Pulsatile Atheroprone Shear Stress Affects the Expression of Transient Receptor Potential Channels in Human Endothelial Cells

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Abstract—The goal of the study was to assess whether pulsatile atheroprone shear stress modulates the expression of transient receptor potential (TRP) channels, TRPC3, TRPC6, TRPM7, and TRPV1 mRNA, in human umbilical vascular endothelial cells. Exposure of cultured vascular endothelial cells to defined shear stress, producing a constant laminar flow (generating a shear stress of 6 dyne/cm²), laminar pulsatile atheroprotective flow (with a mean shear stress of 20 dyne/cm²), or laminar atheroprone bidirectional flow (with a mean shear stress of 0 dyne/cm²) differentially induced TRPC6 and TRPV1 mRNA as measured by quantitative real-time RT-PCR and normalized to GAPDH expression. Thereby, TRPC6 and TRPV1 mRNA expressions were significantly increased after 24 hours of exposure to an atheroprone flow profile compared with an atheroprotective flow profile. Furthermore, the expression of transcription factors GATA1 and GATA4 was significantly correlated with the expression of TRPC6 mRNA. In contrast, after 24 hours of constant laminar flow, the expression of TRPC6 and TRPV1 mRNA was unchanged, whereas the expression of TRPC3 and TRPM7 was significantly higher in endothelial cells exposed to shear stress in comparison with endothelial cells grown under static conditions. There was a significant association between the expression of TRPC6 and tumor necrosis factor-α mRNA in human vascular tissue. No-flow and atheroprone flow conditions are equally characterized by an increase in the expression of tumor necrosis factor-α; however, inflammation-associated endothelial cell reactions may be further aggravated at atheroprone flow conditions by the increase of TRPV1 and TRPC6, as observed in our study. (Hypertension. 2012;59:1232-1240.)

Key Words: TRP channels ■ mRNA expression ■ shear stress ■ endothelial cells

Atherosclerosis correlates with regional hemodynamics. Atherosclerotic lesions are more likely to occur at specific sites, for example, bifurcations, in the arterial system. These sites are characterized by altered shear stress, specifically by atherogenic flow patterns composed of low flow, flow separation, relatively steep shear stress gradients, reversal of flow, and turbulent flow.1 Shear stress, defined as the tensile force tangentially acting on endothelial cells, is elicited by the flowing blood.2 It has been shown that the force and direction of shear stress are essential for atherosclerotic lesions to occur and are, thus, linked to cardiovascular disease and susceptibility for atherosclerosis in particular. So, shear stress of low magnitude and reversing direction changes the endothelial cell’s phenotype toward a chronic inflammatory reaction in the preatherosclerotic endothelium.3 Accordingly, atherosclerosis-susceptible and atherosclerosis-resistant regions of the human carotid arteries are characterized by different pulsatile profiles of wall shear stress. These different profiles are represented by an atheroprone prototypic arterial waveform versus an atheroprotective prototypic arterial waveform, which can be replicated in vitro.4,5

An early reaction of endothelial cells toward shear stress is the activation of flow-sensitive ion channels, proposed to act as flow sensors.6 Moreover, several subtypes of transient receptor potential (TRP) channels, including transient receptor potential cation channel, subfamily C, member 3 (TRPC3) TRPC6, TRPM7, and TRPV1, have been linked to cardiovascular disease, hypertension, and atherosclerosis.7–13 TRP channels are activated by a variety of factors, including chemical stimuli and mechanical stimuli, like shear stress. TRP channels are known to be mechanosensitive, although the specific mechanisms still need to be elucidated.7 In addition, alterations in blood flow are well known to have an
effect on the expression of many genes, which harbor promoter regions responsive to shear stress.1 With respect to TRPCs, different flow types could thus, change endothelial TRPC expression, which, in turn, might change the responsiveness of endothelial cells to flow. In the present study we, therefore, tested the hypothesis that shear stress produced from pulsatile atheroprone, on one hand, or pulsatile atheroprotective, on the other, differentially affects the expression of TRP channel transcripts in human endothelial cells. We, thus, investigated changes of TRPC3, TRPC6, TRPM7, subfamily M member 7 (TRPM7), subfamily V member 1 (TRPV1), tumor necrosis factor-α (TNF-α), GATA binding protein 1 (gobin transcription factor 1; GATA1), GATA binding protein 4 (GATA4), and GAPDH was performed using the following primers: TRPC3 (Reference Sequence database accession No. NM_003305.2), forward, 5′GACCTTGGGAGGGTGTTGCTC3′ and reverse, 5′GTGACGATGCAGAAGCAGA3′; TRPC6 (NM_004621.5), forward, 5′GCCAATG AOCTCTGGAATA3′ and reverse, 5′TGAGCTCAACATGCGAGG3′; TRP7M (NM_017672.4), forward, 5′CCTATGAAGAAGCCGAG C3′; TRPV1 (NM_080704.3, NM_018727.5, NM_080705.3, NM_080706.3), forward, 5′CAACAAGATCGCCAACAGGAG3′ and reverse, 5′TCCT TGCCATCAAGTGTIGTA3′; GATA1 (NM_002049.3), forward, 5′TGGAACCTTGAGACAGACGGCTGAG3′ and reverse, 5′GAAGCTTGAGGAGAGATGATGCGT3′; TNF-α (NM_000594.2), forward, 5′CCCCAGGACCTCTCTCTAT3′ and reverse, 5′ATGAGCTACAGCCTGTAC3′; GATA4 (NM_008092.3), forward, 5′AATGCCTGTOGCTCTTACA3′ and reverse, 5′CTGTT TTGAATCCCTCCTCTT3′; and GAPDH (NM_002046.3), forward, 5′AATCCTGCTAAGACCCCTGG3′ and reverse, 5′ATGACCTTCGCGAACCAGCT3′. Quantitative RT-PCR was performed in a Real-Time Cycler (Rotor-Gene 2000, Corbett Research) using 2 μg of RNA, which was added to a final volume of 20 μL, which contained 10 μL of 2× Quantitect SYBR Green PCR Master Mix (Qiagen, Hilden) and 500 nmol/L of each primer. The reaction was initiated at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing for 30 seconds at 54°C (GAPDH), at 57°C (TRPC6), or at 60°C (TRPC3, TRPM7, TRPV1, TNF-α, GATA1, and GATA4) and extension at 72°C for 15 seconds. After amplification, a melting curve analysis from 50°C to 95°C with a heating rate of 0.2°C per second with continuous fluorescence acquisition was performed to assure correct PCR amplification. Data were recorded on a Real-Time Cycler (Rotor-Gene 2000), and cycle threshold values (crossing points) for each reaction were determined using the Rotor-Gene program (Corbett Research). The relative quantification method was used whereby the change in expression of the target genes (TRPC3, TRPC6, TRPM7, TRPV1, TNF-α, GATA1, and GATA4) relative to the housekeeping gene (GAPDH) was calculated.

**Immunoblotting**

HUVECs were harvested and washed with PBS. Afterward, cells were centrifuged at 4°C for 3 minutes at 3000 rpm and the supernatant discarded. Then, 300 μL of lysis buffer containing 25 mmol/L of Tris-HCl (pH 8.0), 1 mol/L of NaCl, 200 mmol/L of EDTA, 250 mmol/L of Octyl-β-D-glucopyranoside, 1 mol/L of sodium fluoride, and complete mini protease inhibitor mixture (Roche Diagnostics) were added. The homogenate was incubated for 30 minutes on ice. The proteins in the supernatant were concentrated using a centrifugal filter device (Amicon ultra; Millipore), mixed with loading buffer, heated up to 100°C for 3 minutes, separated using a denaturing 10% SDS-PAGE at 150 V for 90 minutes, and transferred to pure nitrocellulose membranes (Trans-Blot transfer medium; Bio-Rad Laboratories) at 14 V overnight. Membranes were blocked with Odyssey blocking buffer (LI-COR Bioscences, Bad Homburg, Germany) for 1 hour at room temperature. Membranes were incubated with primary rabbit antihuman TRPC6 antibody, rabbit antihuman TRPV1 antibody (both Alomone Laboratories, Jerusalem, Israel), or goat antihuman GAPDH antibody (Santa Cruz Biotechnology) at a 1:1000 solution containing 0.1% Tween 20, Odyssey blocking buffer, and PBS for 1 hour, washed 4 times for 5 minutes. Membranes were incubated with the secondary antibody (either IRDye 800 infrared fluorescent dye-conjugated sheep anti-rabbit antibody [Biomol, Hamburg, Germany] or Alexa Fluor 680 infrared fluorescent dye-conjugated donkey anti-goat antibody [Molecular Probes, Eugene, OR]), both at a 1:1000 solution containing 0.1% Tween 20, Odyssey blocking buffer, and PBS for 1 hour and washed 4 times for 5 minutes. Imaging was performed at a wavelength of 700 nm (GAPDH) and 800 nm (TRPC6 and TRPV1).

**Patients**

We analyzed the expression of TRPC6 and TNF-α mRNA in human vascular tissue from 6 chronic kidney disease patients.
Bioinformatics
The core promoter region 2 kb upstream of the reported transcription start site of TRPC6 was analyzed in silico for transcription factor binding sites using the Transcription Element Search System (www.cbil.upenn.edu/cgi-bin/tess/tess). The analysis was limited to the transcription factors GATA1 (accession No. TRANSFAC Database T00306) and GATA4 (T02687).

Statistical Analysis
All of the data were expressed as mean±SEM and were compared using the nonparametric Mann-Whitney test. Relations between variables were investigated using the Spearman correlation. Two-sided P values <0.05 were considered to indicate statistical significance. Where error bars do not appear on the figure, error was within the symbol size. All of the data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA).

Results
Detection of TRP Channel mRNA Using Quantitative RT-PCR
We analyzed the expression of TRPC3, TRPC6, TRPM7, TRPV1, TNF-α, GATA1, GATA4, and GAPDH transcripts in HUVECs using Quantitative RT-PCR. All of the mentioned types of mRNA species could be detected in HUVECs. The specificity of the PCR was demonstrated by the presence of a single peak in the melting curve analysis.

Atheroprone Shear Stress Increases TRPC6 and TRPV1 mRNA
Morphological changes were analyzed according to the type of shear stress applied. HUVECs exposed to atheroprotective shear stress were more elongated than under atheroprone shear stress. HUVECs exposed to atheroprone shear stress were not aligned but were polygonal and randomly oriented (Figure 1A and 1B). We analyzed the expression of TRP channel mRNA in an established in vitro dynamic flow system,4 in which HUVECs are exposed to 2 well-defined types of mechanical stimulation, considered to have direct pathophysiological relevance to atherosclerotic disease in vivo. Specifically, we compared the effect of an atheroprotective flow profile with shear stress ranging from 0 to 40 dyne/cm² and a mean shear stress of 20 dyne/cm², and an
atheroprone bidirectional flow profile with a mean shear stress near 0 dyne/cm² on TRP channel mRNA expression. TRPC6 and TRPV1 mRNA expressions were significantly increased after 24 hours of exposure to an atheroprone flow profile compared with an atheroprotective flow profile (normalized ratio, 185±21 versus 100±26 arbitrary units; \(P=0.04\); each \(n=6\) for TRPC6; normalized ratio, 260±48 versus 100±35 arbitrary units; \(P=0.02\); each \(n=6\) for TRPV1). The inflammatory properties of the atheroprone flow profile were well underlined by the significant higher expression of TNF-α mRNA compared with the atheroprotective flow profile (normalized ratio, 243±69 versus 100±35 arbitrary units; \(P=0.04\); each \(n=6\)). There was a significant correlation between the expression of TNF-α and TRPC6 mRNA \((r=0.58; P<0.05)\), whereas there was no correlation between TNF-α and TRPV1 mRNA (Figure 1C through 1G).

In addition, we compared the effect of an atheroprone flow profile with shear stress ranging from 0 to 40 dyne/cm² and a mean shear stress of 20 dyne/cm² and an atheroprone bidirectional flow profile with a mean shear stress near 0 dyne/cm on TRPC6 and TRPV1 channel protein expression. TRPC6 and TRPV1 protein expressions were not changed after 24 hours of exposure to an atheroprone flow profile compared with an atheroprotective flow profile (normalized ratio, 93±8 versus 100±11 arbitrary units; \(P=1.00\); each \(n=5\) for TRPC6; normalized ratio, 98±14 versus 106±15 arbitrary units; \(P=0.69\); each \(n=5\) for TRPV1; Figure 2).

Transcription factors of the GATA family have been validated experimentally as relevant transcription enhancers for TRPC6. In the present study, the expression of GATA1 and GATA4 mRNA was significantly correlated with the expression of TRPC6 mRNA \((P=0.02\) for GATA1; \(P=0.04\) for GATA4; Figure 3).

**Regular Laminar Shear Stress Increases TRPC3 and TRPM7 mRNA Expression in Endothelial Cells**

Morphological changes were analyzed according to the type of shear stress applied. The static conditions displayed a confluent monolayer with polygonal, randomly oriented, and cobblestone-like HUVECs. Shear stress caused HUVECs to develop leading edges, retract rear edges, become elongated and spindle like, and align parallel in the direction of flow (Figure 4A and 4B).

In contrast to atheroprone shear stress, regular laminar shear stress of ≈6 dyne/cm² was chosen, because it can be present at every part of the vascular tree and represents the typical average level in human blood vessels well between venous (≈1 dyne/cm²) and arterial conditions (≈10 dyne/cm²). As shown in Figure 4, after 24 hours of laminar shear stress, the expression of TRPC6 or TRPV1 mRNA was not significantly changed compared with control conditions \((68±25 versus 100±19\) arbitrary units, \(P=0.93\) for TRPC6; \(136±26 versus 100±16\) arbitrary units, \(P=0.61\) for TRPV1). In contrast, after 24 hours of regular laminar shear stress, the expression of TRPC3 and TRPM7 was significantly higher in HUVECs exposed to shear stress in comparison with HUVECs grown under static conditions (normalized ratio, \(374±126 versus 100±19\) arbitrary units, \(P=0.02\) for TRPC3 and \(177±25 versus 100±19\) arbitrary units, \(P=0.04\) for TRPM7; each \(n=5\)). Again, the inflammatory properties of the static conditions were demonstrated by the significantly higher expression of TNF-α compared with the dynamic flow profile (normalized ratio, \(100±28 versus 16±4\) arbitrary units; \(P=0.03\); each \(n=6\); Figure 4).

To analyze whether the alteration in the expression of TRP channel mRNA expression is PI3K dependent, we repeated the aforementioned experiments with cells that were pre-treated with the PI3K inhibitor LY-294002 and applied a shear stress of 6 dyne/cm² for 4 hours. Even in the presence of LY-294002, laminar shear stress significantly increased TRPC3 in comparison with static conditions (normalized ratio, \(423±132 versus 100±13\) arbitrary units; \(P=0.03\)), whereas, in the absence of LY-294002, neither TRPM7 mRNA nor TRPV1 was significantly changed (normalized ratio, TRPM7, \(150±74 versus 100±40\) arbitrary units; \(P=1.00\); TRPV1, \(167±50 versus 100±46\) arbitrary units; \(P=1.00\)). TRPC6 mRNA was signifi-
cantly reduced by LY-294002 treatment from 100/11006 to 42/11006 (P<0.03; each n=6) in comparison with static conditions. Similar to the experiments without previous inhibition of PI3K, the inflammatory properties of the static conditions were demonstrated by the significantly higher expression of TNF-α compared with the dynamic flow profile (normalized ratio, 100/11006 versus 30/11006 arbitrary units; P<0.03; each n=6; Figure 5). These data reveal that the alterations in TRP channel mRNA expression induced by shear stress are not PI3K dependent.

**In Silico Prediction of Transcription Factor Binding Sites in the TRPC6 Promoter Region**

To indicate possible pathways for the regulation of TRPC6, we analyzed the TRPC6 core promoter and searched for binding sites for GATA transcription factors, which are known to be regulated by shear stress, which are acknowledged to regulate TRPC6 as part of a signaling circuit during pathological cardiac remodeling, and which play a significant role in endothelial cells, for example, in vessel formation, endothelial cell migration, and apoptosis. We, therefore, performed an in silico prediction for GATA transcription factor binding sites and their binding affinities (according to Transcription Element Search System Database) in the core promoter region 2 kb upstream of the ATG start site (chr11: 101454235–101456234; -strand) of the human TRPC6 gene on chromosome 11 using the Transcription Element Search System (Figure 6).

**Expression of TRP Channel mRNA in Atherosclerotic Vessels**

Next we analyzed TRPC6 and TNF-α mRNA expressions in atherosclerotic vessels from humans. Small parts of atherosclerotic arteries were obtained from chronic kidney disease patients (n=6; age, 56±4 years; 3 men, 3 women; body mass index, 25.4±3.0 kg/m²). In line with the findings in vitro, there was a significant association between the expression of TRPC6 and TNF-α mRNA (r=0.89; P<0.05) in vascular tissue from chronic kidney disease patients.

**Discussion**

The present study demonstrates that transcripts of different TRP channels are differentially regulated under various shear stress protocols. Our findings implicate selective TRP gene products as contributors to the vascular remodeling process. We used several well-described shear stress profiles. First, the atheroprone bidirectional flow profile with a mean shear stress near 0 dyne/cm² was described by Dai et al in 2004 and was chosen in the present study because it represents atherosclerosis-susceptible regions of human arteries and is seen at atheroprone sites. This atheroprone shear stress waveform is significantly different from the sinusoid wave-
form, which is also used as a paradigm for atherosclerosis-susceptible regions.4 Second, the atheroprotective flow profile with shear stress ranging from 0 to 40 dyne/cm² and a mean shear stress of 20 dyne/cm² represents regions that are resistant to the development of atherosclerotic lesions. Third, static, that is, no-flow conditions, represent nonperfused vessels and occur in vivo only in sprouting capillaries and after vessel occlusion.16,20–22 Morphological changes represent a late response of endothelial cells on flow exposure.23 The experiments performed confirmed typical morphological changes of human endothelial cells under the influence of atheroprotective shear stress24 versus atheroprone25 and static

Figure 4. Morphology of human umbilical vascular endothelial cells (HUVECs) after flow exposure. HUVECs were exposed to control (static) conditions (A) or dynamic shear stress (6 dyne/cm², 24 hours; B). There was a significant HUVEC alignment in response to the dynamic shear stress applied compared to control (static) conditions for 24 hours. Scale bars indicate 100 μm. Arrow indicates direction of shear stress. Data show changes in mRNA expression by laminar shear stress. HUVECs were exposed to dynamic shear stress (6 dyne/cm², 24 hours) or kept under static control conditions and analyzed for differential expression of mRNA transcripts of (C) TRPC3, (D) TRPC6, (E) TRPM7, (F) TRPV1, and (G) TNF-α by quantitative RT-PCR. *P<0.05 between groups.

Figure 5. Influence of shear stress and inhibition of phosphatidylinositol 3-kinase (PI3K) on expression of mRNA. Human umbilical vascular endothelial cells (HUVECs) were kept under static conditions, dynamic conditions with shear stress of 6 dyne/cm² for 4 hours, or dynamic conditions with shear stress of 6 dyne/cm² for 4 hours after treatment with LY-294002 (10 μmol/L; +Ly), and expression of (A) TRPC3, (B) TRPC6, (C) TRPM7, (D) TRPV1, and (E) tumor necrosis factor (TNF)-α mRNA was measured by quantitative RT-PCR. *P<0.05 between groups.
conditions versus dynamic shear stress. The inflammatory properties of the atheroprotective flow profile were well underlined by the significant higher expression of TNF-α mRNA in the former group in the present study. Finally, regular laminar shear stress of 6 dyne/cm² was chosen because it can be present at every part of the vascular tree and represents the typical average level in human venous (1 dyne/cm²) and arterial vessels (≈10 dyne/cm²). This profile represents well-perfused mature vessels. In our study, the expression of TNF-α mRNA, which was used as a marker of inflammation, tended to be higher under static conditions compared with the dynamic flow profile; however, it failed to reach significance probably because of the lower number of experiments.

Specific flow characteristics and the shear stress produced by them are linked to atherosclerosis, for example, in vivo no-flow conditions occur after vessel occlusion (and in...).
sprouting capillaries), and atheroprotive flow profiles are observed at atheroprotive sites. We showed that a shear stress profile taken from an atheroprotive site increases TRPC6 and TRPV1 mRNA expression in endothelial cells. Although atheroprotive shear stress increased TRPC6 and TRPV1 transcripts, protein levels were not significantly affected within 24 hours. These data indicate that only long-term exposure to atheroprotive shear stress may cause receptor protein modifications. TRPC6 expression in branched vessels has been reported to be associated with increased atherosclerosis. TRPC6 plays a role in vascular endothelial growth factor-mediated microvessel permeability and thrombin-induced endothelial shape change. In addition, it has been shown recently by our group that increased TRPC6 expression is a hallmark of atherosclerosis.

Analysis of the TRPC6 promoter revealed possible binding sites for GATA transcription factors, which are known to be regulated by shear stress, which are acknowledged to play a significant role in endothelial cells, for example, in vessel formation, endothelial cell migration, and apoptosis. Both, the significant association of GATA1 and GATA4 mRNA expression with TRPC6 mRNA expression and the determination of GATA1 binding sites in the promoter region of TRPC6 point to these transcription factors as the underlying mechanisms, which may enhance atheroprotective shear stress.

There was a significant association between the expression of TRPC6 and TNF-α mRNA in vascular tissue from chronic kidney disease patients. The linkage between shear stress and inflammatory gene response has been the subject of recent publications concluding that Jun N-terminal protein kinase 2 promotes endothelial cell alignment under flow, and mitogen-activated protein kinases play an important role in this regard. Moreover, the correlation between TRPC6 and TNF-α experimentally shown in vitro was, thus, similarly shown in vivo.

**Perspectives**

For the first time, we showed that atheroprotective shear stress especially affects TRPC6 and TRPV1 mRNA expression in endothelial cells. Because TRP channels are known to be associated with atherosclerotic effects, such as inflammation and oxidative stress, these results imply that these selective TRP channel gene products are those that contribute to the atherosclerotic process. In line with these findings, Thilo et al. showed recently that TRP channels are regulated by oxidative and antioxidative substances. Increased transplasma membrane calcium influx through TRP channels may aggravate the atherosclerotic processes. Up to now, it is only partly known which TRP channels are in which way linked to atherosclerotic events, including increased proliferation and apoptosis of endothelial cells or smooth muscle cells, vascular remodeling, low-density lipoprotein uptake, and/or lipid synthesis. However, recent interventional studies in mice indicated that modulating TRPV1 channel activity may reduce atherosclerosis. Hence, the present study may indicate that upregulation of TRPC6 and TRPV1 mRNA in human endothelial cells contributes to transduction of hemodynamic events including shear stress at “disturbed” flow conditions and may, thus, turn the system balance toward major cardiovascular diseases.

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**Disclosures**

None.

**References**


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