Histones stimulate von Willebrand factor release *in vitro* and *in vivo*

Histones, nuclear proteins that normally package DNA in cells, are increasingly recognized as important mediators of inflammation and thrombosis, and have recently been linked to death in sepsis.[1] They are released during inflammation by activated leukocytes, primarily neutrophils, as part of neutrophil extracellular traps (NETs).[2] Although NETs play an important role in innate immunity, components within NETs, namely histones, are linked to platelet activation[3] and the development of thrombi.[4] Indeed, mice that are unable to produce NETs have decreased thrombus formation.[5] Although most descriptions of the prothrombotic effects of histones are based on activation of platelets and fibrin formation, less is known about their effects on endothelial cells.

The vascular endothelium plays an integral role in the regulation of blood flow, permeability, and prevention of thrombus formation.[6] When the vascular endothelium becomes activated or damaged, it can result in a hypercoagulable state, mediated in part through the release of ultra-large (UL) von Willebrand factor (VWF) which leads to the capture and activation of platelets and formation of thrombi.[7] Increased plasma VWF and ULVWF has been described in experimental and clinical sepsis,[8,9] and is suggested to contribute to thrombosis in this condition.

Although there are several known stimuli for the release of VWF, one novel stimulus may be through the release of histones from activated neutrophils. We had previously reported a dose-dependent effect of histones on platelet aggregation.[3] However, the role of histones on endothelium, specifically VWF release, is unclear. In this report, we describe the effect of histones on VWF release from endothelial cells *in vitro* and *in vivo*.

We evaluated whether histones induced ULVWF

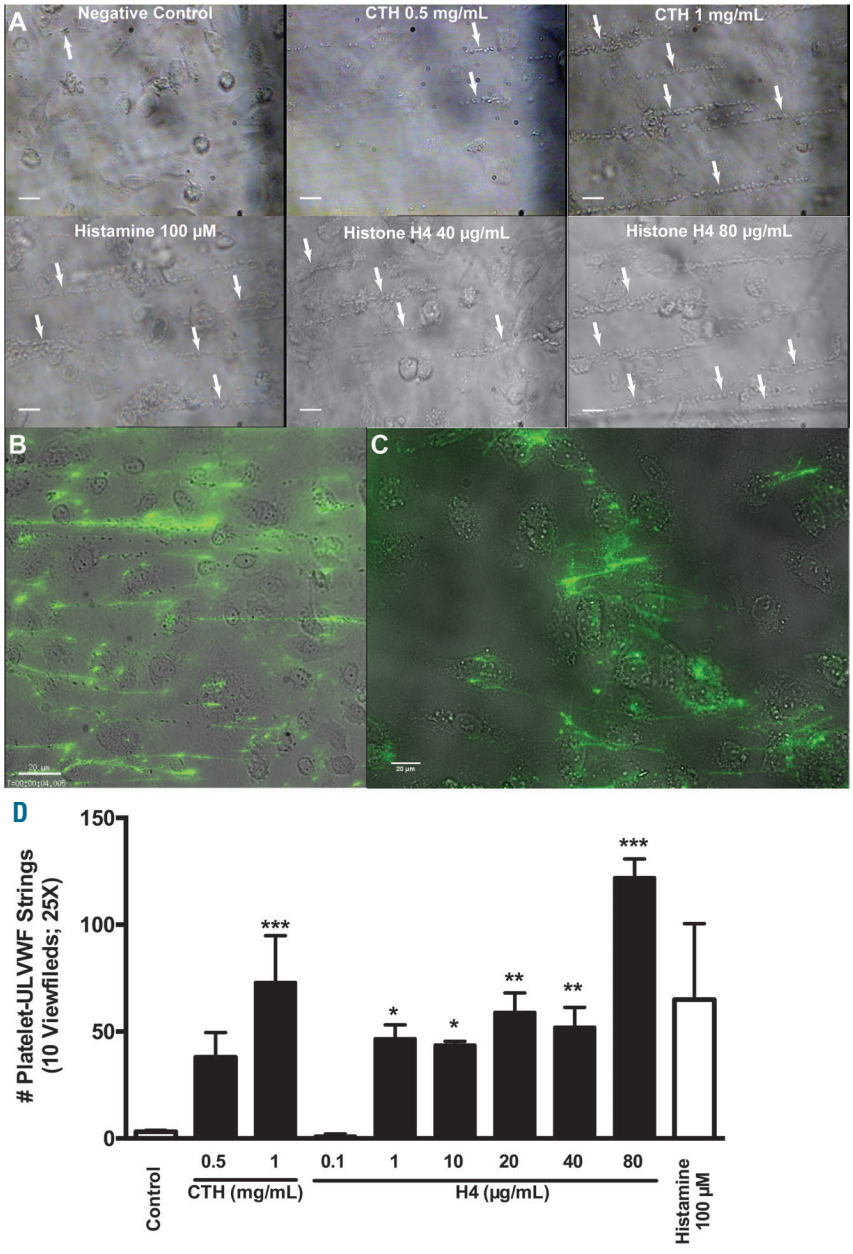

Figure 1. Exogenous histones increase the number of platelet-ultra-large von Willebrand factor (ULVWF) strings (arrows) during parallel-plate flow (700 s^{-1}). (A) Phase contrast images of lyophilized platelet-ULVWF strings over HUVEC stimulated with various agonists. (B and C) Merged differential interference contrast and fluorescence image of ULVWF strings after labeling with an FITC-conjugated anti-VWF antibody in the presence (B) and absence (C) of platelets. Note the co-localization between platelet strings and VWF in (B). (D) Stimulation of HUVEC with calf thymus histone (CTH) or histone H4 significantly increased the number of platelet-ULVWF strings. Scale bar = 20 µm. N=3-7 experiments per group. *P<0.05, **P<0.01, ***P<0.001 *versus* control.

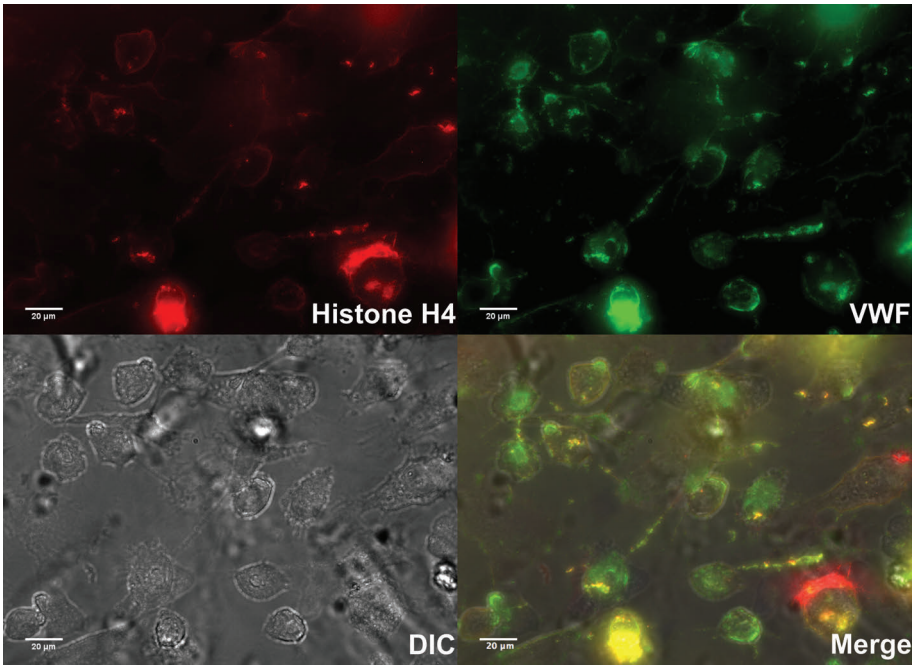

Figure 2. Histone H4 (red) binds to both endothelial cell borders as well as co-localizes (yellow) with external von Willebrand factor (VWF) (green) on histone-stimulated HUVEC during parallel-plate flow (700 s^{-1}). (Top left) AF647-conjugated anti-histone H4 antibody. (Top right) FITC-conjugated anti-VWF antibody. (Bottom left) Differential interference contrast. (Bottom right) Merge. Scale bar = 20 μm.

release from human umbilical vein endothelial cells (HUVEC) using a parallel plate flow chamber system. HUVEC monolayers were stimulated for 5 min with varying doses of recombinant histone H4 (New England Biolabs) or calf thymus histone (CTH; Worthington Biochemical). We used concentrations in the range of plasma histones measured in mice exposed to experimental sepsis and in humans with severe non-thoracic blunt trauma-induced acute lung injury (10-230 μg/mL); these concentrations stimulate aggregation of human platelets, although their effect on VWF release from endothelial cells is unknown.[1,3,10,11] Histamine (100 μM) was used as a positive control for ULVWF release. The stimulated HUVEC were washed in phosphate buffered saline and then ULVWF strings were visualized by perfusing lyophilized platelets over stimulated HUVEC at 700 sec^{-1} for 200 sec. Lyophilized platelets were used because they quickly interact with the released ULVWF strings at that shear rate, do not release VWF, and are not required for VWF release.[12] Ten 25X fields of view using differential interference contrast were video recorded and compared among the different stimuli (Figure 1A). We confirmed the presence of ULVWF strings by perfusing fluorescein isothiocyanate (FITC)-conjugated anti-VWF antibody over histone-stimulated HUVEC in the presence (Figure 1B) and absence (Figure 1C) of lyophilized platelets. This demonstrates that lyophilized platelets are not necessary for the production of ULVWF strings in our model. The addition of histones significantly increased the number of platelet-ULVWF strings as compared to vehicle control in a dose-dependent fashion (Figure 1D). Lyophilized platelets did not adhere to or form strings on culture dishes exposed to histones but devoid of HUVEC (*data not shown*). To determine where histones were binding, we perfused both FITC-conjugated anti-VWF and AF647-conjugated anti-histone H4 antibodies over histone-stimulated HUVEC. We observed that histone bound to both endothelial cell borders and to VWF (Figure 2). The observation of VWF-histone co-localization is consistent with a previously published report that histone binds to the A1 domain of VWF.[13] Based on prior reports that histones induce endothelial cytotoxicity,[1,14] we performed live-dead cell staining of HUVEC exposed to various doses of histones under the same conditions as the flow experiments. Stimulated HUVEC were washed and then labeled with Sytox Green (staining dead cells) either immediately or after 60 min. HUVEC were then washed, fixed with paraformaldehyde, and then counterstained with 4',6'-diamidino-2-phenylindole (DAPI) to label all nuclei. There was no difference in cell death immediately or 60 min after histone stimulation (Figure 3). These data suggest that the increased presence of platelet-ULVWF strings that we observed was due to active release of ULVWF and not to increased cell death.

Based on our *in vitro* observations, we then determined the effects of histones on VWF release *in vivo*. In addition to measuring plasma VWF levels, we also measured circulating platelet counts and thrombin-antithrombin (TAT) levels (Assaypro LLC), which are altered in experimental and clinical sepsis.[9,15] All animal procedures were approved by the Institutional Animal Care and Use Committee. CTH (75 mg/kg)[1] or phosphate buffered saline (control) was injected intravenously into anesthetized male C57BL/6 mice 30 min prior to whole blood collection in 3.2% sodium citrate. The blood was centrifuged to obtain platelet-free plasma and used to measure plasma VWF levels using enzyme linked immunosorbent assay (ELISA; Ramco Laboratories), as we have previously described.[15] Similar to our previous observations in endotoxemic mice,[15] mice exposed to exogenous histones had significantly elevated plasma VWF levels as compared to control animals (171%±28% *vs.* 100%±14% of control values; n=6/grp; mean ± SEM; $P<0.05$). In addition, histone-treated mice also had significantly decreased circulating platelet counts (0.86 ± 0.05x10^6 plt/μL *vs.* 1.12±0.08x10^6 plt/μL; n=6/group; mean±SEM; $P<0.05$), and elevated thrombin-anti-thrombin complexes (4.48±0.41 ng/mL *vs.* 0.56±0.15 ng/mL;

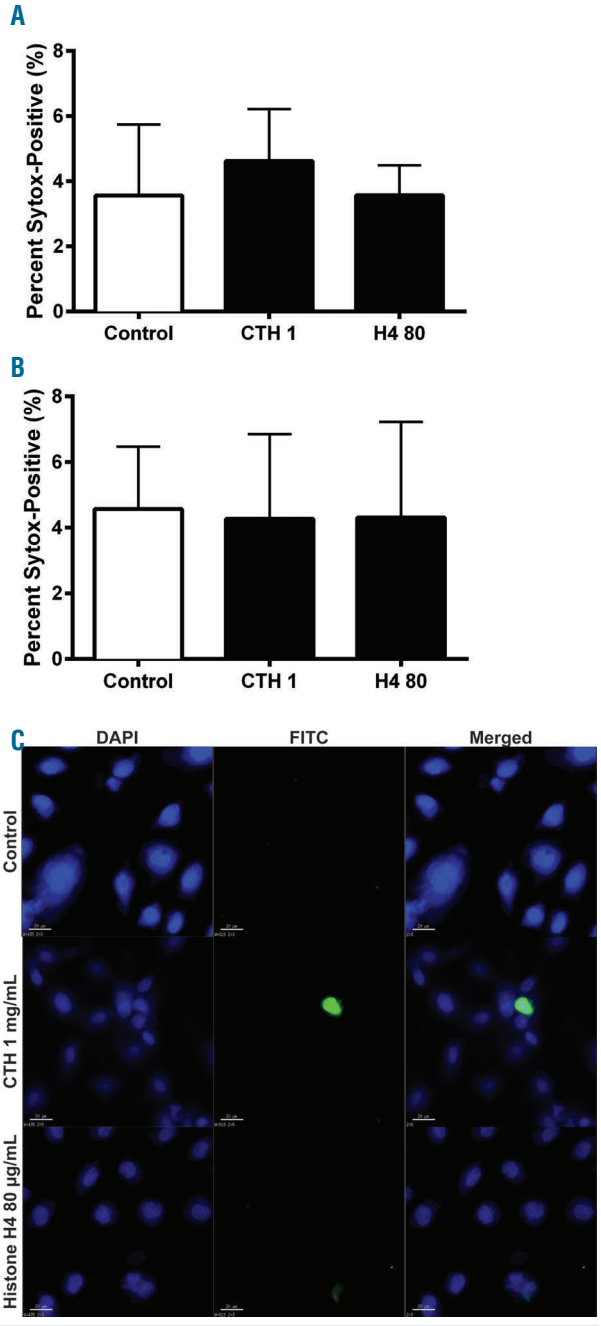

Figure 3. There was no increase in Sytox green-positive HUVEC (dead cells) when observed either immediately (A) or 60 min (B) after a 5-min incubation period with histones as compared to control (calf thymus histones 1 mg/mL, CTH 1; histone H4 80 µg/mL, H4 80; n=3-6 per group). (C) Fluorescence staining with DAPI (left), FITC (middle), and merged images (right). Scale bar = 20 µm.

n=6/group; mean±SEM; $P<0.01$). We did not detect a significant change in high molecular weight VWF multimers in the plasma of mice receiving CTH (*data not shown*).

Our data demonstrate that histones bind to and stimulate endothelial cell release of ULVWF *in vitro* and increase plasma VWF levels *in vivo*. *In vivo*, administration of CTH did not significantly increase the plasma level of high molecular weight VWF multimers. We speculate that this may be due to the effect of circulating a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13, which was absent from our *in vitro* experiments. To our knowledge, this is the first reported observation that histones induce release of ULVWF from endothelial cells and may represent a target to minimize inflammation-induced thrombosis.

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