

Histones Differentially Modulate the Anticoagulant and Profibrinolytic Activities of Heparin, Heparin Derivatives, and Dabigatran

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ABSTRACT

The antithrombin activity of unfractionated heparin (UFH) is offset by extracellular histones, which, along with DNA, represent a novel mediator of thrombosis and a structural component of thrombi. Here, we systematically evaluated the effect of histones, DNA, and histone-DNA complexes on the anticoagulant and profibrinolytic activities of UFH, its derivatives enoxaparin and fondaparinux, and the direct thrombin inhibitor dabigatran. Thrombin generation was assessed by calibrated automated thrombinography, inhibition of factor Xa and thrombin by synthetic substrates, tissue plasminogen activator-mediated clot lysis by turbidimetry, and thrombin-activatable fibrinolysis inhibitor (TAFI) activation by a functional assay. Histones alone delayed coagulation and slightly stimulated fibrinolysis. The anticoagulant activity of UFH and enoxaparin was markedly inhibited by histones, whereas that of fondaparinux was enhanced. Histones neutralized both the

anti-Xa and anti-IIa activities of UFH and preferentially blocked the anti-IIa activity of enoxaparin. The anti-Xa activity of fondaparinux was not influenced by histones when analyzed by chromogenic substrates, but was potentiated in a plasma prothrombinase assay. Histones inhibited the profibrinolytic activity of UFH and enoxaparin and enhanced that of fondaparinux by acting on the modulation of TAFI activation by anticoagulants. Histone H1 was mainly responsible for these effects. Histone-DNA complexes, as well as intact neutrophil extracellular traps, impaired the activities of UFH, enoxaparin, and fondaparinux. Dabigatran was not noticeably affected by histones and/or DNA, whatever the assay performed. In conclusion, histones and DNA present in the forming clot may variably influence the antithrombotic activities of anticoagulants, suggesting a potential therapeutic advantage of dabigatran and fondaparinux over heparins.

Introduction

Extracellular nuclear chromatin has been identified as a novel mediator of thrombosis. Its main constituents, DNA and histones, can be released from dead cells or activated inflammatory cells, especially in the form of neutrophil extracellular traps (NETs), a complex network of chromatin and cytoplasm granule proteins (Chen et al., 2014). Upon release, they markedly affect the hemostatic system. DNA favors the activation of factor XII and factor XI (Kannemeier et al., 2007). Histones activate platelets (Fuchs et al., 2010, 2011) and erythrocytes, inducing the expression of a procoagulant phenotype (Semeraro et al., 2011, 2014), and increase thrombin generation by impairing protein C activation (Ammollo et al., 2011). Several *in vivo* data support the role of DNA and histones in thrombogenesis. In a model of inferior vena cava flow restriction, infusion of DNase I protects mice from deep

vein thrombosis (Brill et al., 2012). Injection of high doses of histones in mice induces a septic-like condition, and neutralization of histone H4 is protective in several sepsis models (Xu et al., 2009). Sublethal doses of histones cause thrombocytopenia (Fuchs et al., 2011), stimulate thrombin generation (Abrams et al., 2013), and foster deep vein thrombosis development in mice (Brill et al., 2012). The importance of these new mediators is further confirmed by the abundance of extracellular histones and DNA in animal (Fuchs et al., 2010) and human (Savchenko et al., 2014) venous thrombi. Moreover, they have also been detected in human arterial thrombi (Longstaff et al., 2013), providing new clues for the pathogenesis of arterial thrombosis.

Anticoagulant drugs are the cornerstone for the prevention and therapy of venous thrombosis and pulmonary embolism and are used in the management of arterial thrombosis together with platelet inhibitors. By inhibiting thrombin activity/generation, they hamper thrombus formation and accretion (Chiu et al., 1977; Van Ryn-McKenna et al., 1989) and favor clot dissolution (Semeraro et al., 2007; Varin et al., 2007; Ammollo et al., 2010), mainly by impairing

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ABBREVIATIONS: CAT, calibrated automated thrombinography; ETP, endogenous thrombin potential; f.d., final dilution; FXa, factor Xa; NET, neutrophil extracellular trap; LMWH, low-molecular-weight heparin; PMN, polymorphonuclear leukocytes; TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated TAFI; t-PA, tissue plasminogen activator; UFH, unfractionated heparin; ZGGR-AMC, Z-Gly-Gly-Arg-7-amino-4-methylcoumarin.

thrombin-activatable fibrinolysis inhibitor (TAFI) activation (Semeraro et al., 2007; Amollo et al., 2010). Heparins are a family of glycosaminoglycans that accelerate antithrombin-mediated inhibition of activated clotting factors [mainly thrombin and factor Xa (FXa)]. The family progenitor is unfractionated heparin (UFH), whereas low-molecular-weight heparins (LMWHs) are derived from the chemical or enzymatic depolymerization of UFH; pentasaccharide represents the smallest heparin sequence with inhibitory activity. These compounds mainly differ in selectivity, UFH exerting equal activity against FXa and thrombin, LMWHs showing a preference for FXa, and pentasaccharide being specific for FXa.

It is renowned that cationic proteins can interact with UFH by electrostatic forces and neutralize its activity, as in the case of protamine sulfate, which is indeed used as heparin antidote. Also histones, as basic proteins, can bind with high affinity to UFH and offset its antithrombin activity (Pal et al., 1983). However, evidence that histones affect the antithrombotic potential of anticoagulants other than UFH is limited to a report showing that nuclear material from cancer cells containing histones interferes with the activity of LMWHs (Morita et al., 2001). Moreover, it is not known whether and how histones can influence the profibrinolytic activity of anticoagulant drugs.

Given the emerging importance of histones and DNA as thrombogenic factors and structural components of thrombi, we systematically evaluated their effect on both anticoagulant and profibrinolytic activities of UFH, the LMWH enoxaparin, the synthetic pentasaccharide fondaparinux, and the novel small direct oral thrombin inhibitor dabigatran.

Materials and Methods

Materials. The following reagents were purchased: single-chain recombinant tissue plasminogen activator (t-PA) (Boehringer Ingelheim GmbH, Florence, Italy); human thromboplastin (Recombiplastin) and chromogenic FXa substrate S-2772 (Instrumentation Laboratory, Milan, Italy); type II-A calf thymus histones, recombinant human histones (H1, H2A, H2B, H3.3, and H4), calf thymus DNA, bovine fibrinogen, potato tuber carboxypeptidase inhibitor, bovine pancreas DNase I, De-*N*-sulfated heparin sodium salt, and calcium ionophore A23187 (Sigma-Aldrich, Milan, Italy); human FXa and α -thrombin (Haemathologic Technologies, Essex Junction, VT); reptilase from *Bothrops atrox* venom (American Diagnostica, Pfungstadt, Germany); fluorogenic thrombin substrate Z-Gly-Gly-Arg-7-amino-4-methylcoumarin (ZGGR-AMC) and thrombin calibrator (Diagnostica Stago, Asnieres, France); and DNA-binding dye Sytox Green (Life Technologies, Monza, Italy). Phospholipid vesicles (20% phosphatidylserine, 60% phosphatidylcholine, 20% phosphatidylethanolamine; Avanti Polar Lipids, Alabaster, AL) were prepared by sonication. DNA was fragmented by sonication to 200–400 bp size, as assessed by agarose gel electrophoresis. Histones and DNA were used at 40 μ g/ml, unless otherwise reported. Where indicated, histones were preincubated with DNA at a 1:1 (w:w) ratio for 10 minutes at room temperature to form complexes. The anticoagulant drugs were studied at the following final concentrations per ml of plasma, unless otherwise specified: sodium heparin, 0.25 IU/ml (heparin BMS; Bristol-Myers Squibb, Anagni, Italy); enoxaparin, 1 IU/ml (Clexane; Sanofi-Aventis, Milan, Italy), which has a mean anti-Xa/anti-IIa activity ratio of 4.3 (Alban, 2005); fondaparinux, 2 μ g/ml (Arixtra; GlaxoSmithKline, Verona, Italy); dabigatran, 1 μ M (provided by Boehringer Ingelheim GmbH, Biberach, Germany).

Plasma Preparation. Peripheral venous blood was collected into 3.8% trisodium citrate from healthy volunteers who gave informed consent. Platelet-poor plasma was prepared by double centrifugation

(1000 and 12,000 $\times g$, 10 minutes each) and stored at -80°C . Where indicated, plasma was defibrinated by reptilase treatment [1:50 final dilution (f.d.)] for 1 hour at 37°C . Plasma from at least three donors was pooled for each experiment.

Isolation of Polymorphonuclear Leukocytes and NET Production. Blood polymorphonuclear leukocytes (PMN) were isolated from healthy volunteers of both sexes by dextran sedimentation followed by density gradient centrifugation on Lympholyte-H (Cedarlane Laboratories, Hornby, ON, Canada) and hypotonic lysis of erythrocytes (Johnston et al., 1975). PMN were seeded in flat-bottomed 96-well microplates at the indicated concentrations in 20 mM Hepes and 150 mM NaCl buffer containing 2.5 mM CaCl_2 and 4 mM glucose, and stimulated with ionophore A23187 (25 μ M) for 2 hours at 37°C in a 5% CO_2 atmosphere. NET extrusion was assessed by staining of extracellular DNA with 4 μ M Sytox Green (not shown). After incubation, the medium was removed and plasma was gently layered onto adherent cells. To degrade NETs, cells were incubated with DNase I (100 U/ml) and 10 mM MgSO_4 for 30 minutes at 37°C and washed afterward. To block histones in NETs, cell monolayers were treated with De-*N*-sulfated heparin (200 μ g/ml) for 1 hour at 37°C and then washed.

Assay of Thrombin Generation. Thrombin generation was assessed by calibrated automated thrombinography (CAT) as described (Hemker et al., 2006). In brief, 80 μ l of plasma was incubated for 5 minutes at 37°C with histones in round-bottomed microplate wells. Then anticoagulants were added along with thromboplastin (6 pM) and phospholipid vesicles (10 μ g/ml). Analysis was started upon addition of 20 μ l of a mixture containing 100 mM CaCl_2 and 2.5 mM ZGGR-AMC in a final volume of 120 μ l. To study prothrombin activation by FXa, thrombin generation was triggered by FXa (0.25 μ g/ml) in FX-depleted plasma (Instrumentation Laboratory). Thrombin generation with PMN was performed in half final volume to minimize the volume-to-surface ratio. Measurements were taken in a Fluoroskan Ascent fluorometer (Thermo Scientific, Dreieich, Germany), data were analyzed using the Thrombinoscope software (Thrombinoscope BV, Maastricht, The Netherlands), and lag time and endogenous thrombin potential (ETP) were calculated. The anticoagulant activity of drugs was expressed as percent reduction of ETP.

Thrombin and FXa Inhibition. Inhibition of clotting factors by anticoagulants was studied by means of a one-stage assay using specific synthetic substrates. Plasma containing histones and anticoagulants was diluted 5-fold in Tris-NaCl buffer and supplemented with the chromogenic substrate S-2772 (100 μ M) or the fluorogenic substrate ZGGR-AMC (100 μ M) for FXa or thrombin evaluation, respectively. Upon addition of FXa (0.3 μ g/ml) or thrombin (2 U/ml), substrate cleavage was followed for 30 minutes at 405 nm or at 390-nm excitation and 460-nm emission, and the final signal was corrected for the background noise. Residual enzyme activity in the presence of anticoagulants was expressed as the percentage of absorbance/fluorescence of the correspondent control.

Microplate Clot Lysis Assay. Clot lysis was studied by a turbidimetric assay (Amollo et al., 2010), modified as follows. Plasma (80 μ l) was preincubated with histones or buffer in microplate wells for 5 minutes at 37°C . Afterward, anticoagulants were added, and clotting-fibrinolysis started with thromboplastin (6 pM), phospholipids (10 μ g/ml), t-PA (40 ng/ml), and CaCl_2 (16.6 mM) in a final volume of 120 μ l. In some instances, clotting was induced by reptilase (1:24 f.d.) in the absence of calcium. Changes in the optical density at 405 nm were monitored in a Multiskan FC spectrophotometer (Thermo Scientific) at 37°C , and clotting times were calculated as the time to half-maximum absorbance increase, whereas lysis times were calculated as the interval between the midpoint of the clear to maximum turbidity transition and the midpoint of the maximum turbidity to clear transition. The profibrinolytic activity of anticoagulants was expressed as the lysis ratio, i.e., the ratio between the lysis time in the absence and in the presence of drugs.

TAFI Activation Assay. TAFI activation was evaluated by a two-stage assay as described (Semeraro et al., 2013). In the first stage,

clotting was induced in defibrinated plasma under the same conditions used for the lysis assay but without t-PA. At intervals, aliquots from the reaction mixtures were transferred into refrigerated tubes containing hirudin (200 U/ml; Abbott GmbH, Ludwigshafen, Germany) to halt TAFI activation and preserve activated TAFI (TAFIa) activity. In the second stage, TAFIa activity was evaluated as the ability to prolong the lysis time of clots formed with purified fibrinogen. Eighty microliters of sample was added to microplate wells along with fibrinogen (830 $\mu\text{g}/\text{ml}$), t-PA (40 ng/ml), and reptilase (1:50, f.d.) as clotting reagent (final volume 120 μl). The plate was read at 405 nm at room temperature to reduce TAFIa decay, and the lysis times were calculated as described earlier. Results were expressed as prolongation of lysis time over parallel samples supplemented with the TAFIa inhibitor potato tuber carboxypeptidase inhibitor.

Statistical Analysis. Results shown are the mean \pm S.E.M. Statistical analysis was performed using GraphPad Prism version 4.01 for Windows (GraphPad Software, San Diego, CA). Multiple comparisons were performed by one-way analysis of variance for repeated measures followed by a post-test for linear trend (for dose response), Dunnett's post-hoc test (for comparing each sample to the control), or Newman-Keuls post-hoc test (for comparing all samples to each other). Student's *t* test for paired data was used where applicable. *P* values <0.05 were considered statistically significant.

Results

Effect of Histones on Coagulation/Fibrinolysis. We first evaluated the influence of histones on coagulation/fibrinolysis under our experimental conditions by means of CAT and clot lysis assay, respectively. In the thrombin-generation assay, histones dose dependently prolonged the lag time without significant modifications of the ETP (Fig. 1A). Consistent with these data, clot formation was significantly delayed by histones in the clot lysis assay (Fig. 1B). As for fibrinolysis, histones induced a weak but significant profibrinolytic effect (Fig. 1B), which was independent of coagulation, as was also observed in reptilase-induced clots (not shown).

Effect of Histones on the Anticoagulant Activity of Drugs. Plasma was supplemented with a fixed concentration of each anticoagulant, producing at least 60% inhibition of ETP, and the CAT assay was performed in the presence of increasing concentrations of histones. As shown in Fig. 2A, the effect of histones varied considerably depending on the drug tested. The anticoagulant activity of UFH and enoxaparin was markedly inhibited by histones, with an IC_{50} of 7.9 ± 2.5 and 31 ± 3.7 $\mu\text{g}/\text{ml}$, respectively. On the contrary, histones did not impair, but rather enhanced, albeit slightly, the anticoagulant activity of fondaparinux. As for dabigatran, no significant modification was observed. Next, we evaluated the effect of 40 $\mu\text{g}/\text{ml}$ histones on different concentrations of drugs. Under this condition, UFH displayed no anticoagulant activity up to 0.5 IU/ml, and only partial activity ($\approx 50\%$ of control) at 1 IU/ml (Fig. 2B). Similarly, enoxaparin showed a detectable activity only at concentrations ≥ 0.5 IU/ml. As for fondaparinux, the enhancement of anticoagulant activity by histones was seen at all concentrations. As expected, dabigatran activity was barely influenced by histones.

To directly evaluate the influence of histones on the neutralization of target enzymes by each drug, FXa (0.3 $\mu\text{g}/\text{ml}$) or thrombin (2 U/ml) was added to diluted plasma containing the anticoagulant and histones, and residual enzymatic activity was determined by chromogenic (FXa) or fluorogenic (thrombin) substrates. The used concentration of drugs reduced the enzymatic activity of either thrombin or FXa

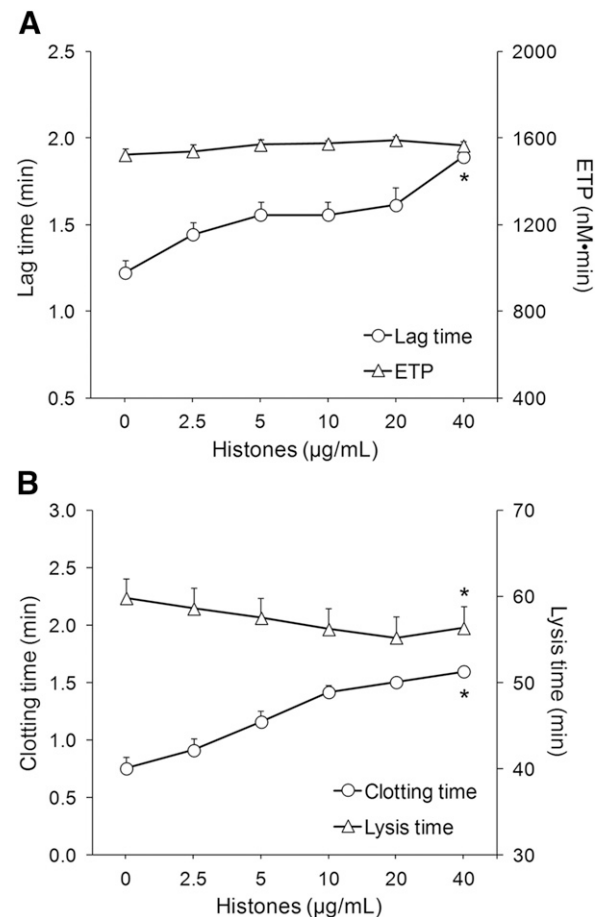


Fig. 1. Effect of calf thymus histones on coagulation and fibrinolysis. Plasma supplemented with increasing concentrations of calf thymus histones (0–40 $\mu\text{g}/\text{mL}$) was clotted with 6 pM thromboplastin and 10 $\mu\text{g}/\text{mL}$ phospholipids, and lysed with 40 ng/ml t-PA. (A) The effect of histones on thrombin generation was evaluated by CAT ($n = 6$). (B) The influence of histones on plasma clotting and fibrinolysis was assessed by turbidimetry ($n = 8$). Results shown are the mean \pm S.E.M. * $P < 0.05$ for linear trend by repeated-measures analysis of variance.

by $\geq 90\%$. The anti-Xa and anti-IIa activities of UFH were nearly completely quenched by histones (Fig. 2, C and D). In the case of enoxaparin, anti-Xa activity was inhibited by only $\approx 11\%$, whereas anti-IIa activity was inhibited by $\approx 85\%$. The anti-Xa activity of fondaparinux (1 $\mu\text{g}/\text{mL}$) was not modified by histones, and the anti-IIa activity of dabigatran (0.5 μM) was unchanged. As the lack of effect of histones on fondaparinux anti-Xa activity was in contrast with the thrombin-generation data, we investigated their influence on the FXa-mediated conversion of prothrombin to thrombin in FX-depleted plasma by CAT. In this experimental setting, which more closely approaches physiologic conditions, histones delayed thrombin formation and enhanced the anti-Xa activity of fondaparinux (Fig. 2E).

Effect of Histones on the Profibrinolytic Activity of Drugs. In the clot lysis assay, the used concentrations of anticoagulants displayed a strong profibrinolytic activity, with a shortening of the lysis time greater than 40%. The profibrinolytic activity of UFH and enoxaparin was inhibited by histones in a concentration-dependent fashion (Fig. 3A). In particular, the activity of UFH was markedly reduced by as little as 2.5 $\mu\text{g}/\text{mL}$ histones, and completely quenched by 10 $\mu\text{g}/\text{mL}$,

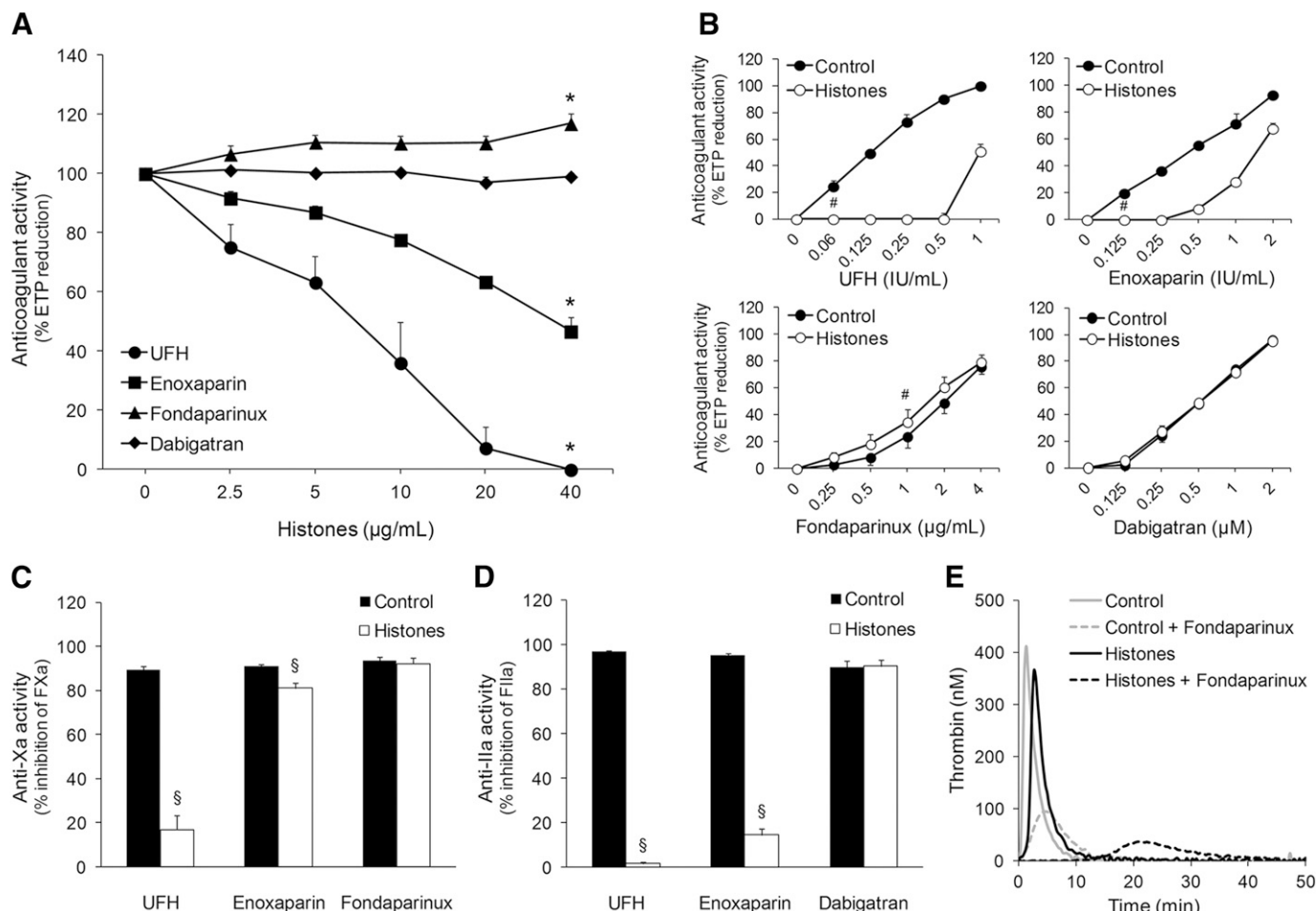


Fig. 2. Effect of calf thymus histones on the anticoagulant activity of drugs. (A) Thrombin generation in plasma incubated with increasing concentrations of histones (0–40 µg/ml) and spiked with UFH (0.25 IU/ml), enoxaparin (1 IU/ml), fondaparinux (2 µg/ml), or dabigatran (1 µM) was evaluated by CAT. The anticoagulant activity of the drugs was measured as percent reduction of the ETP, and the activity registered for each drug in the absence of histones was set at 100% ($n = 4$). (B) Thrombin generation in plasma incubated with buffer (control) or histones (40 µg/ml) and spiked with increasing concentrations of drugs; anticoagulant activity was expressed as percent reduction of ETP ($n = 3–6$). (C and D) Inhibition of human FXa and thrombin by anticoagulants (UFH, 0.5–0.25 IU/ml for FXa and FIIa assays, respectively; enoxaparin, 1 IU/ml; fondaparinux, 1 µg/ml; dabigatran, 0.5 µM) was studied by cleavage of chromogenic (S-2772) or fluorogenic (ZGGR-AMC) substrates with or without 40 µg/ml histones. Bars represent the anti-FXa and anti-IIa activities of drugs, expressed as percent inhibition of FXa and IIa ($n = 3–4$). (E) Representative thrombogram of FXa-depleted plasma triggered by FXa (0.25 µg/ml) in the absence/presence of histones (40 µg/ml) and fondaparinux (0.2 µg/ml; $n = 3$). Unless otherwise specified, results are the mean \pm S.E.M. * $P \leq 0.0001$ for linear trend by repeated-measures analysis of variance; #lowest drug concentration with a significant difference ($P < 0.05$) between histones and control by t test for paired data; § $P < 0.01$ versus control by t test for paired data.

whereas complete inhibition of enoxaparin was observed at 40 µg/ml histones. Calculated IC_{50} values of histones were 2.1 ± 0.3 and 8.2 ± 1.7 µg/ml for UFH and enoxaparin, respectively. The profibrinolytic activity of fondaparinux was enhanced by the highest concentrations of histones, and that of dabigatran was only slightly increased.

When 40 µg/ml histones were challenged with increasing concentrations of anticoagulants, the clot lysis results (Fig. 3B) recapitulated fairly well those obtained in the thrombin-generation assay. In fact, in the presence of histones, UFH failed to stimulate fibrinolysis at concentrations up to 0.5 IU/ml, and exhibited only a modest activity at 1 IU/ml. Enoxaparin displayed a similar behavior, with some profibrinolytic activity appearing at concentrations >0.5 IU/ml. As to fondaparinux, the increase in profibrinolytic activity caused by histones was observed at drug concentrations ≥ 1 µg/ml. The profibrinolytic effect of dabigatran was subtly enhanced by histones.

One of the main mechanisms behind the profibrinolytic activity of anticoagulants is the inhibition of thrombin-mediated TAFI activation. Therefore, we evaluated the influence of histones on drugs' ability to reduce TAFI activation. In accordance with clot lysis data, the inhibitory activities of UFH and enoxaparin were greatly reduced, whereas the ability of fondaparinux to inhibit TAFI activation was enhanced, and that of dabigatran was almost unaffected (Fig. 3C).

Effect of Individual Histones on Anticoagulants. To determine which histone subtype was mainly responsible for the observed effects of the mixture, recombinant individual histones (10 µg/ml) were tested in CAT and clot lysis assays. Among subtypes, histone H1 recapitulated most of the effects of the mixture: it significantly delayed thrombin generation, prolonged clotting time, and stimulated fibrinolysis, although weakly; in addition, it slightly reduced ETP (not shown). In the presence of anticoagulants (Fig. 4), H1 showed the strongest neutralizing activity toward UFH and enoxaparin and the

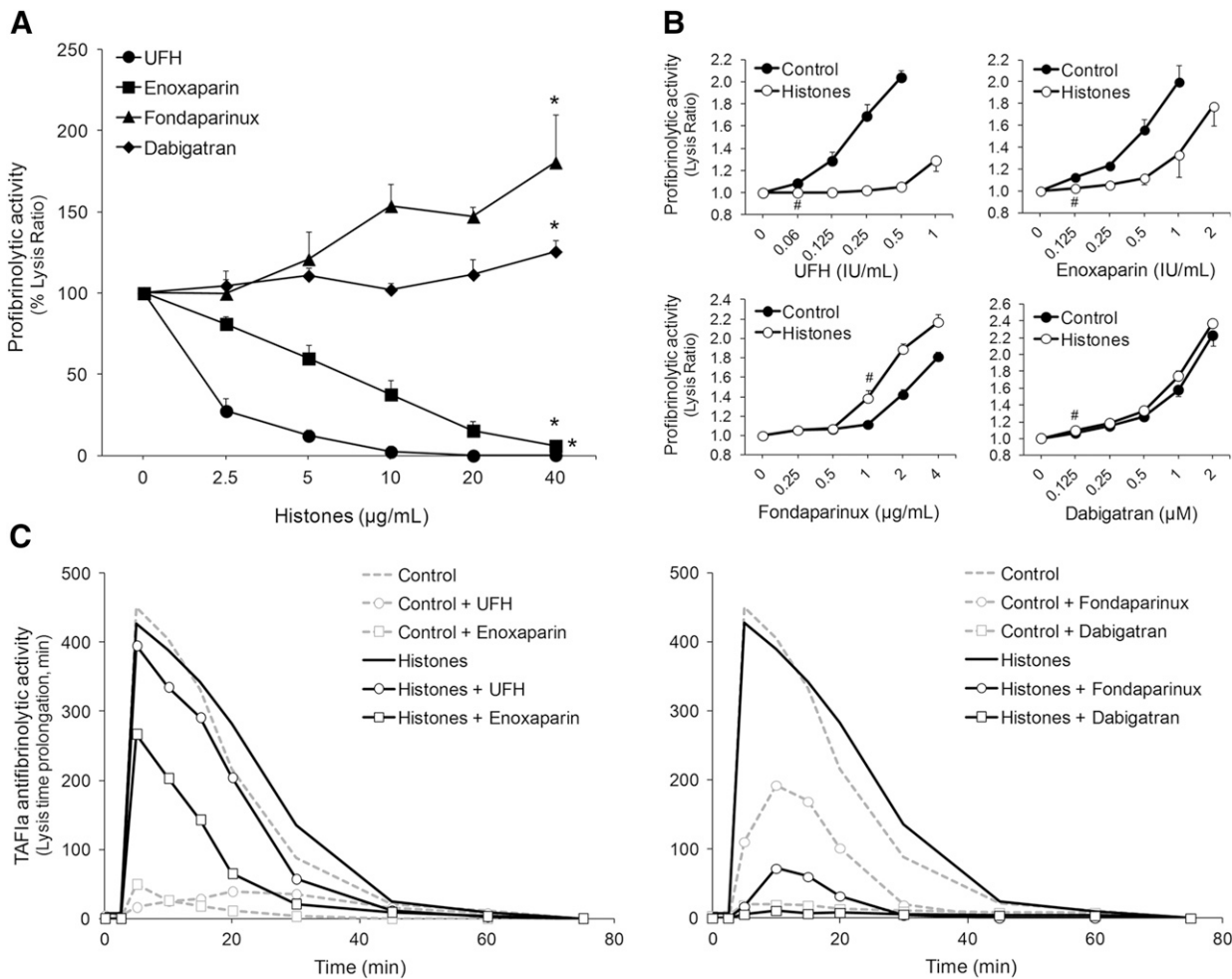


Fig. 3. Effect of calf thymus histones on the profibrinolytic activity and inhibition of TAFIa generation by anticoagulants. (A) Plasma supplemented with increasing concentrations of histones (0–40 µg/ml) was spiked with UFH (0.25 IU/ml), enoxaparin (1 IU/ml), fondaparinux (2 µg/ml), or dabigatran (1 µM). The stimulating activity of each drug on t-PA-mediated clots lysis was assessed by turbidimetry. Data are expressed as percent lysis ratio relative to samples without histones (set at 100%; $n = 4$). (B) Fibrinolysis of plasma clots formed in the presence of buffer (control) or histones (40 µg/ml) and spiked with increasing concentrations of drugs; the profibrinolytic activities were expressed as the lysis ratio ($n = 4–6$). (C) TAFI activation in clotting defibrinated plasma was evaluated at different times by a two-stage fibrin-based TAFIa functional assay as described in *Materials and Methods*, in the presence or absence (control) of 40 µg/ml histones and a single concentration of drugs: UFH, 0.25 IU/ml; enoxaparin, 1 IU/ml; fondaparinux, 2 µg/ml; dabigatran, 1 µM. Results are expressed as lysis time prolongation over potato tuber carboxypeptidase inhibitor-containing samples and are representative of three independent experiments. Unless otherwise specified, results are the mean \pm S.E.M. * $P < 0.01$ for linear trend by repeated-measures analysis of variance; #lowest drug concentration with a significant difference ($P < 0.05$) between histones and control by t test for paired data.

highest stimulating activity toward fondaparinux, followed by H2A and H2B. H3 and H4 caused only a weaker inhibition of UFH. None of the histones influenced dabigatran activity.

Effect of DNA and Histone-DNA Complexes on Anticoagulants. As histones can be released in complex with DNA, we investigated how the presence of DNA modifies the effects of histones. To that purpose, DNA was preincubated with histones (40 µg/ml each) to form complexes. Associated with DNA, histones lost their effects on coagulation and fibrinolysis (not shown). When tested in the presence of anticoagulants in the CAT assay (Fig. 5), DNA partially attenuated the inhibitory effect of histones on both UFH and enoxaparin. Interestingly, DNA blunted the stimulating effect of histones on fondaparinux when thrombin generation was triggered by tissue factor (Fig. 5C) or FXa (not shown). In the clot lysis test (Fig. 5), DNA did not influence the activity of histones on UFH, but partially attenuated the inhibitory effect on enoxaparin. On the contrary, DNA reversed the effect of

histones on fondaparinux, producing a visible reduction in its profibrinolytic activity. A minor reduction in dabigatran profibrinolytic activity was observed in the presence of the complex. The only effect of DNA alone was a reduction in the profibrinolytic activity of fondaparinux.

Effect of NETs on Anticoagulants. To examine the effect of a physiologic source of histone-DNA, PMN were stimulated with calcium ionophore to generate extracellular traps, overlaid with plasma, and then assayed with the various anticoagulants in the CAT assay. Based on the findings of Urban et al. (2009), we calculated that the concentration of NET-associated histones and DNA released in the culture medium by 10^6 neutrophils is on the order of several tens of micrograms per milliliter, which is comparable to the concentrations of purified molecules used throughout the study. In keeping with the results obtained with histone-DNA complexes, NET-expressing PMN interfered with the anticoagulant activity of UFH, enoxaparin, and fondaparinux,

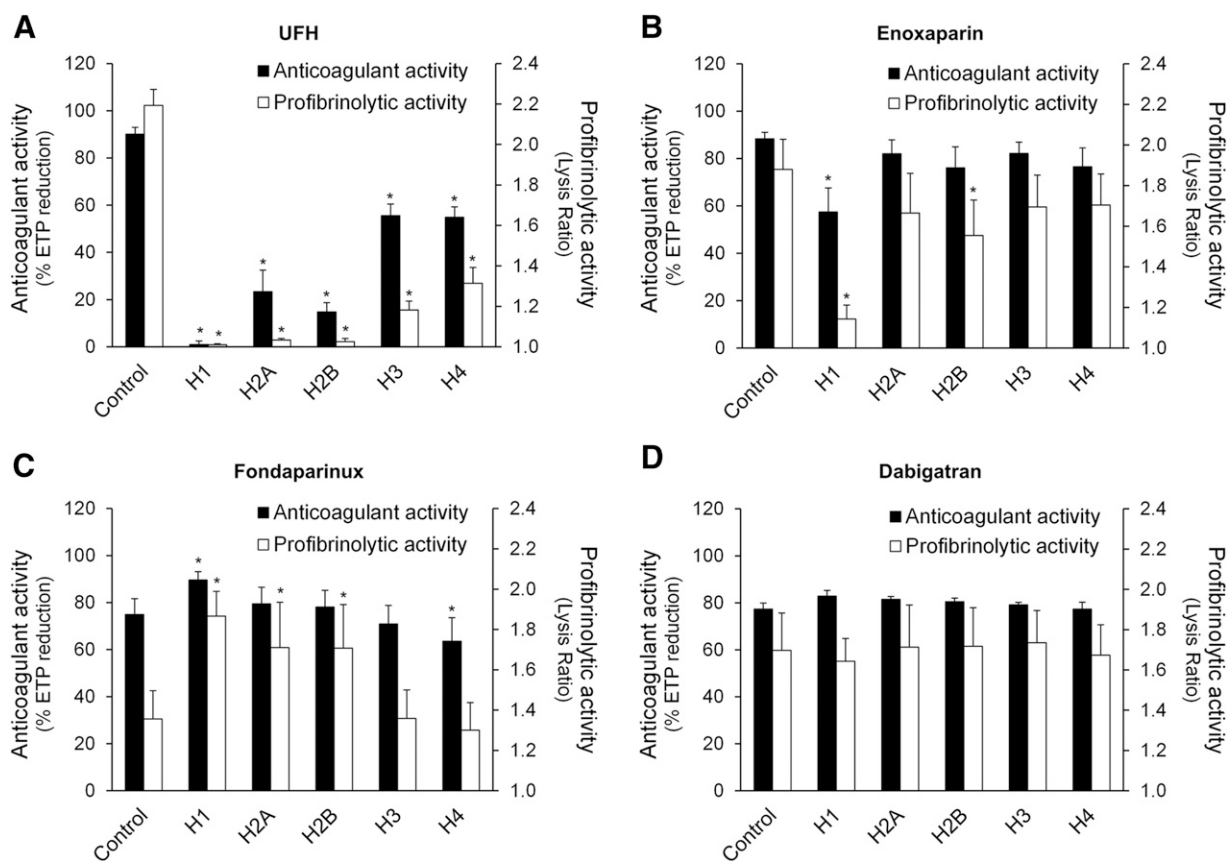


Fig. 4. Effect of recombinant histone subtypes. The anticoagulant and profibrinolytic activities of a single concentration of drugs in the absence/presence of recombinant histones (10 $\mu\text{g}/\text{ml}$) were evaluated by CAT and turbidimetry, and expressed as percent ETP reduction and lysis ratio, respectively ($n = 3-5$). (A) UFH, 0.25 IU/ml; (B) enoxaparin, 1 IU/ml; (C) fondaparinux, 2 $\mu\text{g}/\text{ml}$; (D) dabigatran, 1 μM . Values are the mean \pm S.E.M. * $P < 0.05$ versus control by Dunnett's multiple comparison test.

whereas they did not influence dabigatran (Fig. 6A). In dose-dependence experiments, a significant inhibition of UFH activity was achieved with as low as 6×10^4 stimulated cells per well (Fig. 6B). To ensure that this effect was due to NETs, cells were treated with DNase I to remove NET components prior to UFH challenge. DNase treatment reverted the inhibition at low-medium cell densities, but not at high cell numbers (Fig. 6B), a condition in which DNase was also unable to decrease the fluorescent signal of Sytox Green (not shown), suggesting that high cell densities were unfavorable for DNA degradation. On the contrary, targeting histones in NETs with De-*N*-sulfated heparin, a heparin almost devoid of anticoagulant properties (Amollo et al., 2011), restored most of the anticoagulant activity of UFH even at the highest cell density (Fig. 6C).

Discussion

This study shows that the incorporation of histones and DNA into clots variably influences the anticoagulant and profibrinolytic activities of heparin and heparin-derived anticoagulants, but has no effect on dabigatran, a representative nonheparin anticoagulant.

It has long been known that histones neutralize heparin, but the biologic implications thereof remained elusive, since histones were believed to be exclusively intranuclear proteins (Pal et al., 1983). The considerable progress made in unveiling

the role of extracellular histones in thrombosis brought us to examine the relationship between histones and anticoagulants through global assays, which carefully evaluate coagulation and fibrinolysis under more physiologic conditions. Indeed, we exclusively used plasma systems because histones can interact with plasma proteins (Pemberton et al., 2010) and are susceptible to degradation by plasma proteases (Yamamichi et al., 2011). Moreover, histones were used at concentrations approaching those found in systemic prothrombotic conditions ($\approx 15 \mu\text{g}/\text{ml}$ histone H3 in septic baboons and 10–230 $\mu\text{g}/\text{ml}$ total histones in trauma patients) (Xu et al., 2009; Abrams et al., 2013) or localized thrombotic diseases, in which the high histone content attainable inside the thrombus is reflected by elevated circulating markers of histone release (Brill et al., 2012; Fuchs et al., 2012; Borissoff et al., 2013; van Montfort et al., 2013). Using CAT to measure the anticoagulant activity of therapeutic drug concentrations, we confirmed that histones neutralized UFH and found that they strongly inhibited enoxaparin. These effects directly impacted on the drugs' capacity to stimulate fibrinolysis. The profibrinolytic potential of anticoagulants stems largely from their ability to inhibit thrombin generation/activity and the thrombin-mediated antifibrinolytic mechanisms (Lisman et al., 2003; Varin et al., 2007; Amollo et al., 2009), among which TAFI activation is pivotal. However, the relationship between thrombin generation and fibrinolysis rate is not always straightforward. For instance, the prothrombin

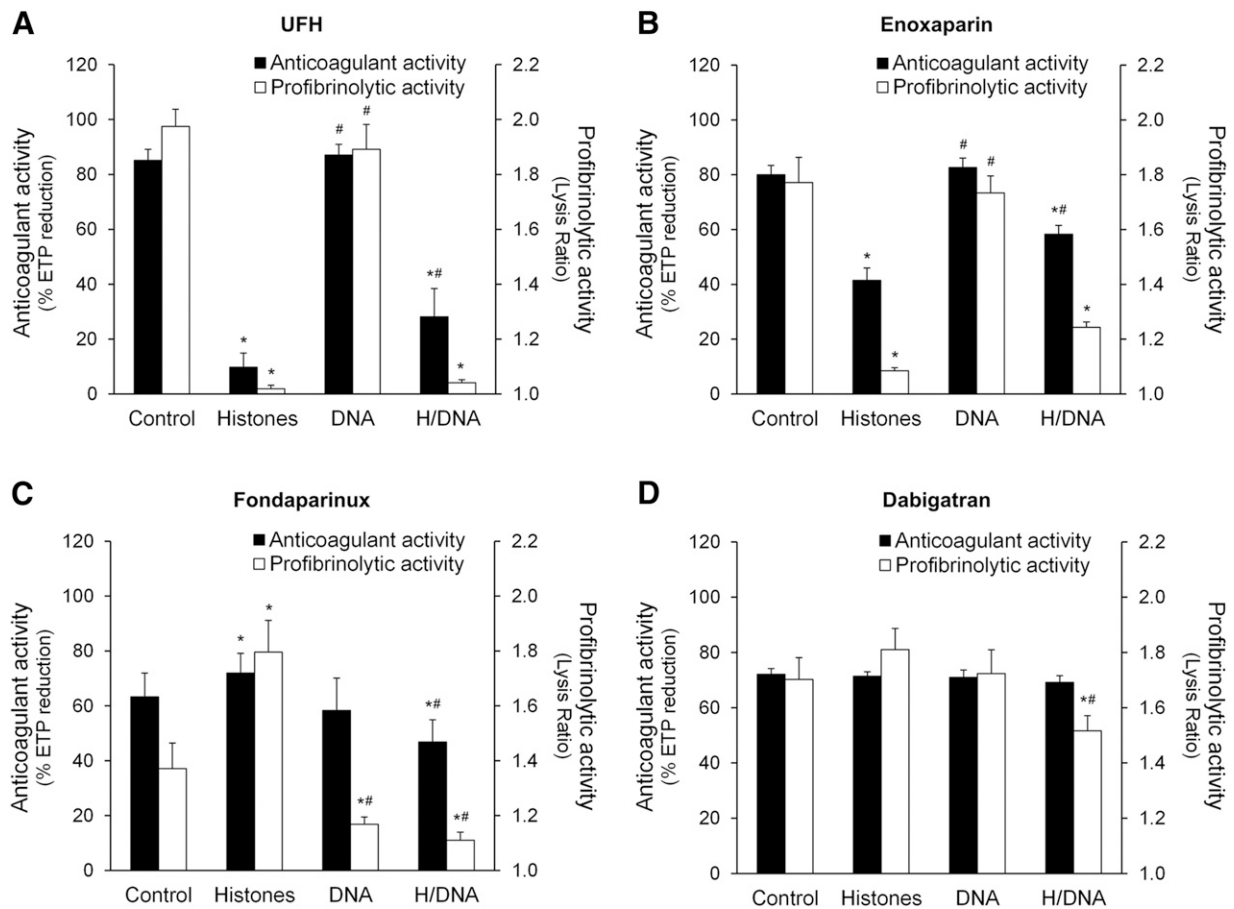


Fig. 5. Effect of calf thymus histones, DNA, and histone-DNA complexes. The anticoagulant and profibrinolytic activities of single drug concentrations in the absence/presence of histones, DNA, or histone-DNA complexes (H/DNA) (40 $\mu\text{g}/\text{ml}$) were evaluated by CAT and turbidimetry and expressed as percent ETP reduction and lysis ratio, respectively ($n = 5-6$). (A) UFH, 0.25 IU/ml; (B) enoxaparin, 1 IU/ml; (C) fondaparinux, 2 $\mu\text{g}/\text{ml}$; (D) dabigatran, 1 μM . Values are the mean \pm S.E.M. * $P < 0.05$ versus control, and # $P < 0.05$ versus histones by Newman-Keuls multiple comparison test.

mutation G20210A was reported to reduce the anticoagulant activity of activated protein C without interfering with its profibrinolytic function (Binetti et al., 2006). Moreover, some thrombin inhibitors (e.g., hirudin) are unable to stimulate fibrinolysis despite their marked anticoagulant activity (Lisman et al., 2003). In the presence of histones, UFH failed to reduce TAFI activation, and enoxaparin was markedly less active; hence, the profibrinolytic activity of UFH was lost and that of enoxaparin greatly impaired, indicating that histones reduced the overall antithrombotic activity of heparins. Based on the IC_{50} calculation, histones proved to be even more effective in inhibiting the profibrinolytic activity of UFH and enoxaparin than in quenching their anticoagulant activity. In agreement with previous works attributing the heparin-neutralizing property mainly to the lysine-rich histone fraction (Pal et al., 1983), we identified histone H1 as holding the strongest antiheparin activity. Because UFH neutralization by histones is due to charge-to-charge interactions (Pal et al., 1983), the lower size and sulfate charge density of enoxaparin can explain its reduced sensitivity to histones, as suggested by previous studies (Morita et al., 2001) and by the similar behavior of other cationic proteins (Lane et al., 1986; Crowther et al., 2002). Using specific enzymatic assays, we found that the anti-Xa and anti-IIa activities of enoxaparin were differentially inhibited by histones (11 and 85%, respectively), which, conversely, blocked both anti-Xa and anti-IIa activities

of UFH, further suggesting that histones are weak antagonists of short heparins endowed with more selective anti-Xa activity. In this regard, it is indicative that fondaparinux, the smallest heparin with exclusive anti-Xa activity, was not only insensitive to histone inhibition, but gained even more anticoagulant/profibrinolytic efficacy in the presence of histones. This unexpected effect does not seem to result from a direct influence of histones on the anti-Xa activity of fondaparinux (see Fig. 2C) but rather an indirect mechanism. Indeed, histones delayed clot formation and thrombin generation (see Fig. 1, A and B), showing a nonspecific anticoagulant effect possibly due to a transient binding to phospholipids (Fürnrohr et al., 2007) and/or vitamin K-dependent clotting factors (Pemberton et al., 2010; Ammollo et al., 2011). Based on the results in the prothrombinase assay (see Fig. 2E), it can be hypothesized that histones interfere with the assembly of the prothrombinase complex and allow more FXa to remain in solution, where it is more accessible to inhibition by fondaparinux-antithrombin (Brufatto et al., 2003). This possibility fits quite well with the observation that the anticoagulant effect of histones and the enhancement of fondaparinux activity were both ascribable to the same histone subtype (H1) and reversed by DNA (see below). Also, the enhancement of the profibrinolytic properties of fondaparinux was probably related to the improvement of its anticoagulant activity, as thrombin-mediated mechanisms (reduction of TAFI

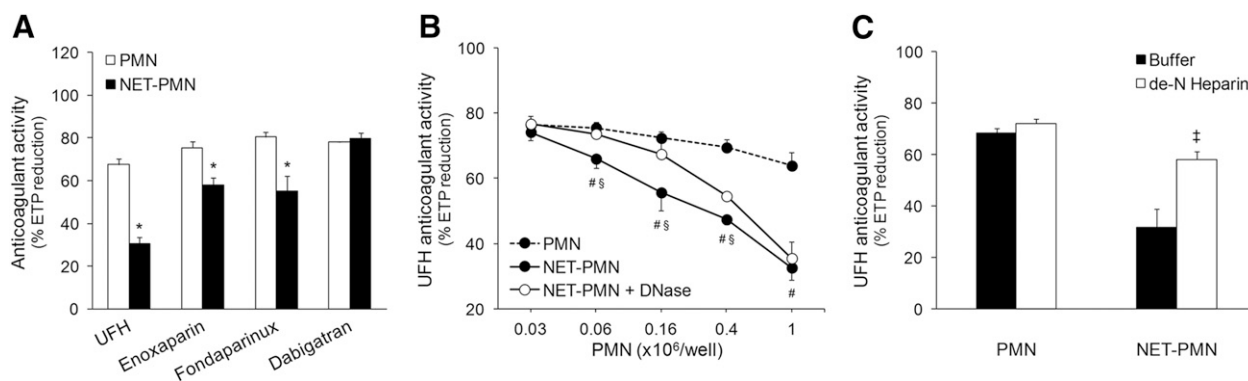


Fig. 6. Effect of NETs on the anticoagulant activity of drugs. Monolayers of resting PMN or NET-releasing PMN (NET-PMN) were overlaid with plasma, and CAT assay was performed with or without anticoagulants. (A) The anticoagulant activity of UFH (0.25 IU/ml), enoxaparin (1 IU/ml), fondaparinux (2 μ g/ml), and dabigatran (1 μ M) was tested in the presence of 1×10^6 PMN or NET-PMN per well ($n = 4-6$). (B) Increasing numbers of PMN, NET-PMN, or NET-PMN pretreated with DNase I (100 U/ml) were challenged with UFH (0.25 IU/ml; $n = 3$). (C) PMN and NET-PMN (1×10^5 /well) were treated with De-N-sulfated heparin (De-N Heparin, 200 μ g/ml) or buffer prior to UFH challenge (0.25 IU/ml). Results are expressed as percent reduction in the ETP ($n = 3$). Values are the mean \pm S.E.M. * $P < 0.05$ versus PMN, and $^{\dagger}P < 0.05$ versus buffer by t test for paired data; # $P < 0.05$ versus PMN, and $^{\S}P < 0.05$ versus NET-PMN + DNase by Newman-Keuls multiple comparison test.

activation; Fig. 3C) were involved. Anyway, the intrinsic profibrinolytic effect of histones could have further contributed to the observed effect. This probably also applies to dabigatran, whose profibrinolytic activity was slightly enhanced by histones even though the anticoagulant potential was unaffected, probably because of its different mechanism of action.

DNA is the natural ligand of histones. Therefore, it was important to assess whether DNA modulates their effects. When complexed with DNA to resemble nucleosomes, histones maintained the inhibitory activity toward UFH and enoxaparin, although it was slightly less pronounced, probably because DNA was displaced by heparins (Longstaff et al., 2013). On the contrary, in the case of fondaparinux, histone-DNA complexes behaved differently from histones alone as they inhibited the anticoagulant and, even more, the profibrinolytic activity of the drug.

In disease, activated neutrophils represent a natural source of nuclear material, released as NETs. The observation that neutrophil-derived NETs recapitulated the effects of histone-DNA on the drugs' anticoagulant activity further strengthens the relevance of our findings. In this respect, it is important to point out that the amount of histones and DNA in our NET preparations (Urban et al., 2009) falls in the concentration range of the purified molecules used in our study.

An additional layer of complication is introduced by the fact that DNA alone inhibited the profibrinolytic activity of fondaparinux without interfering with its anticoagulant function, further suggesting that inhibition of coagulation and stimulation of fibrinolysis (or vice versa) do not always go hand in hand. All the effects of histones, DNA, and histone-DNA on anticoagulants might occur in vivo, because histones and DNA, both circulating and resident in the thrombus scaffold, can exist either in complex or naked (Fuchs et al., 2010; Abrams et al., 2013; Longstaff et al., 2013), most probably as a result of a selective degradation by proteases and DNases.

Our findings may have pathophysiological and clinical implications. The binding of histones to heparan sulfate in the capillaries (Freeman et al., 2013) may reduce the antithrombotic potential of endothelial cells and may represent an additional prothrombotic mechanism of histones, particularly H1, which is usually considered the least dangerous subtype (Xu et al., 2009;

Fuchs et al., 2010; Amollo et al., 2011; Semeraro et al., 2011, 2014). From a clinical standpoint, histones add to other heparin-binding proteins as inducers of heparin resistance, a phenomenon occurring in a considerable proportion of patients with venous thromboembolism and acute coronary syndromes (Hirsh et al., 2001). In this regard, the reported interindividual variability in NET production (Fuchs et al., 2007) might represent a contributing factor. The drugs less affected by histones and DNA might have a therapeutic advantage in the management of thrombosis. Some clinical studies have suggested the superiority of treatment with dabigatran or fondaparinux over LMWHs and, in turn, of LMWHs over UFH in terms of better antithrombotic efficacy or reduced bleeding complications in clinical settings, such as major orthopedic surgery, cancer, and acute coronary syndromes (Siragusa et al., 1996; Agnelli, 2004; Yusuf et al., 2006; Eriksson et al., 2011; Silvain et al., 2012), where release of nuclear material occurs (Borissoff et al., 2013; Demers and Wagner, 2014; Dahl et al., 2015).

In interpreting our data, the possible beneficial effects resulting from the inhibition of histones by heparins must also be considered. Indeed, UFH, heparin-derived, and other negatively charged, nonanticoagulant molecules are known to block histone-mediated deleterious effects in vitro and in vivo (Amollo et al., 2011; Fuchs et al., 2011; Semeraro et al., 2011, 2014; Wildhagen et al., 2014). Allegedly, the neutralization of histones by heparins may be expected to result in a net positive effect in disorders where histone release is a major mechanism of harm, such as severe infection and inflammation (Levi, 2014). In the clinic, heparin is commonly administered to treat or prevent thrombosis, which is triggered by multiple mechanisms, such as the increase in tissue factor expression, the decrease of natural anticoagulants, the impairment of fibrinolysis, and, as pointed out earlier, the release of histones. Thus, it is plausible that the advantage deriving from the neutralization of histones might be overwhelmed by the loss of the effects of heparins on coagulation and fibrinolysis.

In conclusion, histones and DNA present in the ensuing fibrin clot may modulate the profibrinolytic and anticoagulant activities of antithrombotic drugs, thus possibly influencing the thrombus fate toward accretion or stabilization/resolution,

depending on the drug used. Direct thrombin inhibitors such as dabigatran and very short heparin molecules such as fondaparinux are less susceptible to influence than long-chain heparins, suggesting a possible therapeutic advantage.

Authorship Contributions

Participated in research design: Ammollo, F. Semeraro, Colucci.

Conducted experiments: Ammollo, F. Semeraro.

Performed data analysis: Ammollo, F. Semeraro, Colucci.

Wrote or contributed to the writing of the manuscript: Ammollo, F. Semeraro, Carratù, N. Semeraro, Colucci.

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