumar A, Balcells M, Sinha S, Katz J, Feinberg MW, Gerzsten RE, Edelman ER, Jain MK (2007) Kruppel-like factor 4 regulates endothelial inflammation. *J Biol Chem* 282:13769–13779
- Jain MK, Sangwung P, Hamik A (2014) Regulation of an inflammatory disease: Kruppel-like factors and atherosclerosis. *Arterioscler Thromb Vasc Biol* 34:499–508
- Lee JS, Yu Q, Shin JT, Sebzda E, Bertozzi C, Chen M, Mericko P, Stadtfeld M, Zhou D, Cheng L, Graf T, MacRae CA, Lepore JJ, Lo CW, Kahn ML (2006) Klf2 is an essential regulator of vascular hemodynamic forces in vivo. *Dev Cell* 11:845–857

30. Villarreal G Jr, Zhang Y, Larman HB, Gracia-Sancho J, Koo A, Garcia-Cardena G (2010) Defining the regulation of KLF4 expression and its downstream transcriptional targets in vascular endothelial cells. *Biochem Biophys Res Commun* 391:984–989
31. Zhou G, Hamik A, Nayak L, Tian H, Shi H, Lu Y, Sharma N, Liao X, Hale A, Boerboom L, Feaver RE, Gao H, Desai A, Schmaier A, Gerson SL, Wang Y, Atkins GB, Blackman BR, Simon DI, Jain MK (2012) Endothelial Kruppel-like factor 4 protects against atherothrombosis in mice. *J Clin Invest* 122:4727–4731
32. Wani MA, Means RT Jr, Lingrel JB (1998) Loss of LKLF function results in embryonic lethality in mice. *Transgenic Res* 7:229–238
33. Hernandez-Olmos V, Abdelrahman A, El-Tayeb A, Freudendahl D, Weinhausen S, Muller CE (2012) N-substituted phenoxazine and acridone derivatives: structure-activity relationships of potent P2X4 receptor antagonists. *J Med Chem* 55:9576–9588
34. Minami T, Aird WC (2005) Endothelial cell gene regulation. *Trends Cardiovasc Med* 15:174–184
35. Dai G, Kaazempur-Mofrad MR, Natarajan S, Zhang Y, Vaughn S, Blackman BR, Kamm RD, Garcia-Cardena G, Gimbrone MA Jr (2004) Distinct endothelial phenotypes evoked by arterial waveforms derived from atherosclerosis-susceptible and -resistant regions of human vasculature. *Proc Natl Acad Sci U S A* 101:14871–14876
36. Ray FR, Huang W, Slater M, Barden JA (2002) Purinergic receptor distribution in endothelial cells in blood vessels: a basis for selection of coronary artery grafts. *Atherosclerosis* 162:55–61
37. Boon RA, Fledderus JO, Volger OL, van Wanrooij EJ, Pardali E, Weesie F, Kuiper J, Pannekoek H, ten Dijke P, Horrevoets AJ (2007) KLF2 suppresses TGF-beta signaling in endothelium through induction of Smad7 and inhibition of AP-1. *Arterioscler Thromb Vasc Biol* 27:532–539
38. Parmar KM, Nambudiri V, Dai G, Larman HB, Gimbrone MA Jr, Garcia-Cardena G (2005) Statins exert endothelial atheroprotective effects via the KLF2 transcription factor. *J Biol Chem* 280:26714–26719
39. Walshe TE, dela Paz NG, D'Amore PA (2013) The role of shear-induced transforming growth factor-beta signaling in the endothelium. *Arterioscler Thromb Vasc Biol* 33:2608–2617
40. Wang W, Ha CH, Jhun BS, Wong C, Jain MK, Jin ZG (2010) Fluid shear stress stimulates phosphorylation-dependent nuclear export of HDAC5 and mediates expression of KLF2 and eNOS. *Blood* 115:2971–2979
41. Li J, Fountain SJ (2012) Fluvastatin suppresses native and recombinant human P2X4 receptor function. *Purinergic Signal* 8:311–316
42. Stokes L, Surprenant A (2009) Dynamic regulation of the P2X4 receptor in alveolar macrophages by phagocytosis and classical activation. *Eur J Immunol* 39:986–995
43. Qureshi OS, Paramasivam A, Yu JC, Murrell-Lagnado RD (2007) Regulation of P2X4 receptors by lysosomal targeting, glycan protection and exocytosis. *J Cell Sci* 120:3838–3849
44. Burnstock G, Knight GE (2004) Cellular distribution and functions of P2 receptor subtypes in different systems. *Internat Rev Cytol* 240:31–304
45. Gardinier J, Yang W, Madden GR, Kronbergs A, Gangadharan V, Adams E, Czymbek K, Duncan RL (2014) P2Y2 receptors regulate osteoblast mechanosensitivity during fluid flow. *Am J Physiol Cell Physiol* 306:C1058–67
46. Cosentino S, Banfi C, Burbiel JC, Luo H, Tremoli E, Abbracchio MP (2012) Cardiomyocyte death induced by ischaemic/hypoxic stress is differentially affected by distinct purinergic P2 receptors. *J Cell Mol Med* 16:1074–1084
47. Hochhauser E, Cohen R, Waldman M, Maksin A, Isak A, Aravot D, Jayasekara PS, Muller CE, Jacobson KA, Shainberg A (2013) P2Y2 receptor agonist with enhanced stability protects the heart from ischemic damage in vitro and in vivo. *Purinergic Signal* 9:633–642
48. Kessler S, Clauss WG, Fronius M (2011) Laminar shear stress modulates the activity of heterologously expressed P2X(4) receptors. *Biochim et Biophys Acta* 1808:2488–2495
49. Khakh BS, Proctor WR, Dunwiddie TV, Labarca C, Lester HA (1999) Allosteric control of gating and kinetics at P2X(4) receptor channels. *J Neurosci* 19:7289–7299
50. Toulme E, Soto F, Garret M, Boue-Grabot E (2006) Functional properties of internalization-deficient P2X4 receptors reveal a novel mechanism of ligand-gated channel facilitation by ivermectin. *Mol Pharmacol* 69:576–587
51. Zemkova H, Tvrdonova V, Bhattacharya A, Jindrichova M (2014) Allosteric modulation of ligand gated ion channels by ivermectin. *Physiol Res* 63(1):S215–224
52. Parmar KM, Larman HB, Dai G, Zhang Y, Wang ET, Moorthy SN, Kratz JR, Lin Z, Jain MK, Gimbrone MA Jr, Garcia-Cardena G (2006) Integration of flow-dependent endothelial phenotypes by Kruppel-like factor 2. *J Clin Invest* 116:49–58
53. Clark PR, Jensen TJ, Kluger MS, Morelock M, Hanidu A, Qi Z, Tatake RJ, Pober JS (2011) MEK5 is activated by shear stress, activates ERK5 and induces KLF4 to modulate TNF responses in human dermal microvascular endothelial cells. *Microcirculation* 18:102–117
54. Ohnesorge N, Viemann D, Schmidt N, Czymai T, Spiering D, Schmolke M, Ludwig S, Roth J, Goebeler M, Schmidt M (2010) Erk5 activation elicits a vasoprotective endothelial phenotype via induction of Kruppel-like factor 4 (KLF4). *J Biol Chem* 285:26199–26210
55. Yan C, Takahashi M, Okuda M, Lee JD, Berk BC (1999) Fluid shear stress stimulates big mitogen-activated protein kinase 1 (BMK1) activity in endothelial cells. Dependence on tyrosine kinases and intracellular calcium. *J Biol Chem* 274:143–150
56. Korenaga R, Yamamoto K, Ohura N, Sokabe T, Kamiya A, Ando J (2001) Sp1-mediated downregulation of P2X4 receptor gene transcription in endothelial cells exposed to shear stress. *Am J Physiol Heart Circ Physiol* 280:H2214–2221
57. Gu BJ, Sun C, Valova VA, Skarratt KK, Wiley JS (2010) Identification of the promoter region of the P2RX4 gene. *Mol Biol Rep* 37:3369–3376
58. Linnemann AK, O'Geen H, Keles S, Farnham PJ, Bresnick EH (2011) Genetic framework for GATA factor function in vascular biology. *Proc Natl Acad Sci U S A* 108:13641–13646
59. Nithianandarajah-Jones GN, Wilm B, Goldring CE, Muller J, Cross MJ (2014) The role of ERK5 in endothelial cell function. *Biochem Soc Transac* 42:1584–1589
60. Huddleson JP, Ahmad N, Lingrel JB (2006) Up-regulation of the KLF2 transcription factor by fluid shear stress requires nucleolin. *J Biol Chem* 281:15121–15128
61. Fang Y, Davies PF (2012) Site-specific microRNA-92a regulation of Kruppel-like factors 4 and 2 in atherosusceptible endothelium. *Arterioscler Thromb Vasc Biol* 32:979–987
62. Wu W, Xiao H, Laguna-Fernandez A, Villarreal G Jr, Wang KC, Geary GG, Zhang Y, Wang WC, Huang HD, Zhou J, Li YS, Chien S, Garcia-Cardena G, Shyy JY (2011) Flow-dependent regulation of Kruppel-like factor 2 is mediated by MicroRNA-92a. *Circulation* 124:633–641
63. Takumi T, Yang EH, Mathew V, Rihal CS, Gulati R, Lerman LO, Lerman A (2010) Coronary endothelial dysfunction is associated with a reduction in coronary artery compliance and an increase in wall shear stress. *Heart* 96:773–778
64. Yao Z, Yoon S, Kalie E, Raviv Z, Seger R (2010) Calcium regulation of EGF-induced ERK5 activation: role of Lad1-MEKK2 interaction. *PLoS One* 5:e12627
65. Gielen S, Schuler G, Hambrecht R (2001) Exercise training in coronary artery disease and coronary vasomotion. *Circulation* 103:E1–6

66. Tinken TM, Thijssen DH, Hopkins N, Dawson EA, Cable NT, Green DJ (2010) Shear stress mediates endothelial adaptations to exercise training in humans. *Hypertension* 55:312–318
67. Zhang J, Friedman MH (2013) Adaptive response of vascular endothelial cells to an acute increase in shear stress frequency. *Am J Physiol Heart Circ Physiol* 305:H894–902