Functional responses and molecular mechanisms involved in histone-mediated platelet activation

Agostina Carestia[1*]; Leonardo Rivadeneyra[1*]; María Albertina Romaniuk[1]; Carlos Fondevila[2]; Soledad Negrotto[1]; Mirta Schattner[1]

[1]Laboratory of Experimental Thrombosis, Institute of Experimental Medicine, CONICET-National Academy of Medicine, Buenos Aires, Argentina; [2]Service of Hematology, Bazterrica Clinic, Buenos Aires, Argentina

Summary

Histones are highly alkaline proteins found in cell nuclei and they can be released by either dying or inflammatory cells. The recent observations that histones are major components of neutrophil extracellular traps and promote platelet aggregation and platelet-dependent thrombin generation have shown that these proteins are potent prothrombotic molecules. Because the mechanism(s) of platelet activation by histones are not completely understood, we explored the ability of individual recombinant human histones H1, H2A, H2B, H3 and H4 to induce platelet activation as well as the possible molecular mechanisms involved. All histones were substrates for platelet adhesion and spreading and triggered fibrinogen binding, aggregation, von Willebrand factor release, P-selectin and phosphatidylserine (PS) exposure and the formation of platelet-leukocyte aggregates; however, H4 was the most potent. Histone-mediated fibrinogen binding, P-selectin and PS exposure and the formation of mixed aggregates were potentiated by thrombin. Histones induced the activation of ERK, Akt, p38 and NFκB. Accordingly, histone-induced platelet activation was significantly impaired by pretreatment of platelets with inhibitors of ERK (U 0126), PI3K/Akt (Ly 294002), p38 (SB 203580) and NFκB (BAY 11–7082 and Ro 106–9920). Preincubation of platelets with either aspirin or dexamethasone markedly decreased fibrinogen binding and the adhesion mediated by histones without affecting P-selectin exposure. Functional platelet responses induced by H3 and H4, but not H1, H2A and H2B, were partially mediated through interaction with Toll-like receptors –2 and –4. Our data identify histones as important triggers of haemostatic and proinflammatory platelet responses, and only haemostatic responses are partially inhibited by anti-inflammatory drugs.

Keywords

Histones, platelets, neutrophil extracellular traps, inflammation

Correspondence to:
Mirta Schattner
Instituto de Medicina Experimental, CONICET-ANM
Pacheco de Melo 3081, Buenos Aires 1425, Argentina
Tel.: +54 11 4805 5759 ext. 301, Fax: +54 11 4805 0712
E-mail: mschattner@hematologia.anm.edu.ar

* These authors contributed equally to this work.

Financial support:
This study was financially supported by a grant from the National Agency for Scientific and Technological Promotion (Argentina) 2011–0733.

Received: February 26, 2013
Accepted after major revision: July 24, 2013
Prepublished online: August 22, 2013

doi:10.1160/TH13-02-0174
Thromb Haemost 2013; 110: 1035–1045

Introduction

Although the primary role of platelets is to prevent blood loss, many basic and clinical research studies have shown that these enucleated cells are also critically involved in several physiopathologic cellular processes, including inflammation, angiogenesis, cancer and the immune response (1-3). The ability of platelets to participate in so many different biological scenarios is related to the fact that once activated, they synthetise, express or secrete a wide array of proteins that allow their interaction with and/or activation of many types of vascular cells, including endothelial cells, fibroblasts, smooth muscle cells and circulating leukocytes (4). In this latter context, it has been recently demonstrated that platelets, detect Toll-like receptor (TLR)-4 ligands in the blood via TLR-4 and bind to adherent neutrophils; this results in their activation and the formation of neutrophil extracellular traps (NETs) (5). Through this mechanism, platelets participate in the innate immune response by enhancing the confinement of bacteria in blood vessels. NETs were initially described as a novel mode of neutrophil pathogen killing (6). In contrast to the classical mechanism of intracellular phagocytosis of microbes, neutrophils can also release nuclear content and form DNA strands after stimulation, which also contain histones and microbicidal proteins such as elastase, cathepsin G, and myeloperoxidase (5, 7, 8). Once attached to these networks, histones, as well as other antimicrobial proteins, degrade virulence factors and kill pathogens. The NET not only prevents the spread of pathogens from the initial site of infection but also concentrates the antimicrobial proteins at the site of infection (6).

Histones are basic low-molecular-weight proteins that are responsible for DNA organisation. In humans, there are five main types: the linker histone H1 and the core histones H2A, H2B, H3 and H4 that with DNA, form the nucleosomes that constitute the primary component of chromosomes (9).

The role of extracellular histones is an important emerging question. It is known that these proteins are a double-edged sword because they have both antimicrobial actions and cytotoxic properties, as histones have been observed in the circulation and have been shown to contribute to death in septic mice (10). Fur-

thermore, elevated plasma nucleosome levels have been detected in septic patients, and in some cases, these levels correlate with sepsis and mortality (11).

NETs not only exhibit antibacterial functions but also strongly induce procoagulant activity (12, 13). Together with elastase, extracellular histones are relevant mediators of the prothrombotic activity of NETs (14, 15). DNA strands decorated with histones provide a scaffold for platelet adhesion, aggregation and prothrombinase activity that allows for fibrin deposition and thrombus formation (16). These findings revealed that NETs and histones represent a link between inflammation and thrombosis. Increasing evidence suggests that the interplay between neutrophils/monocytes, platelets and NET formation inside the vasculature, both in infections and non-infectious diseases, is intimately involved in the initiation and amplification of deep vein thrombosis (13).

Given the increasing interest regarding histones and the importance of platelets in thrombosis and inflammation, we explored the ability of individual recombinant human H1, H2A, H2B, H3 and H4 to induce several platelet activation responses and investigated the mechanisms involved in these phenomena. We observed that each histone triggers haemostatic and proinflammatory platelet responses through activation of the ERK, AKT, p38 and NFκB signalling pathways and that anti-inflammatory drugs partially attenuate only the haemostatic response.

Materials and methods

Reagents

Human α-thrombin was purchased from Enzyme Research Labs (Swansea, UK). Fibrinogen, TRITC-phalloidin, the FITC-Annexin-V Apoptosis Kit, acetyl salicylic acid (aspirin, ASA) and dexamethasone (DEX) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Alexa 488-fibrinogen was purchased from Invitrogen (Eugene, OR, USA). Recombinant human H1, H2A, H2B, H3 and H4 were purchased from New England Biolabs (Ipswich, MA, USA). PE-conjugated anti-human CD62P, FITC-anti-CD45, PE-anti-CD61, FITC, PE and unlabeled irrelevant IgG1, mouse anti-IκB-α and mouse anti-β-actin were obtained from BD Biosciences (San José, CA, USA). Mouse anti-human CD62P, mouse anti-phospho ERK1/2 (Tyr 204), rabbit anti-phospho p38, mouse anti-phospho NFκB p65 (Ser311) and HRP-conjugated anti-rabbit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-phospho Akt (Tyr473) was purchased from Abcam (Cambridge, UK). Blocking antibodies against human TLR-2 (clone T2.5) and TLR-4 (HTA125) were obtained from eBioscience (San Diego, CA, USA).

The MEK inhibitor bis[amino[(2-aminophenyl)thio]methylene]butanedinitrile (U 0126), the PI3 kinase (PI3K) inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (Ly 294002), the p38 MAP kinase inhibitor 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1H-imidazole-5-yl]pyridine (SB 203580) and the NFκB inhibitor 3-[(4-methylphenyl)sulfonyl]-(2E)-propenenitrile (BAY 11–7082) were obtained from Biomol (Plymouth Meeting, PA, USA) and the NFκB inhibitor 6-(phenylsulfinyl)tetrazolo[1,5-b]pyridazine (Ro 106–9920) was purchased from Tocris (Ellisville, MO, USA). These drugs were dissolved in DMSO. The final DMSO concentration (0.5% v/v) did not show a toxic effect.

Preparation of human platelets

Blood samples were obtained from healthy donors who had not taken any non-steroidal anti-inflammatory drugs for 10 days prior to sampling. This study was performed according to institutional guidelines (National Academy of Medicine, Buenos Aires, Argentina) and was approved by the Institutional Ethics Committee. Written consent was obtained from all subjects. Blood was drawn directly into plastic tubes containing 3.8% sodium citrate. Platelet-rich plasma (PRP) from normal donors was obtained by the centrifugation of blood samples (180 x *g* for 10 minutes [min]). For washed platelet (WP) suspensions, PRP was centrifuged in the presence of 75 nM prostacyclin (PGI_2) (Cayman, Ann Arbor, MI, USA). After washing in wash buffer, the WP were resuspended in Tyrode's buffer at $3x10^8$/ml. Platelet suspensions were kept at room temperature (RT) for 30 min prior to experimentation. Unless otherwise stated, Ca^{2+} (1 mM) was added prior to platelet stimulation.

Platelet spreading

Glass slides were coated with either recombinant histones (1 μM) or fibrinogen (100 μg/ml) and blocked with 2% BSA for 2 hours. WP ($5x10^7$/ml) were then plated and incubated for 20 min. In some experiments, WP were pretreated with various inhibitors (10 μM U 0126, 5 μM Ly 294002, 50 μM SB 203580, 25 μM BAY 11–7082, 12.5 μM Ro 106–9920) for 5 min, anti-inflammatory drugs (1 mM ASA and 10 μM DEX) for 30 min or mAbs against human TLR-2 (50 μg/ml) and TLR-4 (50 μg/ml) for 20 min at RT. Adherent platelets were fixed (4% PFA), permeabilised (0.1% Triton X-100) and stained with TRITC-phalloidin. The images were analysed using ImageJ (NIH, Bethesda, MD, USA).

Flow cytometry analysis and binding assays

WP ($3x10^8$/ml) were stimulated, fixed and stained with a PE-CD62P (anti-P-selectin) or an equivalent amount of isotype-matched control Ab in phosphate-buffered saline (PBS) containing 0.1% foetal bovine serum (FBS). In selected experiments, WPs were pretreated with the inhibitors U 0126 (10 μM), Ly 294002 (5 μM), SB 203580 (50 μM), BAY 11–7082 (25 μM) and Ro 106–9920 (12.5 μM) for 5 min, anti-inflammatory drugs (1 mM ASA and 10 μM DEX for 30 min or mAbs against human TLR-2 (50 μg/ml) and TLR-4 (50 μg/ml) for 20 min at RT. To measure fibrinogen binding, platelets were stimulated in the presence of Alexa-488 fibrinogen (10 μg/ml). To measure phosphatidylserine (PS) exposure, stimulated platelets were labelled with FITC-annexin V for 10 min at RT in the dark. Samples were analysed by flow cytometry on a FACSCalibur flow cytometer using CELLQUEST soft-

ware (BD Biosciences, Franklin Lakes, NJ, USA), and the results were expressed as the mean fluorescence intensity (MFI), except in the case of the PS exposure results which were expressed as % positive cells.

Measurement of von Willebrand factor (vWF) levels

WP ($3x10^8$/ml) were stimulated with either thrombin (0.05 U/ml) or recombinant histones (1 μM) for 15 min. The reaction was halted by the addition of PBS containing 75 nM PGI_2. The samples were centrifuged twice at 1,100 x *g* for 5 min followed by 9,300 x *g* for 5 min, and the supernatants were stored at –80°C until assayed. vWF release was determined by ELISA as previously described (17) using human vWF Ab and HRP-conjugated human vWF Ab as the primary and secondary Ab, respectively.

Platelet-neutrophil mixed aggregate assays

Human neutrophils were prepared by Ficoll-Hypaque gradient centrifugation and dextran sedimentation as previously described (18). Equal volumes of WPs ($2x10^8$/ml) and neutrophils ($5x10^6$/ml) were incubated together in Tyrode's buffer for 20 min, followed by stimulation with either Thr (0.05 U/ml) or histones (1 μM) for 5 min. When it was indicated platelets were pretreated with anti-human CD62P for 20 min. The samples were fixed with 1% PFA followed by staining with either FITC-CD45 and PE-CD61 Abs or equivalent amounts of FITC and PE-labelled isotype-matched controls and then analysed by flow cytometry. The results are expressed as the percentage of $CD45^+/CD61^+$ events within the total $CD45^+$ population.

Immunoblotting

WP were stimulated with histones (1 μM) at 37°C, and the reaction was halted by adding loading buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% 2-mercaptoethanol). Equal amounts of proteins were electrophoresed on a 10% acrylamide gel by SDS-PAGE and electrotransferred to nitrocellulose membranes (GE Healthcare, Buckinghamshire, UK). After blocking, the membranes were incubated overnight at 4°C with primary Ab followed by an HRP-conjugated secondary Ab. The protein bands were visualised using the ECL reaction. The immunoblot results were quantified using Gel-Pro Analyzer 3.1 software and the values from reprobing the blot were used for monitoring equal protein loading.

Statistical analysis

The data are expressed as the means ± SEM. ANOVA and the Newman-Keuls test were employed to determine the significance of differences between groups. A p-value less than 0.05 was considered to be statistically significant.

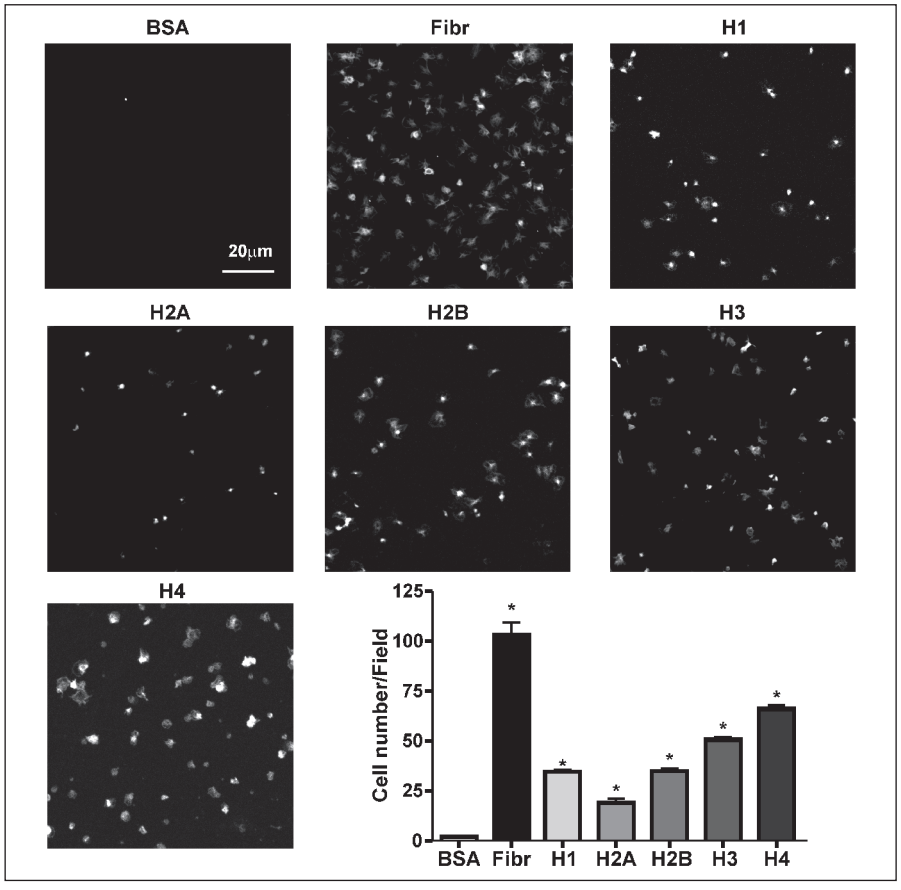

Figure 1: Platelet adhesion and spreading mediated by histones. WP ($5x10^7$/ml) were plated on either 1 μM histone- or 100 μg/ml fibrinogen (Fibr)-coated slides for 20 min, fixed and stained with TRITC-phalloidin. BSA was used as negative control. Platelet spreading was visualised by confocal microscopy. The images are representative of three independent experiments (*p<0.05 vs BSA).

Results

Histones promote platelet adhesion and spreading

It has been shown that NETs are good substrates for platelet adhesion (16, 19); therefore, we first investigated whether immobilised histones were capable of stimulating platelet adhesion and spreading. As shown in ►Figure 1, platelet adhesion was observed on slides coated with equimolar concentrations of each histone (1 μM; H1: 20.8 μg/ml, H2A: 14.1 μg/ml, H2B: 13.7 μg/ml, H3: 15.4 μg/ml, H4: 11.2 μg/ml), showing that each histone was capable of independently inducing platelet adhesion. Platelet spreading was only observed on slides coated with 1 μM H2B, H3 and H4. Higher concentrations of H1 and H2A (50 μg/ml) were required to induce spreading (data not shown).

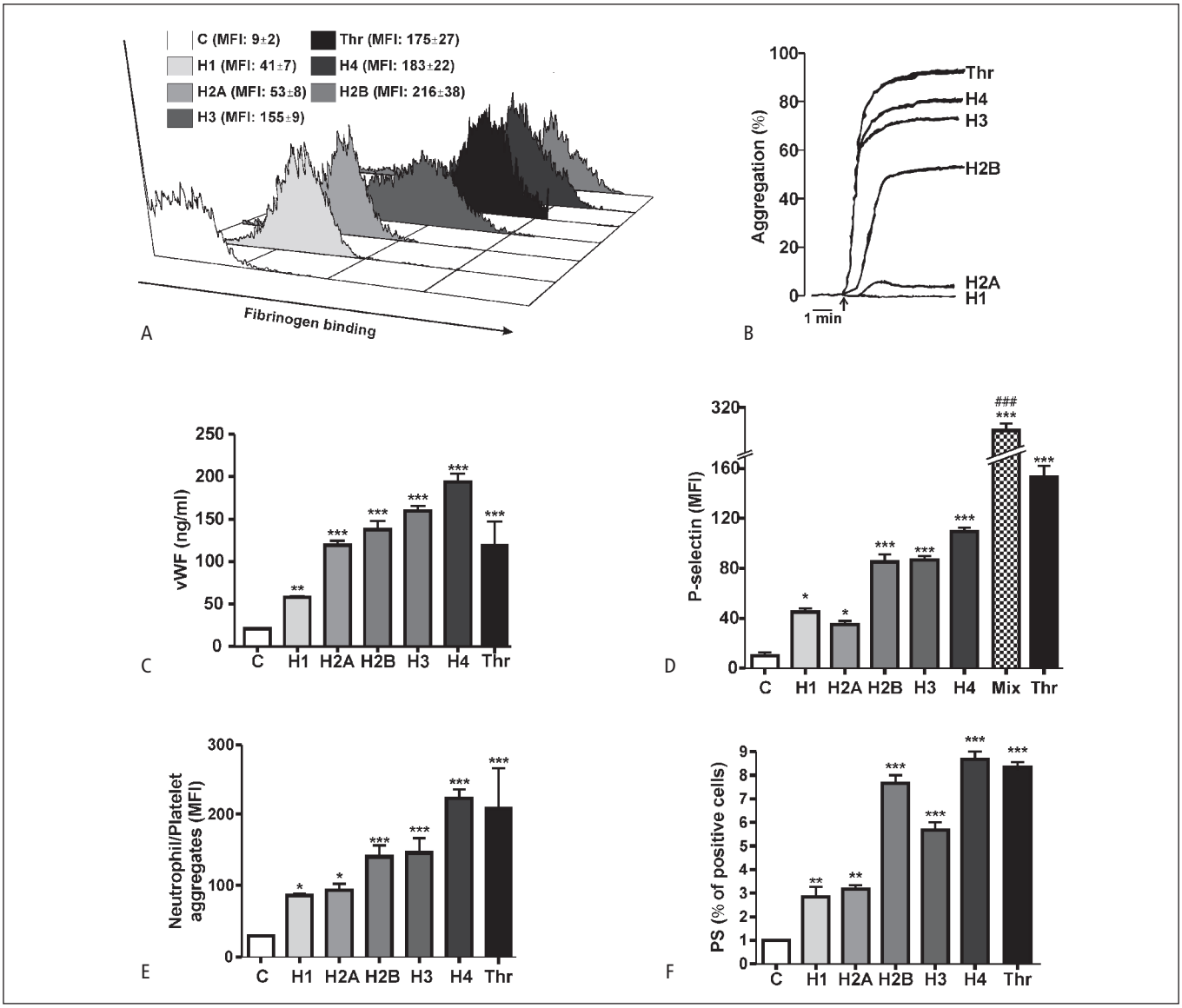

Figure 2: Histones trigger fibrinogen binding, platelet aggregation, vWF release, P-selectin expression, the formation of platelet-leukocyte aggregates and PS exposure. WPs ($3x10^8$/ml) were stimulated with either thrombin (Thr, 0.05 U/ml), histones (1 μM) or a mix of histones (each one at 1 μM) followed by the measurement of (A) fibrinogen binding, (B) platelet aggregation, (C) vWF release (ELISA) and (D) P-selectin expression. E) Equal volumes of WPs ($2x10^8$/ml) and neutrophils ($5x10^6$/ml) were incubated together in Tyrode's buffer for 20 min, followed by stimulation with either Thr (0.05 U/ml) or histones (1 μM) for 5 min. The results are expressed as the percentage of $CD45^+/CD61^+$ events within the total $CD45^+$ population. F) WPs ($3x10^8$/ml) were stimulated with either thrombin (Thr, 0.05 U/ml) or histones (1 μM), and PS exposure was measured. The samples shown in (A), (D), (E) and (F) were analysed by flow cytometer on a FACSCalibur flow cytometer using CELLQUEST software. The images are representative of five independent experiments (*p<0.05, **p<0.01 and ***p<0.001 vs C (control); ###p<0.001 vs histones (H1, H2A, H2B, H3 and H4).

Histones trigger fibrinogen binding, platelet aggregation, vWF release, P-selectin expression, the formation of platelet-leukocyte aggregates and PS exposure

It has been previously demonstrated that histones induce platelet aggregation in a fibrinogen-dependent manner (19). We evaluated the effects of individual recombinant human histones on platelet fibrinogen binding. Flow cytometry analysis showed that all five histones triggered fibrinogen binding, with H2B and H4 being the most potent (►Figure 2A). Unexpectedly, the high level of fibrinogen binding induced by H2B was not reflected in the platelet aggregation assays; the platelet aggregation triggered by H2B was significantly lower than that induced by H3 or H4 (►Figure 2B).

Because it has been suggested that the stimulation of vWF secretion could be one of the mechanisms responsible for the observed *in vivo* prothrombotic effect of histones (20), we explored the ability of histones to induce the release of vWF stored in platelet α-granules. The supernatants from platelets stimulated with all five histones contained a significantly greater amount of vWF than the control samples (►Figure 2C).

P-selectin is another cell adhesion molecule that is stored in α-granules. Upon platelet activation, P-selectin is exposed on the platelet membrane and mediates interactions with both leukocytes and endothelial cells (21). Because platelet-leukocyte interactions are increasingly considered to be key mediators of thrombus formation (13) and previous studies have shown that histones can trigger P-selectin expression (14), we examined the effect of each histone on P-selectin expression and correlated these effects with the formation of mixed platelet-leukocyte aggregates. As shown in ►Figure 2D and E, both platelet responses were triggered by H1, H2A, H2B, H3 and H4. Because histones bind to many cell types, we examined whether the formation of platelet-leukocyte aggregates was mediated by P-selectin or histones using an Ab to block P-selectin. Under these conditions, the formation of mixed aggregates ocurred in 15%, 8%, 20%, 18% and 25% of control samples for H1, H2A, H2B, H3 and H4, respectively, indicating that the histone-mediated induction of platelet-leukocyte aggregates was primarily dependent on P-selectin exposure.

When platelets were stimulated with a combination of the five histones (1 μM each), the MFI of cells that expressed P-selectin was higher than that induced by H4 alone (►Figure 2D).

Another interesting effect of histones on platelet activation is the induction of platelet procoagulant activity through the expression of PS and FV/Va (14). Thus, we analysed the ability of each histone to induce the expression of PS on the platelet surface. Flow cytometry studies showed that platelet stimulation with histones resulted in a significant increase in the percentage of Annexin-V positive cells. Similar to the fibrinogen binding assays results, H2B and H4 were the most potent histones (►Figure 2F).

Histones potentiate platelet activation mediated by thrombin

Because it was demonstrated that histone-activated platelets possess a procoagulant phenotype that drives thrombin generation (14), we examined the effect of the combination of threshold concentrations of thrombin and histones on platelet stimulation. A

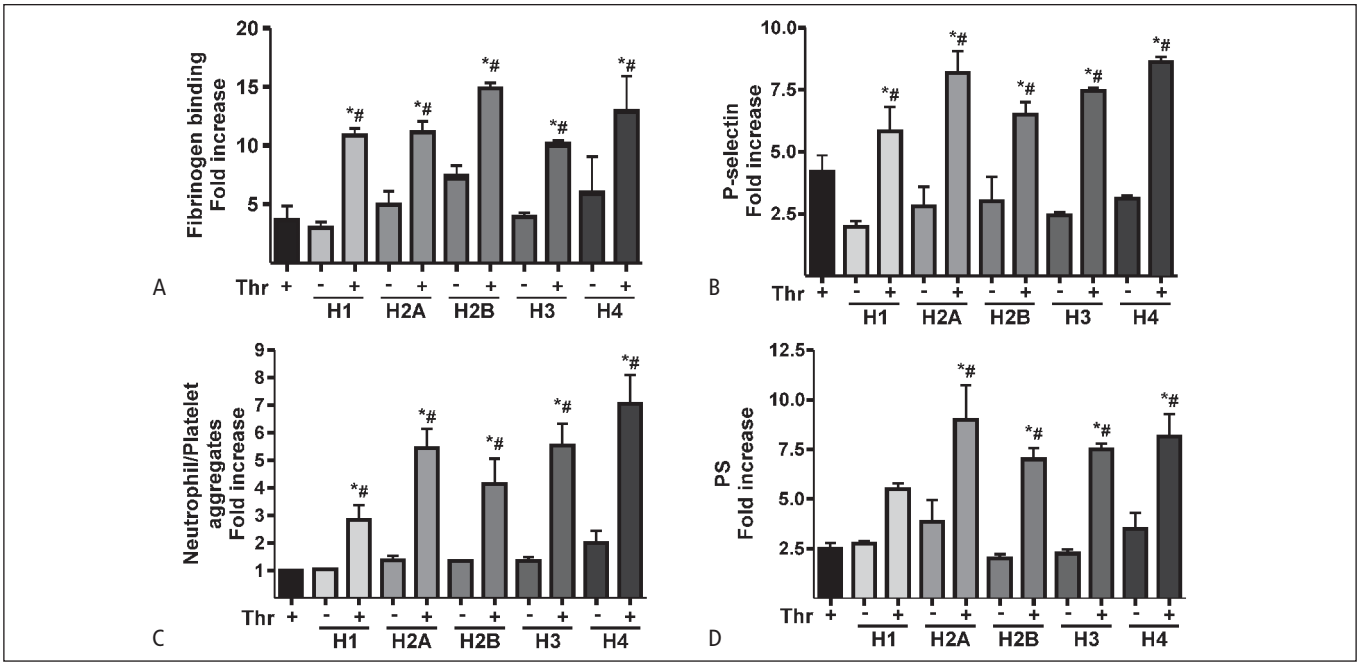

Figure 3: Histones potentiate platelet activation mediated by thrombin. WPs (3x10^8/ml) were stimulated with threshold concentrations of histones (H1 and H2A: 10 μg/ml; H2B, H3 and H4: 3 μg/ml) and thrombin (Thr: 0.01 U/ml), followed by the measurement of (A) fibrinogen binding, (B) P-selectin expression, (C) neutrophil/platelet aggregates and (D) PS exposure. Samples were analysed by flow cytometry (n=5) (*p<0.05 vs Thr, #p<0.05 vs histones (H1, H2A, H2B, H3 and H4)).

concentration-response curve was performed for each histone to determine the threshold concentrations of the agonists (see Suppl. Figure 1, available online at www.thrombosis-online.com).

The fibrinogen binding, P-selectin exposure, platelet-leukocyte aggregates and PS externalisation mediated by the combination of thrombin and each histone was significantly higher than that induced by each agonist alone. While the effects on fibrinogen binding and P-selectin were additive, the effect on mixed aggregate formation and PS exposure were synergistic (►Figure 3A-D).

Signalling pathways involved in platelet activation mediated by histones

To identify the molecular basis of platelet activation mediated by histones, we analysed the contributions of the major signalling pathways involved in activation triggered by classical platelet agonists.

Platelet stimulation with each histone induced the phosphorylation of ERK (►Figure 4). Unexpectedly, H1 was as potent as H4. Additionally, all of the histones efficiently induced the phosphorylation of Akt and p38 (►Figure 4).

Platelet procoagulant activity triggered by histones appears to be mediated in part by TLR-2 and –4 (14). In nucleated cells, NFκB is an effector molecule downstream of TLR-2 and -4 (22). Because we have observed that the activation of NFκB is involved in platelet activation (23), we also evaluated this signalling pathway in platelets stimulated by histones. ►Figure 4 shows that both the phosphorylation of the p65 subunit of NFκB and the degradation of the NFκB inhibitor, IκB, occured upon platelet activation mediated by H1, H2A, H2B, H3 and H4.

To further understand the functional effect of this phosphorylation pattern, we evaluated the roles of ERK, PI3K/Akt, p38 and NFκB on histone-induced platelet activation by treating the platelets with specific inhibitors (23-26). In these experiments, the platelet activation responses were triggered using the EC_{50} of each histone that was extrapolated from the concentration-response curves mentioned above (see Suppl. Figure 1, available online at www.thrombosis-online.com).

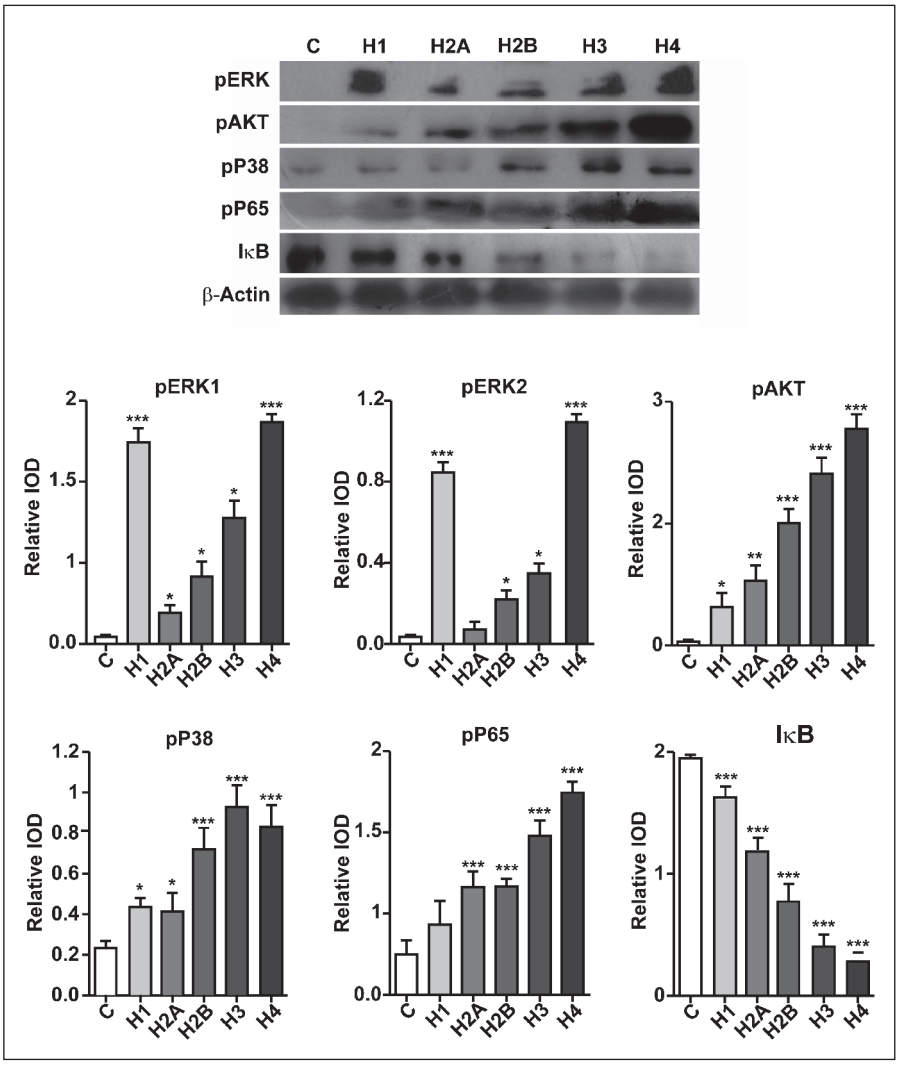

Figure 4: Signalling pathways involved in platelet activation mediated by histones. WPs ($1x10^9$/ml) were stimulated with histones (1 μM) at 37°C and the reaction was halted by adding loading buffer. The levels of ERK, AKT, p38 and p65 phosphorylation and IkB degradation were determined in cell lysates. Each membrane was reprobed with anti-β-actin antibody to calculate the relative IOD using Gel-Pro Analyzer 3.1 software. The images are representative of three independent experiments (*p<0.05, **p<0.01 and ***p<0.001 vs C (control)).

The pharmacological inhibition of these proteins resulted in a significant decrease of fibrinogen binding (►Figure 5A). Platelet adhesion and spreading stimulated by H4 were also significantly inhibited by blocking the ERK, PI3K/Akt, p38 and NFκB activation pathways (►Figure 5B). Similar results were obtained for the other histones (data not shown). Additionally, P-selectin expression mediated by all of the histones was inhibited in a concentration-dependent manner (►Figure 5C and Suppl. Figure 2, available online at www.thrombosis-online.com).

The effects of anti-inflammatory drugs on platelet activation mediated by histones

Considering that NET formation and histone release occur during inflammation, we analysed the effects of two of the most commonly used anti-inflammatory drugs (aspirin and dexamethasone) on histone-mediated platelet activation. The fibrinogen binding (►Figure 6A) and platelet adhesion and spreading (►Figure 6B) induced by each of the histones were markedly decreased when platelets were preincubated with either aspirin or dexamethasone; however, the expression of P-selectin was not modified (►Figure 6C).

The role of TLRs in histone-mediated platelet activation

It has been shown that TLR-2 and -4 partially mediate the generation of platelet prothrombinase activity induced by a mixture of histones (14). Thus, we investigated the roles of these receptors in platelet activation induced by recombinant histones using Abs to block TLR-2 and –4. Interestingly, the expression of P-selectin triggered by histones appeared to be selectively regulated. Although the preincubation of platelets with an Ab against TLR-2 and -4 inhibited the expression of P-selectin as well as the fibrinogen binding triggered by either H3 or H4, the Ab treatment showed no effects on P-selectin upregulation or fibrinogen binding mediated by H1, H2A or H2B (►Figure 7A and B). Regarding platelet adhesion and spreading, these responses were moderately but signifi-

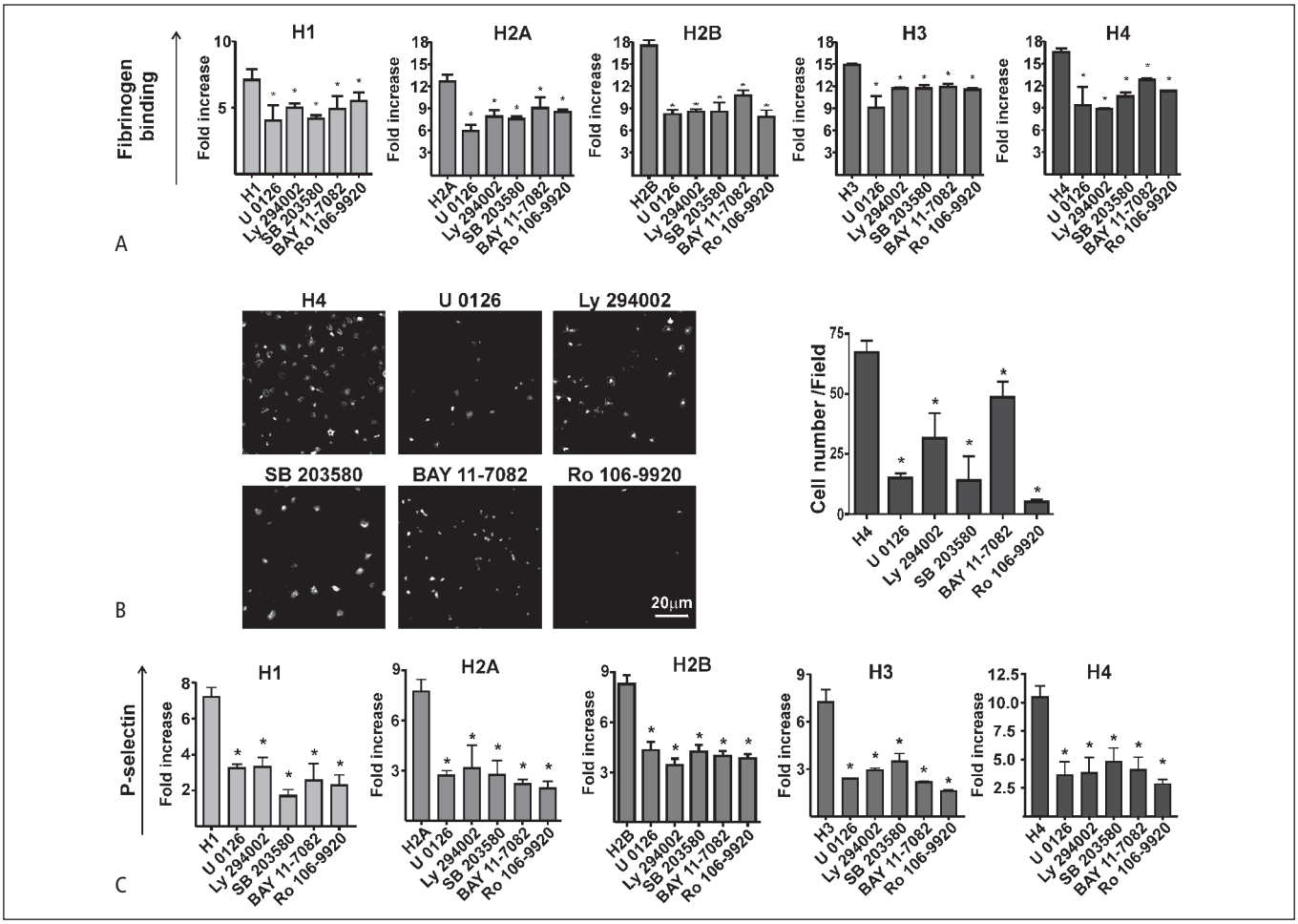

Figure 5: Pharmacological inhibition of histone-stimulated platelets. WPs ($3x10^8$/ml) were pretreated with vehicle (DMSO), U 0126 (10 μM), Ly 294002 (5 μM), SB 203580 (50 μM), BAY 11–7082 (25 μM) or Ro 106–9920 (12.5 μM) for 5 min, followed by treatment with the EC_{50} of each histone (H1 and H2A: 50 μg/ml; H2B, H3 and H4: 10 μg/ml) and (A) fibrinogen binding, (B) platelet spreading and (C) P-selectin expression were measured. In (B), only H4 is shown, as similar effects were observed for all the other histones (n=4). (*$p<0.05$ vs histones (H1, H2A, H2B, H3 and H4)).

cantly impaired in platelets that were preincubated with anti-TLR-2 or –4 Abs and stimulated with H4 but not with a control IgG2a (►Figure 7C).

Discussion

It was recently demonstrated that histones promote platelet aggregation (16, 19) and procoagulant activity (14). In this study, we analysed the action of each histone individually on platelet activation. We demonstrate that the stimulation of platelets with recombinant human H1, H2A, H2B, H3 or H4 resulted not only in haemostatic responses (adhesion and spreading, fibrinogen binding, platelet aggregation and the release of vWF) but also in proinflammatory (P-selectin and the formation of platelet-leukocytes aggregates) and procoagulant (PS exposure) platelet-mediated responses. Although all histones were capable of triggering platelet functional responses, H4, H3 and H2B were more potent than H2A and H1. These differences might be associated to the different aminoacid sequences of the histones, and thus their different tertiary structures, which could cause differences in their interactions with platelet receptors. In this sense, although the four core histones (H2A, H2B, H3 and H4) have similar three-dimensional structures, the histone fold regions of H2B, H3 and H4 exhibit the

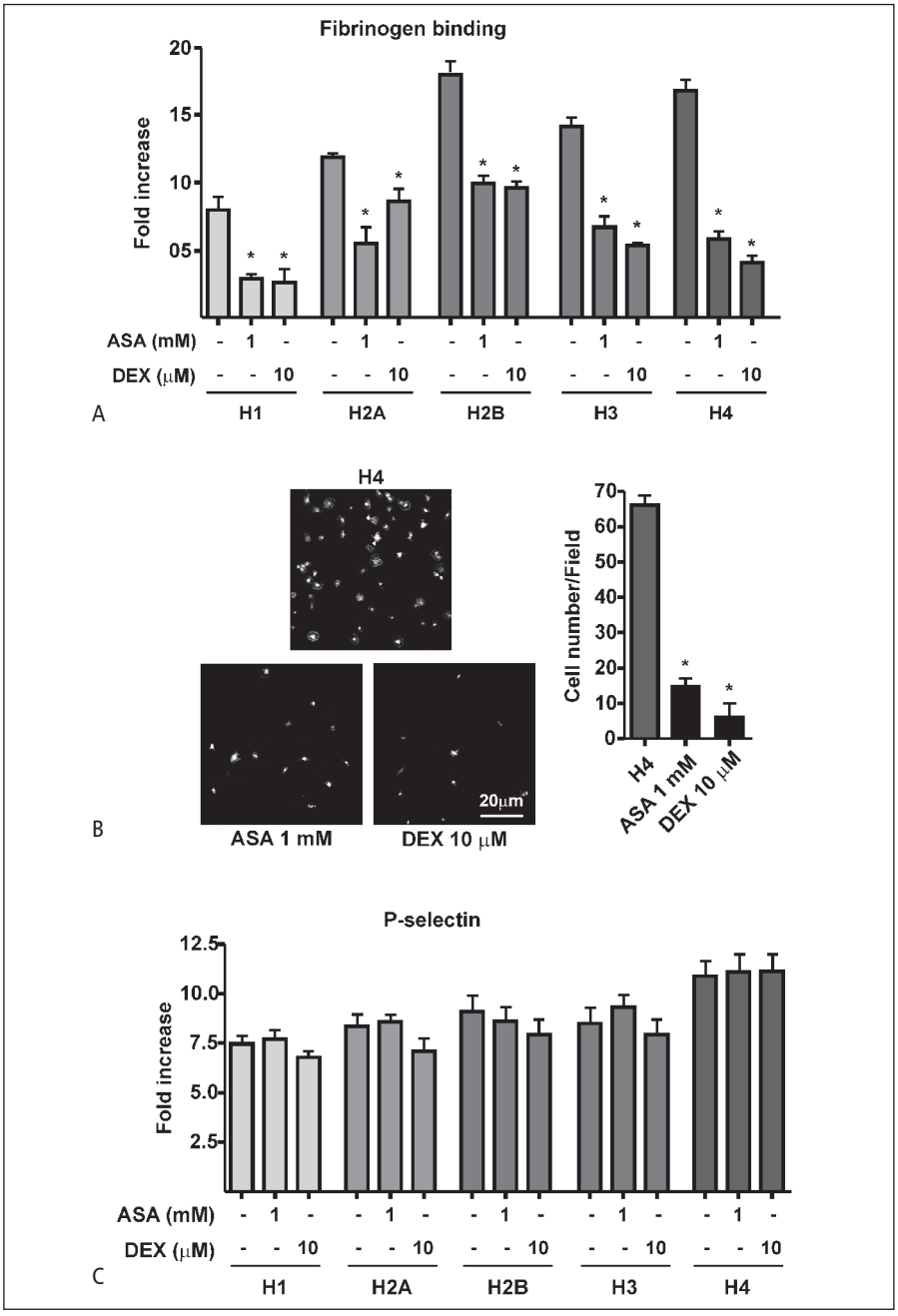

Figure 6: The effects of anti-inflammatory drugs. WPs ($3x10^8$/ml) were pretreated with either ASA (1 mM) or DEX (10 μM) for 30 min followed by stimulation with the EC_{50} of each histone (H1 and H2A: 50 μg/ml; H2B, H3 and H4: 10 μg/ml), and (A) fibrinogen binding, (B) platelet spreading and (C) P-selectin expression were measured. In (B), only H4 is shown, as similar effects were observed for all the other histones (n=4) (*p<0.05 vs histones (H1, H2A, H2B, H3 and H4)).

greatest similarity and H2A differs considerably from the other three histones (27). Furthermore, the linker histone H1 does not contain the histone fold motif (28). Alternatively, the difference in potency among these proteins might indicate that each histone binds to different platelet receptors. Although it remains unknown whether different types of infection are associated with a differential histone release, it has been reported that H3 and H4 are major mediators of tissue damage in different models of sepsis (10). However, the levels of other histones were not measured in that study, and the physiopathological roles of each histone in different diseases thus remain an interesting issue to be investigated. The ability of the histones present in NETs to trigger platelet aggregation is considered a major event linking infection and thrombosis (16). Our present data show that histones are efficient substrates for the initial steps involved in platelet activation, such as adhesion and spreading, providing further support for this theory. The contribution of platelets to the inflammatory response is due to the interaction of platelets with leukocytes and endothelial cells, which is primarily mediated by the expression of P-selectin (29). We now demonstrate that histones promote not only P-selectin expression on platelets but also the formation of mixed aggregates between platelets and polymorphonuclear leukocytes. Although histones bind to different cells, the generation of these mixed aggregates was almost entirely suppressed by a blocking Ab against P-selectin, indicating that this protein has a major role as a linker between platelets and leukocytes. With regard to sepsis, the bind-

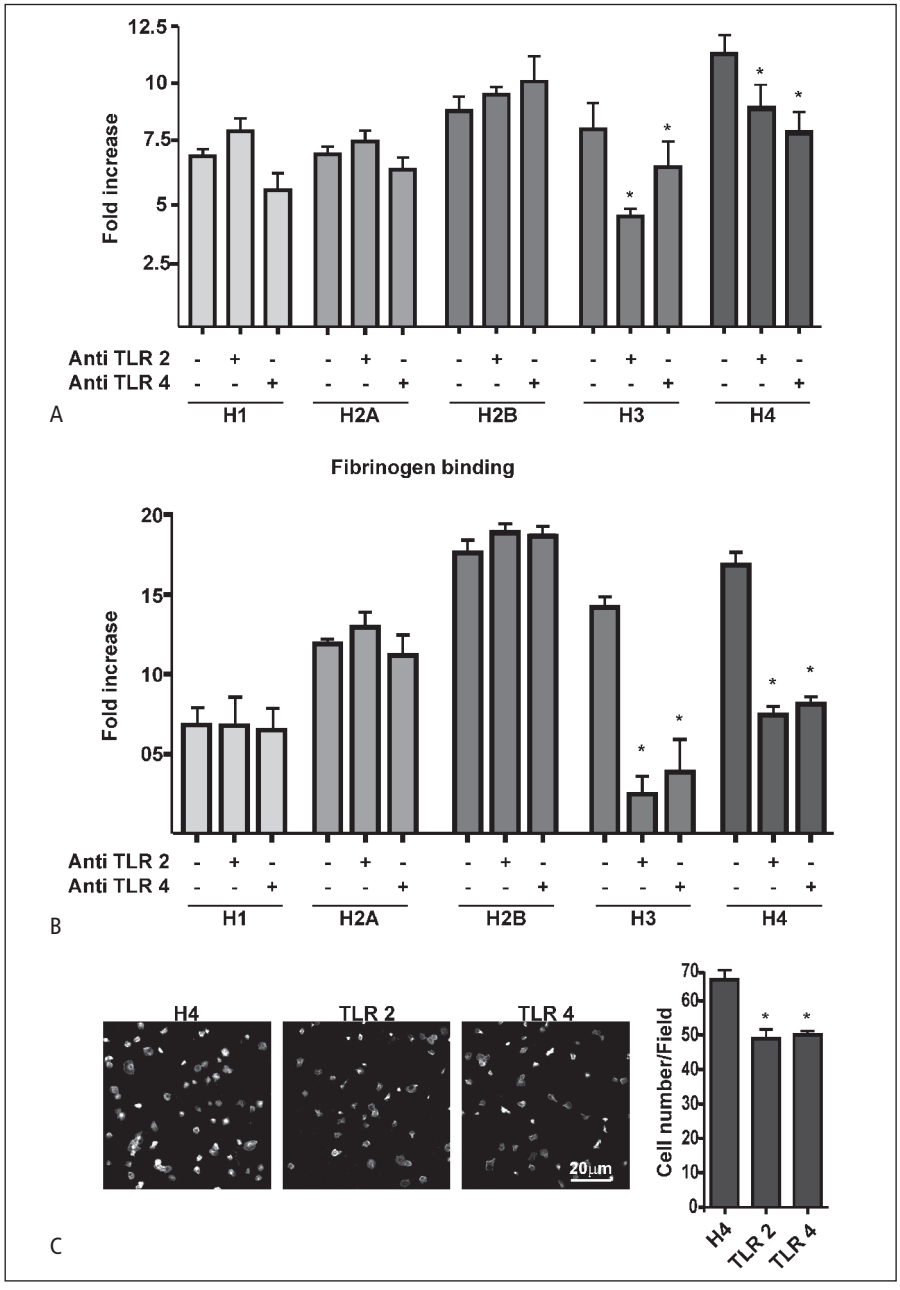

Figure 7: The role of TLRs. WPs ($3x10^8$/ml) were pretreated with blocking mAbs against human TLR-2 and TLR-4 (50 µg/ml) or isotype control for 20 min followed by treatment with the EC_{50} of each histone (H1 and H2A: 50 µg/ml; H2B, H3 and H4: 10 µg/ml), (A) P-selectin expression and (B) fibrinogen binding were observed (n=4). C) Platelet spreading was visualised in platelets pretreated with the mAb and activated with H4 (10 µg/ml). A similar effect was observed with the other histones. The images are representative of three independent experiments (*p<0.05 vs histones (H1, H2A, H2B, H3 and H4)).

ing of lipopolysaccharide to platelet TLR-4 appears to be a relevant event in the activation of neutrophils and the consequent formation of NETs (5). Therefore, the exposure of P-selectin and the formation of mixed cell aggregates triggered by histones might represent not only relevant molecular events fostering NET formation but also another potential mechanism by which platelets contribute to inflammation and the regulation of the innate immune response. Moreover, the direct activation of neutrophils by histones could further contribute to the amplification of the inflammatory response. In this regard, the presence of extracellular histones in damaged tissue is a potent stimulus for leukocyte recruitment (30). It remains unknown whether histones promote other neutrophil effector responses.

Interestingly, we observed that all histones enhanced thrombin-triggered fibrinogen binding, P-selectin and PS exposure and the formation of mixed aggregates formation. Since it was demonstrated that fibrinogen may directly bind to histones (31), it is still not clear whether this effect or the activation of the αIIbβ3 integrin account for the increased fibrinogen binding found in platelets stimulated by the combination of histones and thrombin. Because histones stimulate thrombin formation through platelet-dependent mechanisms (including P-selectin expression) (14), the interaction between traces of thrombin and histones may exacerbate platelet activation and inflammation in the microenvironment of the forming NET.

The phosphorylations of ERK, PI3K/Akt and p38 are among the primary signalling events involved in platelet activation and are mediated by most classical platelet agonists (32). Similar patterns of protein phosphorylation were observed when platelets were stimulated with H1, H2A, H2B, H3 or H4. Surprisingly, although H1 was the least potent platelet agonist, it was as potent as H4 in triggering the phosphorylation of ERK. However, the relevance of this finding remains to be established. Although platelets are enucleated cells, they express several transcription factors that are involved in non-genomic responses (33). We have previously reported not only that platelets express NFκB but also that the activation of this molecule is another signalling pathway involved in platelet activation mediated by classical agonists (23). We now show that the pretreatment of platelets with two structurally non-related NFκB inhibitors significantly impaired the platelet responses mediated by histones indicating that in addition to its role in the platelet activation mediated by classical agonists, NFκB also mediates histone-mediated platelet activation.

As mentioned above, the release of histones into the extracellular milieu occurs primarily under inflammatory conditions. We observed that the platelet adhesion and fibrinogen binding triggered by all of the histones are efficiently inhibited by pretreating platelets with either aspirin or dexamethasone, suggesting that platelet inhibition might be considered a potential therapy for diseases associated with NET formation. In fact, it has recently been demonstrated in an experimental transfusion-related acute lung injury model that targeting platelet activation with either aspirin or a glycoprotein αIIbβ3 inhibitor decreases NET formation and lung injury (34). Importantly, we also observed that in contrast to fibrinogen binding, adhesion and spreading, P-selectin exposure was not modified by treatment with anti-inflammatory drugs. Although the reason for these differences is not clear it could be that histones trigger the activation of signalling pathways involved in P-selectin exposure and that these pathways are not susceptible to aspirin or dexamethasone inhibition. Interestingly, a similar differential inhibition in tumor cell-induced platelet aggregation and P-selectin expression was recently described using an aspirin prodrug (35).

Extracellular histones induce tissue damage by binding to TLRs and they have been recognised as TLR agonists (10, 36). The platelet procoagulant activity mediated by histones has also been partly attributed to their interactions with TLR-2 and -4 (14). By analysing the activities of individual histones and using blocking Abs against these receptors, we confirmed that H3- and H4-induced platelet activation is partly mediated by TLR-2 and -4. Whether the inhibitory effect of the blocking Ab was associated with interference in histone binding to the platelet surface remains to be investigated. In contrast, the binding of fibrinogen and the expression of P-selectin mediated by H1, H2A or H2B was unaffected by the blockade of TLR-2 and –4. Together these findings suggest that besides acting on TLR-2 and –4, histones can regulate platelet functional responses through a different recognition molecule(s). Interestingly, it has been shown that mice lacking the αIIbβ3 integrin are protected from histone-induced death (19). Therefore, this integrin could be another histone receptor. It is also known that histones are small and basic proteins. Other small, positively charged molecules such as galectins, interact with specific glycosaminoglycans (GAGs) present on the platelet surface leading to platelet activation (17, 37). Therefore, GAGs could also

What is known about this topic?

- Either dying or activated inflammatory cells can release histones.
- Histones promote platelet aggregation and platelet-dependent thrombin generation.
- TLR-2 and -4 partially mediate the generation of platelet prothrombinase activity induced by a mixture of calf thymus-derived histones.

What does this paper add?

- Recombinant human H1, H2A, H2B, H3 and H4 trigger haemostatic (platelet adhesion, fibrinogen binding and vWF release), proinflammatory (P-selectin exposure and platelet-leukocyte aggregates) and procoagulant platelet responses (PS exposure) through activation of the ERK, PI3K/AKT, p38 and NFκB signalling pathways.
- Histones promote platelet activation in synergy with thrombin.
- Anti-inflammatory drugs such as aspirin and dexamethasone prevent the generation of haemostatic but not proinflammatory platelet responses triggered by all histones.
- H3- and H4-induced platelet activation (but not H1, H2A or H2B) is partly mediated by TLR-2 and -4.

be potential binding sites involved in platelet activation mediated by histones.

In summary, we have shown that H1, H2A, H2B, H3 and H4 are potent platelet agonists that act directly and in synergy with thrombin. They activate platelets via classical signalling pathways as well as through NFκB activation. Anti-inflammatory drugs such as aspirin and dexamethasone prevent the generation of haemostatic platelet responses triggered by all histones highlighting these drugs as potential treatment to ameliorate the prothrombotic activity of histones.

Conflicts of interest

None declared.

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