

## Original Paper

# The Effect of Shiga Toxin on Weibel-Palade Bodies in Primary Human Endothelial Cells

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## Key Words

Shiga toxin · Weibel-Palade bodies · von Willebrand factor · Endothelial cells

## Abstract

**Background/Aims:** Diarrhea-associated hemolytic uremic syndrome is associated with the presence of Shiga toxin (Stx1, Stx2 and several variants) in the circulation. The aim of this study is to examine the possible triggering effect of Stx1 on the exocytosis of Weibel-Palade bodies (WPBs). **Methods:** Cultured human umbilical venous endothelial cells (HUVECs) and glomerular microvascular endothelial cells (GMVECs) were stimulated by thrombin and Stx1 in both static and flowing conditions. The amount of secreted von Willebrand factor (VWF) in the supernatant as well as the remaining intracellular fraction was determined. **Results:** In HUVECs and in 2 out of 4 GMVECs, the stimulation of Stx1 in flow at 1 dyne/cm<sup>2</sup> resulted in a decrease of intracellular VWF. This is contrary to the results of Stx1 applied in static conditions. At a higher flow rate of 5 dyne/cm<sup>2</sup>, no effect in GMVECs was observed. **Conclusion:** Stx1 can contribute, via an effect on WPBs, to the exocytosis of WPBs in flow conditions in HUVECs and probably in GMVECs. This results in the release of VWF, suggesting an initiating role of the coagulation system in the pathogenesis.

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## Introduction

The exotoxin Shiga toxin (Stx) is the central virulence factor in diarrhea-associated hemolytic uremic syndrome (D + HUS). The underlying pathogenic mechanism is endothelial damage of the glomeruli and arterioles in the kidney by Stx [1]. Much research has been

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performed to elucidate possible disturbances in the coagulation system. von Willebrand factor (VWF) antigen and P-selectin were found at an increased level in serum of patients in the acute phase of D + HUS [2, 3]. A loss of large multimers and an increase of small multimers were observed. This may be due to abnormal shear stress in the microvascular circulation [3].

VWF is involved in direct adhesion and aggregation of platelets on damaged endothelium, whereas it also functions as a chaperone for coagulation factor VIII. The Weibel-Palade bodies (WPBs) are rod-shaped cytoplasmic structures with a tubular composition. After perturbation of the endothelial cell, WPBs can be exocytosed through two different mechanisms [4]. One pathway is activated by an increase on intracellular calcium and leads to exocytosis of the WPBs (thrombin). The other pathway is regulated by cAMP and also leads to exocytosis (vasopressin and epinephrine).

Its constituents are involved in hemostasis (VWF and tissue-type plasminogen activator), induce vasoconstriction (endothelin-1, endothelin-converting enzyme), and inflammation (P-selectin, eotaxin-3, IL-8, angiopoietin-2, CD63,  $\alpha$ 1,3-fucosyltransferase VI, osteoprotegerin) [4]. Mass spectrometry analysis of purified WPBs revealed the presence of 35 novel candidate residents [5].

Since WPBs contain regulators for both hemostasis and inflammation, we evaluated whether stimulation of primary human endothelial cells [cultured human umbilical venous endothelial cells (HUVECs) and glomerular microvascular endothelial cells (GMVECs)] with a subtoxic dose of Stx1 could trigger exocytosis of WPBs. For this purpose, we have determined the effect of Stx1 on the quick release of the hemostatic VWF. The effect on another component of the WPBs, angiopoietin 2 (Ang-2) was included. In a seminal study, Nolasco et al. [6] could demonstrate that Stx1 or Stx2 stimulated the rapid secretion of unusually large VWF multimeric strings from HUVECs or GMVECs. Perfused normal human platelets immediately adhered to the secreted strings. A monoclonal antibody to Gb3 receptors blocked the effect of Stx stimulation. In our study, the effect of Stx1 was studied using a different technique and it was compared with the results obtained in static conditions. In addition, GMVECs were tested at a higher flow rate, usually present in glomeruli.

## Methods

### *Culture of Endothelial Cells*

HUVECs were harvested after collagenase treatment. HUVECs in passage 2–4 were used. GMVECs were obtained from human kidneys. Glomeruli were isolated under sterile conditions using a gradual sieving procedure followed by digestion with collagenase [7]. GMVECs in passages 7–10 were used. Gb3 was present in cultured glomeruli [8].

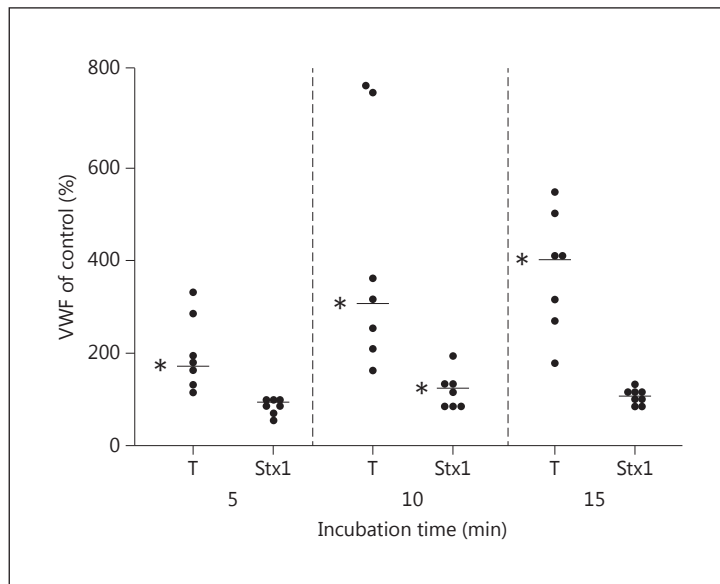
### *Quantification of VWF in Supernatant Endothelial Cells*

To study the exocytosis of WPBs in the supernatant of endothelial cells, the cells were grown to confluency in 24 well plates. While the cells were incubated with thrombin (5 U/ml) or Stx1 (10 nM) for 5, 10 and 15 min, Stx1 was kindly provided by Dr. M. Karmali, Toronto, Ont., Canada, and was endotoxin-free, as determined with a Limulus assay at 37°C. Cells remained viable during this incubation, as determined with a  $^3\text{H}$ -leucine incorporation assay (data not shown). The amount of released VWF in the supernatant from endothelial cells was measured by ELISA.

### *Quantification of Intracellular VWF and Ang-2 in Endothelial Cells*

Endothelial cells were cultured as described in the above section. Stimulation was performed in both flowing and static conditions. In both setups, cells were exposed to

**Fig. 1.** VWF in the supernatant of HUVECs after incubation with thrombin or Stx1 in static conditions. HUVECs ( $n = 7$ ) were incubated with thrombin (T) or Stx1 for 5, 10 and 15 min, respectively. The negative control was set at 100%. Thrombin clearly triggers the secretion of VWF in HUVECs, whereas Stx1 only induces a small release after 10 min. The bar depicts the median. The asterisk indicates a significant effect ( $p < 0.05$ ).



thrombin (1 U/ml), Stx1 (10 nM) or phorbol 12-myristate 13-acetate (PMA; 100 ng/ml) during 15 min (flowing conditions) or 1 h (static conditions). To apply shear stress, cells were incubated with the stimulants in a parallel Focht flow perfusion chamber with well-described characteristics [9]. The applied shear stress was 1 dyne/cm<sup>2</sup>, similar to Nolasco et al. [6]. In addition, a shear stress of 5 dyne/cm<sup>2</sup> was applied on GMVECs based on the data of Remuzzi et al. [10]. After the incubation with the stimulants, the cells were lysed [11]. The lysates were then analyzed for the presence of VWF and Ang-2. Furthermore, cells were prepared and analyzed by confocal laser scanning microscopy.

### Statistics

All data presented are expressed as a range with the median. Significance of the quantitative data was analyzed using the Wilcoxon signed ranks test. The statistical level of significance was defined as  $p < 0.05$ .

## Results

### Quantification of VWF in the Supernatant of Endothelial Cells

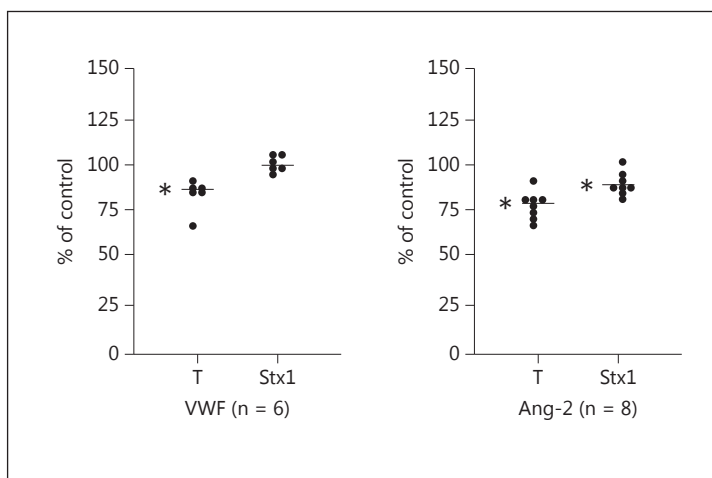
To study the possible exocytosis of WPBs from human endothelial cells after incubation with a subtoxic dose of Stx1, the presence of VWF in the supernatant of stimulated endothelial cells (HUVECs and GMVECs) was determined. Thrombin was used as a positive control.

Figure 1 presents the data on the level of VWF in the supernatant. The results are expressed as percentages of the negative control. Thrombin was able to induce a significant release of VWF after 5, 10 and 15 min in HUVECs. After 10 min of incubation of HUVECs with Stx1, there was a small but significant increase of VWF (median control value 100 vs. 125% with Stx1).

### Quantification of Intracellular VWF and Ang-2 in Endothelial Cells

To determine the level of intracellular VWF after incubation with Stx1, cell lysates were analyzed by ELISA. If Stx1 is able to induce exocytosis, the amount of VWF should be decreased compared to a negative control. In addition, the presence of Ang-2 was determined.

**Fig. 2.** Intracellular VWF in HU-VECs after incubation with thrombin or Stx1 in static conditions. HUVECs were incubated with thrombin (T) and Stx1 in static conditions. After incubation, the intracellular amounts of VWF and Ang-2 were calculated. Thrombin is capable of decreasing the intracellular amount of VWF and Ang-2 in HUVECs. Stx1 induced a small decrease in Ang-2. The asterisk indicates a significant effect ( $p < 0.05$ ).



**Fig. 3.** Intracellular VWF and Ang-2 in HUVECs after incubation with thrombin or Stx1 in flow conditions. HUVECs were incubated with thrombin (T) and Stx1 in flowing conditions (1 dyne/cm<sup>2</sup>). After incubation, the intracellular amounts of VWF and Ang-2 were calculated. Stx1 is capable of decreasing the intracellular amount of VWF, whereas thrombin has a smaller and nonsignificant effect. However, thrombin significantly decreases Ang-2. Stx1 did not produce a significant effect on Ang-2. The asterisk indicates a significant effect ( $p < 0.05$ ).

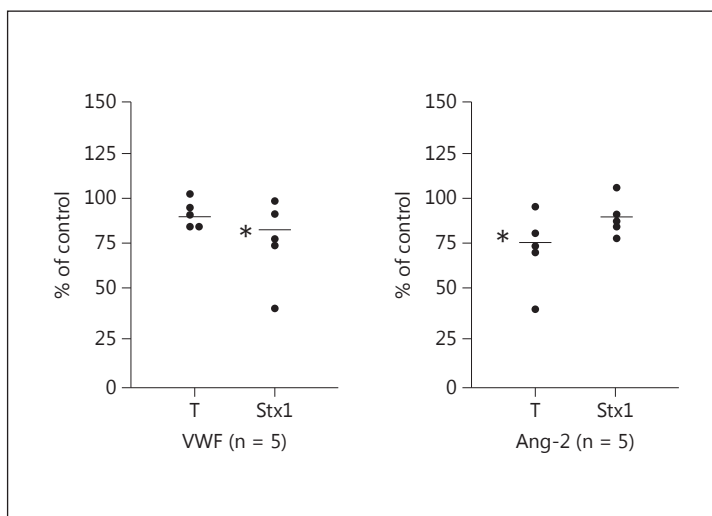
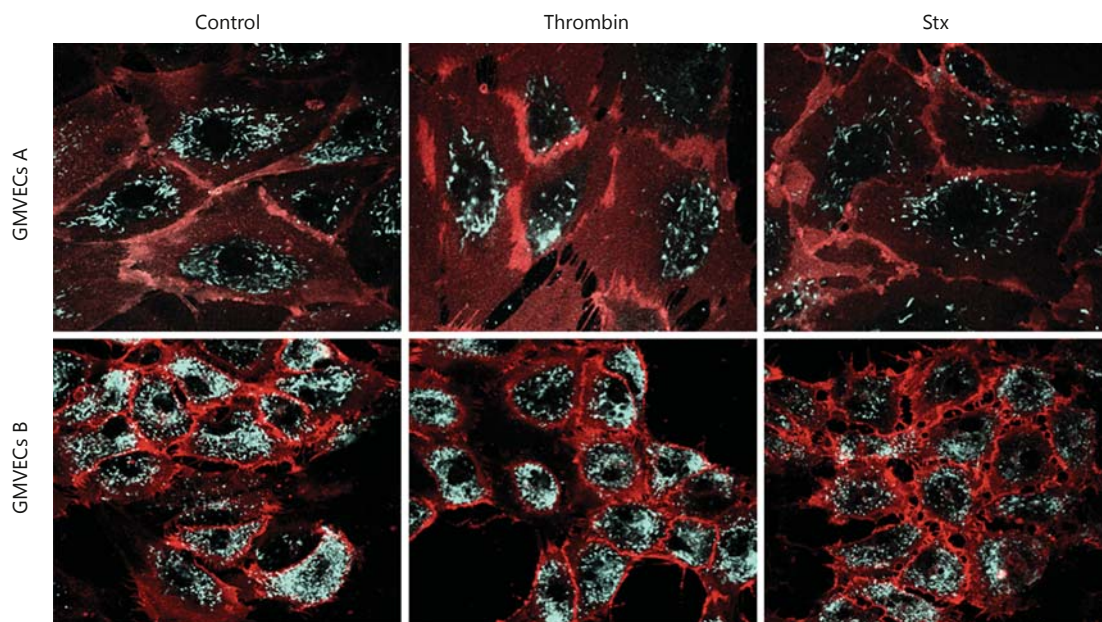


Figure 2 represents the data of the experiments performed in static conditions. HUVECs stimulated by the positive control thrombin contained a clear decreased level of VWF and Ang-2. In contrast, Stx1 was not able to exocytose VWF although a small decrease of Ang-2 in HUVECs was observed. GMVECs were not sensitive for the effect of Stx in static conditions. No decrease in VWF or Ang-2 could be detected in this small study ( $n = 4$ ).

The experiments were also performed in flowing conditions simulating the *in vivo* situation. Figure 3 represents our data of HUVECs incubated with thrombin or Stx1 in flow ( $n = 5$ ). In this setup, Stx1 triggered the endothelial cells into a significant decrease of VWF. Ang-2 was also decreased, but nonsignificantly. Table 1 demonstrates our data gathered after the incubation of GMVECs in flow conditions. Four GMVECs donors were used. Donors 1 and 3 responded with a decrease of VWF and Ang-2 after incubation with both PMA and Stx1. Only in these experiments, PMA was used as a stimulus. Thrombin had a similar but less pronounced effect. Figure 4 demonstrates 2 different GMVEC donors with a clear effect of Stx1. When 5 dyne/cm<sup>2</sup> were applied, no effect was observed in the 4 studied GMVECs.



**Fig. 4.** Confocal microscopy of GMVECs incubated with thrombin or Stx1 in flow conditions. GMVEC donors A and B were incubated in flow with thrombin and Stx1 (1 dyne/cm<sup>2</sup>). The cells were fixed and stained for VWF (green) and CD31 (red). The merge of fluorescent signals is shown. GMVECs B show less effect of thrombin than GMVECs A. A noticeable effect can be detected in both donors after exposure to Stx1.

**Table 1.** VWF and Ang-2 in GMVECs incubated in flow

Stimulus	VWF (% of control)						Ang-2 (% of control)					
	donor				statistics		donor				statistics	
	1	2	3	4	median	p value	1	2	3	4	median	p value
PMA	75	99.1	65	99.3	87.1	0.068	76.4	84	83.8	95.8	83.9	0.068
Stx1	82	104	88.9	93.3	91.1	0.144	82.6	112.3	93.1	102.2	97.7	0.715

## Discussion

The effect of Stx1 on the exocytosis of the WPBs was studied. In HUVECs, there is a clear effect of thrombin, and Stx1 induces only a small release of VWF after 10 min. Since this effect is quick, it can be attributed to the release of WPBs. The intracellular VWF was not decreased by Stx-1. HUVECs and GMVECs were incubated with a positive control (thrombin or PMA) and Stx1 in flow. After these incubations, the intracellular amount of VWF and Ang-2 was measured. It is important to realize that apart from VWF, subsets of WPBs do not contain the same amount of Ang-2 [4]. At a low flow rate, HUVECs could be triggered into a significant decrease of VWF by Stx1, confirming the experimental results of Nolasco et al. [8]. Perfusion in flow conditions without Stx has no or a minimal effect on VWF release.

In static conditions, Stx1 could not stimulate the release of WPBs in GMVECs. In GMVECs, at a low shear stress rate, Stx1 could induce a release of WPBs in 2 out of 4 cell lines, indicating an intact signalling pathway [12]. This is a confirmation of the results obtained by Nolasco et



al. [6]. At a high shear stress of 5 dyne/cm<sup>2</sup>, no effect was obtained. The lack of effect after perfusion at 5 dyne/cm<sup>2</sup> could be due to the short contact of Stx1 with Gb3. In vivo, endothelin-1, also a constituent of WPBs, acts as a potent vasoconstrictor of the afferent arteriole and may limit the velocity in the glomerular capillaries. Serum endothelin-1 levels in serum are increased in the early phase of D + HUS [13].

With the current applied technique, still no perfect imitation of the in vivo situation can be obtained. The influence of platelets, leucocytes, monocytes, erythrocytes and cytokines or chemokines is ignored. The short incubation with Stx in flow conditions, however, reflects more adequately the in vivo situation compared with the continuous exposure during 24 h. In sera of patients with D + HUS, only negligible amounts of free Stx are present, as detected by the inhibition of protein synthesis [14]. Serum Stx2 levels measured with a chemiluminescence assay showed the highest values shortly after bloody diarrhea onset with a rapid decrease before the onset of HUS, but they are extremely low (5–20 pg/ml) and present in a minority of the patients [15]. Short repeated Stx peaks, taking into account the rapid clearance from blood within minutes, is an appealing concept.

From these experimental data, an adapted concept of the pathogenesis of D + HUS may emerge. What is first, complement or coagulation activation?

Short stimulation by Stx induces the release of WPBs, resulting in local coagulation with adhesion and activation of platelets. With the exception of clusterin (an inhibitor of membrane attack complex), WPBs do not contain complement components by the proteomic technique [5]. Recently, Rayes et al. [16] have demonstrated the presence of factor H in WPBs. This factor H inhibited the ADAMTS13-mediated proteolysis of VWF and showed high cofactor activity for factor I-mediated downregulation of complement activation. The coagulation process apparently precedes the activation of the complement system [17]. Local activation of the coagulation system will enhance the activation of the complement system via multiple pathways, one of them being P-selectin [18, 19]. Adherent platelets promote the secondary capture of neutrophils, mediating endothelial injury.

From the experimental studies about the effect of Stx on endothelial cells under flow conditions, including the results from Nolasco et al. [6], it can be suggested that the activation of the coagulation system (including VWF and P-selectin) is the initial factor of the injury of these cells. Interference in the coagulation system in a more effective way than with heparin, namely by hirudin or perhaps thrombomodulin combined with monoclonal antibodies to Stx, is an appealing possibility [20]. Thrombin bound to a thrombus is inaccessible by heparin [21]. No short-term benefit was obtained in the treatment of D + HUS by eculizumab, a monoclonal antibody directed against C5 [22]. In the acute phase of D + HUS, immunofluorescence microscopy showed staining for C1q but not for C3, C4 and properdin in renal tissue [23]. Proteomic analysis of laser-microdissected glomeruli should confirm these findings. A recent study by Lee et al. [24] underpins the proposed concept. In the nonhuman primate, HUS induced only by Stx challenge shows the full spectrum of HUS, including glomerular platelet-rich thrombi, but the complement system was not activated.

## Acknowledgment

This study was supported by the Dutch Kidney Foundation (PC153).

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