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Effect of Frequency of Uniform and Disturbed Pulsatility Flow on Tissue Factor Expression of Human Umbilical Vein Endothelial Cells

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Abstract

We analyzed the effect of change in frequency of uniform or disturbed pulsatile flow on human umbilical vein endothelial cells (HUVECs) in the presence or absence of thrombin (Th). We found that tissue factor (TF) RNA expression in HUVECs exposed to 60 cycles per minute (cpm) of disturbed and uniform flow was significantly higher than that induced by 30 cpm. HUVECs exposed to disturbed flow have significantly higher TF RNA expression than HUVECs exposed to uniform flow at either frequency. We conclude that frequency of pulsatile flow is a critical independent factor in TF RNA expression in HUVECs.

Keywords: Human umbilical vein endothelial cells; Shear stress; Tissue factor; Pulsatile flow; Frequency

Abbreviations: TF: Tissue Factor; HUVEC: Human Umbilical Vein Endothelial Cell; Th: Thrombin; EC: Endothelial Cell; TFF: To and Fro Flow; TFF: Pulsatile Forward Flow, PF; CPM: Cycle Per Minute

Introduction

Numerous studies demonstrate that atherosclerotic lesion development occurs at the site of the blood vessel that is subjected to disturbed flow [1-3], such as the lesser curve of the aortic arch or at bifurcations. These observations have led researchers to hypothesize that different types of blood flow have different effects on the function of endothelial cells (ECs) that line the blood vessels and are directly exposed to hemodynamic forces. The major mechanical force is fluid shear stress, the frictional force on ECs generated by blood flow. Disturbed flow occurs when the fluid shear stress is not constant in magnitude or frequency.

Recent evidence also suggests that tissue factor (TF) is abundant in atherosclerotic plaques, and its content seems to predict plaque thrombogenicity [4,5]. TF has been demonstrated to contribute to the hyperthrombotic state of human atherosclerotic vessels [6], and an excessive expression of TF results in an acute thrombotic event, leading to complications of atherosclerosis.

Our laboratory has investigated the role of mechanical forces on ECs [7-10], and we have previously reported a significant increase in TF expression of ECs exposed to disturbed flow [7,11,12]. The purpose of this study is to compare the effect of frequency of disturbed and uniform flow on EC expression of TF.

Materials and Methods

Cell culture

Primary cultures of human umbilical vein endothelial cells (HUVECs) were obtained from the laboratory of Dr Jordan Pober (Department of Pathology, Yale School of Medicine). Cells were cultured with M-199 medium enriched with 20% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), 10μg/mL heparin, 50 μg/mL EC growth supplement (BD Biosciences, Bedford, MA), and penicillin-streptomycin antibiotic combination (both 100 μg/mL), in a 5% CO_2 incubator at 37°C. After reaching confluence, 0.25% trypsin ethylene diamine tetra acetic acid was used for detachment, and passage 2 to 4 cells were seeded on fibronection (BD Biosciences) coated glass slides (7 ×38 mm: Fisher Scientific, Pittsburgh, PA).

Mechanical stress exposure

HUVECs were exposed to shear stress, utilizing a parallel-plate flow chamber system (Cytodyne, San Diego, CA) as previously described [7,10]. Flow of the perfusion medium was regulated by a computer-controlled syringe pump (PHD 2000 and PHD Ultra Programmable; Harvard Apparatus, Holliston, MA). To generate pulsatile forward flow (PFF), an automated switch clamp (Auto-Fill valve box; Harvard Apparatus) is placed between the syringe pump and the flow chamber, and between the syringe pump and the culture medium reservoir. Synchronous activation of both switch clamps with the cycle allows unidirectional flow. In the to and fro flow (TFF) model, the switch clamp is not used. The flow chambers were directly attached to the flow loop circuit including the flow reservoir, which enabled culture medium in the flow chamber to be exchanged by every to-fro impulse. Approximately one-tenth of the culture media in the flow chamber was exchanged at least every 1 sec. The shear stresses were 1) 60 cycle per minute (cpm) of PFF, i.e. a forward square wave impulse for 0.5 sec alternating with no flow for 0.5 sec, 2) 30 cpm of PFF, i.e. a forward square wave impulse for 0.25 sec alternating with no flow for 0.25 sec, 3) 60 cpm of TFF, i.e. a forward square wave impulse for 0.5 sec alternating with a backward square wave impulse for 0.5 sec and 4) 30 cpm of TFF, i.e. a forward square wave impulse for 0.25 sec alternating with a backward square wave impulse for 0.25 sec. The magnitude of shear was kept constant at 14 dyne/cm^2.

Chemical stress exposure under static and flow conditions

M199 culture medium with 1% FBS and 4 U/mL of thrombin

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(Th) (Sigma-Aldrich, St. Louis, MO) was applied to HUVEC under static condition, and to HUVEC exposed to either TFF or PFF, and was present throughout the entire experiment. This dose of thrombin results in reproducible stimulation of TF expression [7,11-14]. These experiments applying mechanical and chemical stress together were performed simultaneously with the experiments applying only mechanical stress, as paired experiments.

RNA isolation and measurement of TF RNA levels

RNA was isolated using the RN easy Mini kit (Qiagen Sciences, Germantown, MD) according to the manufacturer's instruction. Two micrograms of total RNA were reverse-transcribed into cDNA using the iScript reverse transcription kit (Bio-Rad Laboratories, Hercules, CA). TF RNA levels were estimated by quantitative real-time PCR with β -actin serving as the house-keeping gene. The iQ SYBR Green Supermix (Bio-Rad Laboratories) was used in quantitative real-time PCR and all RT-PCR reactions were performed in a CFX96 Thermal Cycler machine (Bio-Rad). Primer sequences for TF were: forward 50-GCC AGG AGAAAG GGG AAT-30; reverse 50-CAG TGC AAT ATA GCA TTT GCA GTA GC-30. Sequences for β -actin were: forward 50-TCA CCC ACA CTG TGC CCA TCT ACG A-30; reverse 50-CAG CGG AAC CGC TCATTG CCA ATG G -30. Primers were purchased from Integrated DNA Technologies (Coralville, IA). The Pfaffl method was used to calculate fold changes in TF mRNA expression levels [15].

Statistical analysis

The results are presented as mean ± standard error (SEM) of at least three separate experiments. Statistical significance was determined by analysis of variance (ANOVA) followed by a post-hoc analysis of Bonferroni, or analysis of covariance (ANCOVA) when appropriate. Statistical significance was defined as $P<0.05$. Statistical analysis was performed using Prism 6 for Mac OS X software package (GraphPad Software Inc., La Jolla, CA).

Results

TF RNA expression induced by uniform and disturbed flow

Figure 1 shows TF RNA expression levels in HUVECs exposed to uniform (PFF) (Figure 1A) or disturbed (TFF) flow (Figure 1B) for up to

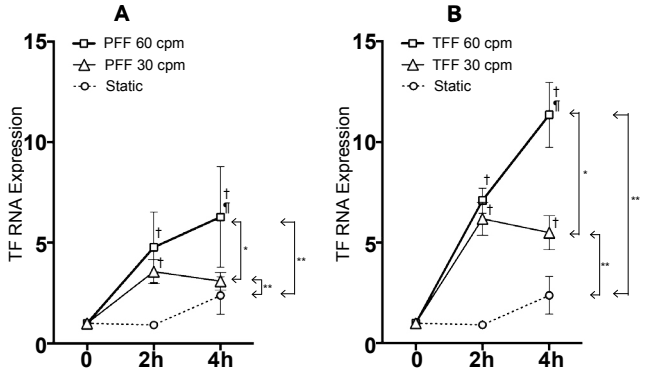

Figure 1: TF RNA expression in HUVECs exposed to PFF (Figure 1A) or TFF (Figure 1B). HUVECs were subjected to either 60 cpm or 30 cpm, 14 dyne/cm² TFF or PFF for up to 4 hours. TF RNA expression was expressed as fold change compared with static control values at 0 h as a baseline. Values are means ± SE; n=3-8. *P < 0.05, calculated by ANOVA. **P<0.05, calculated by ANOVA, compared to static. ¶ P<0.05, compared to PFF, †P<0.05, compared to static, calculated by post hoc analysis of Bonferoni.

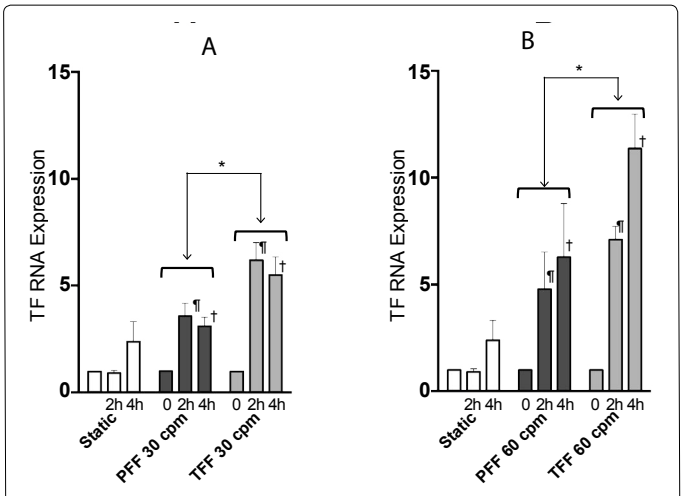

Figure 2: TF RNA expression in HUVECs exposed to 30 cpm flow (Figure 2A) or 60 cpm flow (Figure 2B). HUVECs were subjected to 14 dyne/cm² TFF or PFF for up to 4 hours. TF RNA expression levels were expressed as fold change compared with static control at 0 h as a baseline. Values are means ± SE; n = 3–8. *P < 0.05, calculated by ANOVA. TFF vs. PFF. ¶, †, P<0.05, calculated by post hoc analysis of Bonferoni.

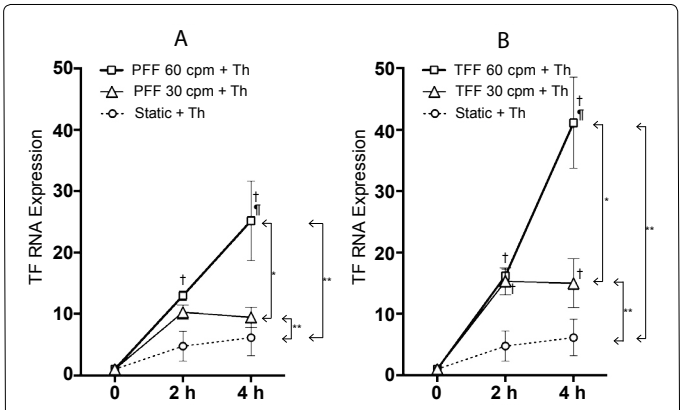

Figure 3: TF RNA expression in HUVECs exposed PFF (Figure 3A) or TFF (Figure 3B) in the presence of 4 U/ml Th. HUVECs were subjected to 60 cpm and 30 cpm, 14 dyne/cm² TFF or PFF and 4 U/ml Th for up to 4 hours. TF RNA expression was expressed as fold change compared with static control at 0 h as a baseline. Values are means ± SE; n = 3-8. * P<0.05, calculated by ANOVA. **P<0.05, calculated by ANOVA, compared to static. ¶ P<0.05, compared to PFF, †P<0.05, compared to static, calculated by post hoc analysis of Bonferoni.

4 hours compared with static conditions. We have previously reported the time course of TF expression of ECs exposed to laminar flow and this started by 2 hr, peaked at 4 hrs and then started to decline by 8 hrs. [7,11,12]. All mechanical stimuli conditions induced higher TF RNA expression compared with static conditions (ANOVA and post-hoc analysis of Bonferroni, $P<0.05$). TF RNA expression in HUVECs exposed to 60 cpm flow of either PFF (Figure 1A) or TFF (Figure 1B) were significantly higher than that induced by 30 cpm (ANOVA, $P<0.05$). HUVEC exposed to TFF always had significantly higher TF RNA expression than that of HUVECs exposed to PFF with either 60 cpm or 30 cpm (Figure 2).

TF RNA expression induced by simultaneous mechanical and chemical stimuli

Figure 3 shows TF RNA expression levels in HUVECs exposed to

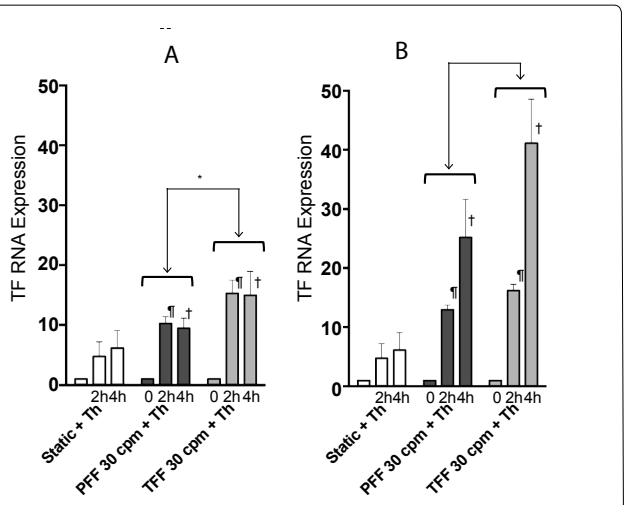

Figure 4: TF RNA expression in HUVECs exposed to 30 cpm flow (Figure 4A) or 60 cpm flow (Figure 4B) in the presence of 4 U/ml Th. HUVECs was subjected to 14 dyne/cm^2 TFF or PFF for up to 4 hours. TF RNA expression was expressed as fold change compared with static control at 0 h as baseline. Values are means ± SE; n=3–8. * P < 0.05, calculated by ANOVA. TFF vs PFF. ¶, †, P<0.05, calculated by post hoc analysis of Bonferoni.

both fluid flow and Th stimuli for up to 4 hours compared with static cells exposed to Th. HUVECs exposed to any mechanical stimuli and Th induced higher TF RNA expression compared with static HUVECs treated with Th (ANOVA and post-hoc analysis of Bonferroni, P<0.05). TF RNA expression in HUVEC exposed to 60 cpm flow of either PFF (Figure 3A), or TFF (Figure 3B) in addition to Th were significantly higher than that induced by 30 cpm (ANOVA, P<0.05).HUVEC exposed to TFF and Th had significantly higher TF RNA expression than HUVEC exposed to PFF and Th with either frequency (Figure 4). The increase in TF RNA expression in HUVEC exposed to 60 cpm TFF and Th (as measured by the slope between 2 and 4 hours in figure 3B) was significantly higher than that in HUVEC exposed to 30 cpm with Th between 2 and 4 hours (ANCOVA, P<0.05).

Discussion

Mechanical forces that are associated with blood flow have been shown to be major initiating factors of vascular pathology [16]. Regions of the vasculature where shear stress is low is more likely to develop atherosclerosis [17]. In addition, specific patterns of flow disturbance induced by flow separation, gradients, flow reversal and turbulence contribute to plaque formation [18].

It is now widely accepted that although there may be similar responses by EC to different hemodynamic forces, there are temporal and/or magnitude differences in these responses [2,19,20]. It is also now evident that sites of atherosclerosis correlate with areas of disturbed fluid dynamics and links the observations that temporal or spatial gradients in these hemodynamic forces are more potent stimuli for ECs than the actual magnitude of the force [21]. ECs exposed to unidirectional flow, especially high laminar shear, have been reported to express atheroprotective genes [2]. Low and oscillatory shear stress are closely associated with atherogenesis.

We used TFF as a model of disturbed flow because the oscillatory direction of flow change is analogous to that seen at regions high at risk for atherosclerotic plaque development such as the carotid bulb. Oscillatory flow is characterized by time-averaged fluctuations in shear stress during the cardiac cycle due to forward-reverse flow cycles and disrupted flows. In this study, we specifically analyzed the effect of different frequencies of pulsatile flow, on both disturbed (TFF) and uniform flow (PFF),on TF RNA expression of HUVECs.

Our results demonstrate that although the maximum magnitude of shear and the total time that HUVECs are exposed to flow stimuli are the same, the 60 cpm frequency induces a higher TF RNA expression than for 30 cpm frequency. This dependence on frequency was observed in HUVECs exposed to either TFF or PFF.

The mechanism of these findings is not clear, but suggests that the EC mechanotransducer is exquisitely sensitive to spatial and temporal gradients of a mechanical force. For instance, we previously reported that an acute change in cyclic strain from 60 cpm to 100 cpm results in activation of IP_3 [20]. In addition, an acute decrease from 100 cpm to 60 cpm also resulted in an immediate increase in IP_3 [19]. Bao et al. [21] demonstrated that EC exposed to different shear segments of either step flow, ramp flow, impulse flow or pulsatile shear had variable activation of signaling pathway. Likewise, we showed that bovine aortic ECs exposed to pulsatile flow frequencies of 0.5 and 1.0 Hz, but not 1.5 Hz, exhibited elongated morphologies and oriented with the direction of flow [22]. Our laboratory also demonstrated that 1 Hz but not 0.1, 0.5, or 1.5 Hz was optimal for bovine aortic ECs proliferation when exposed to PFF [8]. Interestingly, frequency had no effect on proliferation of bovine aortic ECs exposed to TFF [8]. Other investigators have also reported that anti-inflammatory responses of porcine aortic ECs exposed to 1 Hz forward flow are reversed at 2 Hz [23]. In addition, proinflammatory responses evoked by the 2 Hz but not 1 or 3 Hz were greater when ECs were exposed to reversing and oscillatory flow [23]. Taken together, these studies and our present results strongly suggest that the frequency of pulsatile flow is a critical independent factor in EC response to shear.

Our study also showed that TF RNA expressions at 60 cpm in both combined stimulation of TFF and PFF with Th induces higher TF RNA expression that those of 30 cpm with Th. Our previous report showed that there might be synergistic interaction between mechanical and chemical stimuli since the combination of mechanical and chemical stimulations yielded greater TF RNA expression levels than the theoretical values calculated by adding the results of mechanical stress alone values with chemical stress alone values [12]. The current study confirms those findings and in addition demonstrates that during simultaneous mechanical and chemical stimulation of HUVECs increasing the frequency enhanced TF RNA expression. In the cells that are subjected to disturbed flow under chemical stimuli, the reaction to the change of the frequency seems to result in a non-linear increase in TF RNA expression.TF RNA expressions of HUVECs exposed to 60 cpm TFF with Th is still increasing significantly by 4 hours, while those exposed to 30 cpm reach peak expression by 2 hours. This is consistent with previous studies that demonstrate that, EC activated by laminar flow are down regulated over time, on the order of 1 hour. Disturbed shear, however, activates the same pathways in a sustained manner [24].

In conclusion, our study provides important information on the role of frequency of shear in the modulation of EC that are exposed to uniform or disturbed flow. This adds to our current knowledge of how flow patterns contribute to atherogenesis.

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