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Inhibition of Clotting Factor XIII Activity by Nitric Oxide[1]

Maria Valeria Catani, Francesca Bernassola, Antonello Rossi, and Gerry Melino[2]
IDI-IRCCS, Biochemistry Laboratory c/o Department of Experimental Medicine, D26/F153, University of Rome Tor Vergata, Via Tor Vergata 135, 00133 Rome, Italy

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The plasma factor XIII (FXIII) is a transglutaminase which catalyzes the cross-linking of fibrin monomers during blood coagulation. S-nitrosylation of protein sulfhydryl groups has been shown to regulate protein function. Therefore, to establish whether nitric oxide (NO) affects the enzymatic activity of FXIII, we studied the effect of the NO-donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) in a blood coagulation test *in vitro*. High concentrations of SNAP were found to have inhibitory effects on clot formation. Moreover, specific formation of γ-dimers through the action of FXIII is selectively inhibited by high concentrations of SNAP, as revealed by Western blot. Purified activated FXIII and plasma preparations were then exposed to NO-donor compounds and the enzyme activity was assayed by measuring the incorporation of [^{3}H] putrescine into dimethylcasein. The NO donors, SNAP, spermine-NO (SPER-NO) and 3-morpholinosydnonimine (SIN-1), and the NO-carrier, S-nitrosoglutathione (GSNO), inhibited FXIII activity in a dose-dependent manner, in both purified enzyme and plasma preparations. Titration of -SH groups of FXIII with [^{14}C] iodoacetamide has shown that the number of titratable cysteines per monomer of FXIII decreased from 1 (in absence of NO donors) to 0 (in the presence of NO donors). These results demonstrate that blood coagulation FXIII is a target for NO both *in vitro* and *in vivo*, and that inhibition occurs by S-nitrosylation of a highly reactive cysteine residue. In conclusion, we show that inhibition of FXIII activity by NO may represent an additional regulatory mechanism for the formation of blood clot with physio-pathological implications.

Key Words: **factor XIII; nitric oxide; S-nitrosylation; transglutaminase.**

FXIII is the last enzyme of the coagulation cascade [1]. In the plasma, it is present as an inactive heterotetramer of paired A and B subunits, whereas in platelets, monocytes, megakaryocytes and placenta, it is found as a potentially active A_2 homodimer. Activation of the pro-enzyme in blood is achieved by the proteolytic action of thrombin [2], which promotes the dissociation of the A and B subunits. The active form of FXIII rapidly crosslinks fibrin monomers and thus stabilizes the blood clot, rendering it resistant to mechanical and chemical attack. Furthermore, activated FXIII plays a crucial role in regulating fibrinolysis by crosslinking fibrin to α2-antiplasmin, the most potent inhibitor of plasmin, thereby protecting fibrin from elimination by plasmin. FXIII belongs to the transglutaminase (TGase) family. At least six members of this family have been identified: FXIII, TGase 1, TGase 2, TGase 3, TGase 4, and band 4.2. These Ca^{2+}-dependent enzymes catalyze acyl transfer reactions among proteins by establishing either ϵ-(γ-glutamyl)lysine or N, N bis (γ-glutamyl)polyamine isopeptide bonds. All TGases have a cysteine residue in the active site, as do the cysteine proteases. In particular, the thioester acyl enzyme intermediate of FXIII occurs at cysteine 314 [3]. We have recently shown that NO inhibits TGase 2 activity by S-nitrosylation [4].

NO is a short-lived free radical which is generated by the enzymatic oxidation of L-arginine into citrulline through the action of NO synthase [5]. NO is a major messenger molecule involved in a wide range of biological processes, including regulation of vascular tone and blood pressure by relaxing blood vessels, neurotransmission, cell-mediated immune response and cell death [6]. NO exerts its biological effect by interacting with a variety of molecular targets, mainly using metals such as iron, thiols and oxygen as reactive groups. In the vascular system, for example, blood pressure and the modulation of platelet aggregation are influenced by NO via activation of cytosolic guanylate cyclase. NO has also been shown to interact with other haeme- and non-haeme iron-containing proteins, thus regulating their activity. Nitrosonium ion (NO^+) can react with protein thiol groups causing S-nitrosylation, thus af-

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[2] To whom correspondence should be addressed at IDI-IRCCS, Biochemistry Lab, c/o Dept. Experimental Medicine, D26/F153, University of Rome Tor Vergata, Via Tor Vergata 135, 00133, Rome, Italy. Fax: +39 6 20427290. E-mail: gerry.melino@uniroma2.it.

Abbreviations: FXIII, factor XIII; NO, nitric oxide; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SPER-NO, spermine-NO; SIN-1, 3-morpholinosydnonimine; GSNO, *S*-nitrosoglutathione; -SH groups, sulfhydryl groups; DTT, dithiothreitol; TCA, trichloroacetic acid; PBS, phosphate buffered saline.

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fecting the function of these proteins. S-nitrosylation occurs in some receptors [7], several cytosolic proteins [8] and some redox-sensitive transcription factors [9-10]. Here, we show that FXIII can be modulated by S-nitrosylation.

MATERIALS AND METHODS

Immunoblot. Plasma citrate from three healthy volunteers was diluted 1:1 with water and SNAP (Alexis Biochemicals, Läufelfingen, Switzerland), at the indicated concentrations, was added. After 5 minutes, plasma was incubated with 1.2 NIH-U/ml human thrombin (Sigma Chemical, St. Louis, MO) and 20 mM $CaCl_2$ at 37°C. The incubation was continued 5 min after clotting and then the fibrin clot was solubilized by the addition of 2% SDS and 2% β-mercaptoethanol. After 5 minutes of incubation at 90°C, Western blot was carried out on solubilized samples. An aliquot of 15 μg of total proteins was subjected to SDS-PAGE on a 8% polyacrylamide gel [11] and proteins were electroblotted onto nitrocellulose membranes. The blots were first blocked with 3% bovine serum albumin, 0.3% gelatin from porcine skin, 0.1% Tween-20 in PBS for 2 hours. They were then probed for 12-16 hours with monoclonal anti-fibrinogen-containing ascites (Sigma Chemical, St. Louis, MO). After two 10-min. and three 5-min. washings in PBS/0.1% Tween-20, a horseradish peroxidase-conjugated antimouse secondary antibody (Bio-Rad, Munchen, Germany) was added. Finally, the membranes were incubated with enhanced chemiluminescence detection solution and exposed to Hyperfilm ECL film (Amersham, Arlington Heights, IL).

Activation of FXIII. Purified coagulation FXIII (A_2 subunit from human platelets; Calbiochem, La Jolla, CA) or plasma citrate from three healthy volunteers was activated by 18-hour incubation with agarose gel-immobilized thrombin (FXIII:thrombin ratio was 1:0.1 mg) in 50 mM Tris-HCl buffer, pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$, at 25°C. The immobilized thrombin was then removed by low speed centrifugation and activated samples were stored at −20°C.

Enzyme assay. TGase activity was determined by measuring the incorporation of [^{3}H] putrescine into dimethylcasein [12]. The reaction mixture contained 150 mM Tris-HCl buffer pH 8.3, 90 mM NaCl, 30 mM dithiothreitol (DTT), 15 mM $CaCl_2$, 12.5 mg N,N'-dimethylcasein/ml, 0.2 mM putrescine containing 1 μCi [^{3}H] putrescine (Amersham, Arlington Heights, IL). Proteins from activated plasma preparations (0.1 mg) or 100 ng of activated coagulation FXIII were incubated at 37°C with the reaction mixture in a final volume of 150 μl. After 20 minutes of incubation, the reaction was stopped by spotting 100 μl quadruplicate aliquots onto Whatman 3 MM filter paper. Unbound [^{3}H] putrescine was removed by washing with large volumes of 15%, 10% and 5% trichloroacetic acid (TCA) and absolute ethanol. Filters were then air-dried and the radioactivity was measured by liquid scintillation counting. One enzyme unit was defined as the amount of enzyme binding 1 nmol of putrescine to N,N'-dimethylcasein per hour per mg of protein. The effect of NO-releasing compounds (Alexis Biochemicals, Läufelfingen, Switzerland) was assessed by adding the agents to the enzyme preparation just prior the beginning of the reaction.

Titration of -SH groups. The highly reactive active site cysteine residue was titrated by alkylation with iodoacetamide. Reaction mixtures containing 0.1 M Tris-acetate pH 7.5, 20 mM $CaCl_2$, 0.5 mM [^{14}C] iodoacetamide (Amersham, Arlington Heights, IL; 59 mCi/mmol) and 0.5 μg of FXIII, either before or after activation, were incubated 5 minutes at 23°C. Reactions were terminated by the addition of 100 mM DTT and 20 mM EDTA. Aliquots were spotted on 3MM filter paper. Filters were washed in TCA 20%, TCA 10%, TCA 5%, ethanol, and then dried and counted.

RESULTS AND DISCUSSION

To investigate whether NO regulates the formation of blood clots, we studied the effect of the NO-donor SNAP on coagulation *in vitro* in duplicate using blood samples from three healthy volunteers. Neither the mechanism of nitrosylation nor the foremost mechanism for NO release (possibly a metal-catalyzed reaction) *in vivo* is known. Clearly these mechanisms depend on the biological milieu, and release free NO concentrations four orders of magnitude lower than the donor concentrations added [9].

Ten mM SNAP completely suppressed clot formation, whereas 0.1, 1, 2 mM SNAP dissolved the blood clot after the addition of 2% SDS and 2% β-mercaptoethanol (not shown). We evaluated the effect of NO on the γ-chain cross-linking in fibrin gels by immunoblot (Fig. 1A). In the plasma, two bands reacting with an

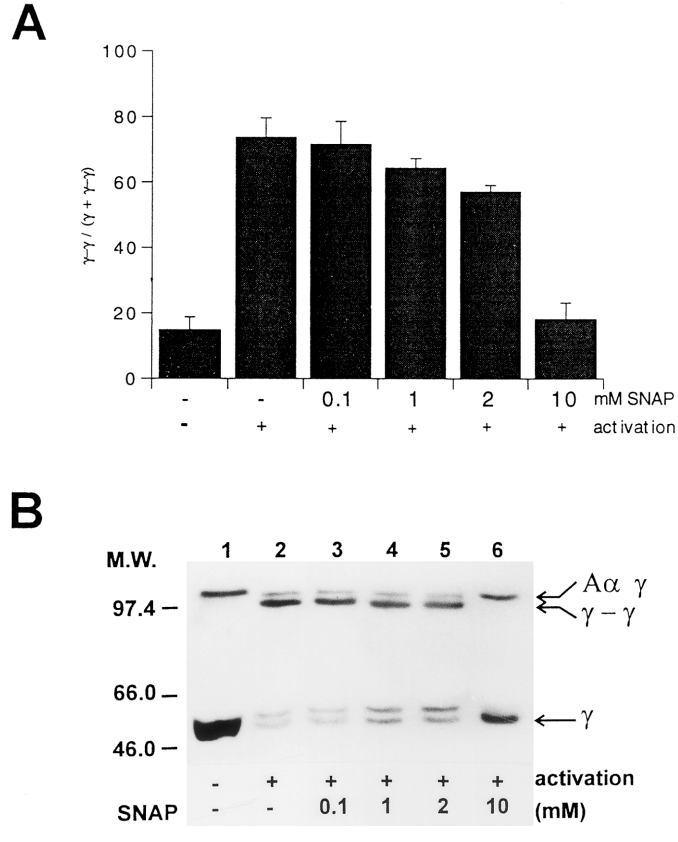

FIG. 1. Effect of SNAP on FXIII-catalyzed crosslinking of fibrin. (A) Western blot, quantified by gel scanning densitometry, was carried out on aliquots of plasma clotted by the addition of 1.2 NIH-U/ml human thrombin, before and after incubation with increasing concentrations of the NO-donor. The γ dimer formation was calculated as the ratio (density) of the γ dimer band over the sum of dimers plus monomers. Data are the means of three separate experiments performed in duplicate. (B) Immunoblot showing FXIII-catalyzed crosslinking of the γ-chain. Lane 1, control plasma; lane 2, activated control plasma; lanes 3-6, activated plasma samples incubated in the presence of 0.1, 1, 2 and 10 mM SNAP, respectively.

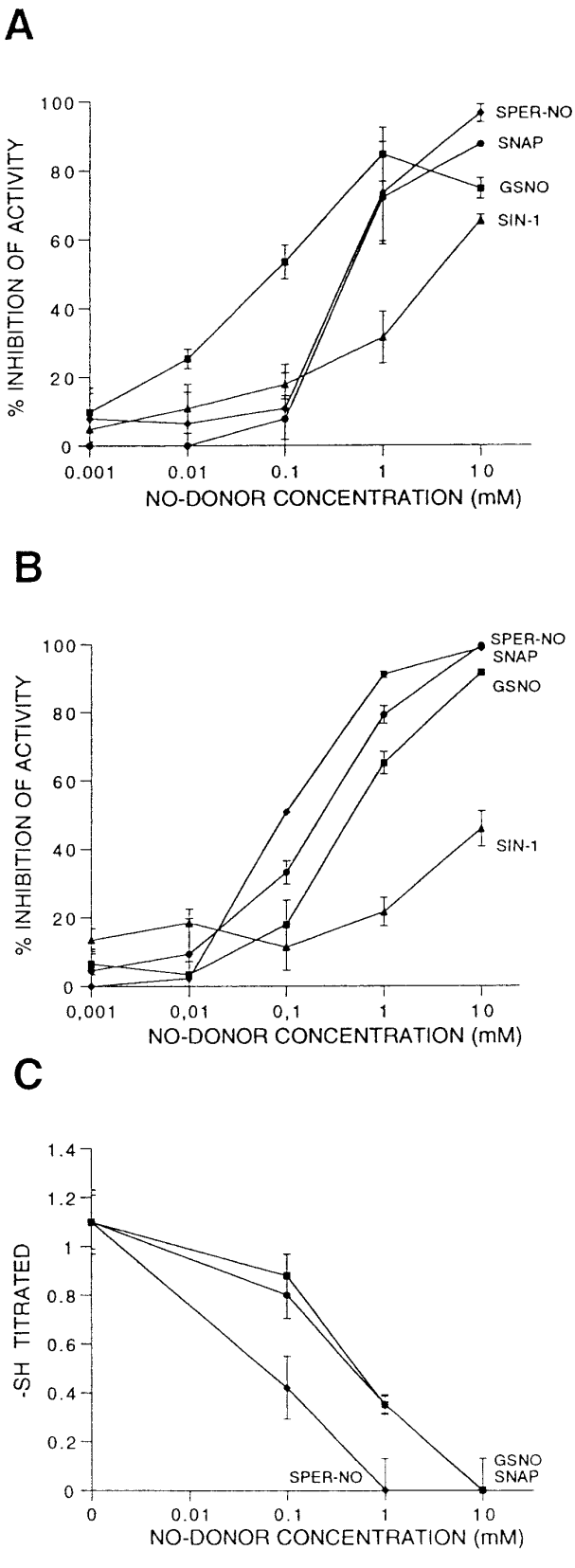

FIG. 2. Effect of NO-donors on FXIII activity and titration of -SH groups. (A) Inhibition of FXIII activity by NO-donors in blood samples. Activated plasma preparations (0.1 mg protein) were incubated with increasing concentrations of SNAP, GSNO, SPER-NO and SIN-1 for 20 min. The NO-donors were added to the enzyme preparation immediately before the assay. Activity was evaluated as [^{3}H]-putrescine incorporated into dimethylcasein. Data are the means of quadruplicate determinations carried out with the plasma obtained from three healthy volunteers. (B) Inhibition of purified FXIII activity by NO-donors. Activation was obtained by 18h incubation with a FXIII:thrombin ratio 1:0.1 μg and removal of immobilized thrombin. Purified FXIII (100 ng) was exposed immediately before the assay to different concentrations of SNAP, GSNO, SPER-NO and SIN-1 and incubated in the presence of 30 mM DTT for 20 min. Data are the means of quadruplicate determinations of three separate experiments. (C) Reaction of NO with the active cysteine residue of FXIII. Titration was evaluated by alkylation with [^{14}C]-iodoacetamide. Purified enzyme was incubated with increasing concentrations of the indicated NO-donors and titration was performed after 20 min. Data are the means of quadruplicate determinations carried out in two separate experiments.

antibody raised against the γ-chain of fibrinogen were revealed (Fig. 1B, lane 1): one band, 47 kDa, corresponds to the γ-chain of fibrinogen, and the second, 105 kDa, corresponds to intramolecular cross-linked Aα-γ chain heterodimers present in circulating fibrinogen molecules [13]. After activation by thrombin, the disappearance of the monomeric γ-chain and the appearance of γ-dimers were clearly detected (lane 2). Following the addition of 10 mM SNAP (lane 6) the formation of γ-dimers was completely inhibited, whereas lower concentrations seemed to be less effective (lanes 3-5). Quantification of immunoblots by densitometry is shown in Fig. 1A.

We studied the effect of NO on FXIII enzymatic activity in blood samples from healthy volunteers using activated plasma preparations incubated with increasing concentrations of NO-donors. As shown in Fig. 2A, all NO-donors inhibited FXIII activity in a dose-dependent manner, with GSNO being the most effective inhibitor (half-maximal inhibition: 76 μM GSNO). SIN-1 was less effective in inhibiting FXIII activity. SIN-1 also releases $O_2^{-\cdot}$, which reacts with NO to produce peroxinitrite, thus reducing the NO available to inhibit FXIII. This might indirectly suggest that NO^+, and not $ONOO^-$, is the reactive species.

Purified FXIII was also shown to be inhibited by NO-donors. The activity was inhibited in a dose-dependent manner by SNAP (Fig. 2B). When DTT was omitted from the reaction mixture, the basal activity was reduced from 1700 $\pm$ 718 to 150 $\pm$ 73 (90 $\pm$ 5% inhibition) nanomoles [^{3}H] putrescine/hr/mg of protein and more NO was required to produce a given percent of inhibition. The half-maximal inhibition of FXIII, in fact, occurred with 230 μM SNAP in the presence of DTT and 8.3 mM in the absence of DTT. The shift of the dose-response curve in the absence of DTT may be explained by the fact that NO is able to S-nitrosylate a cysteine crucial for the catalytic activity of FXIII. DTT mantains this residue in a reduced active state and thus in-

creases the ability of SNAP to inhibit FXIII, allowing NO to interact with the active -SH group. SPER-NO was the most potent inhibitor, with half-maximal inhibition at 96 μM. FXIIIA has 9 cysteines. We evaluated solvent accessibility using the WHATIF program with the crystallographic pdb-data, and determined that only one of these cysteines is likely to be exposed to the solvent. We titrated the reactive cysteine -SH groups with iodoacetamide before and after the reaction of FXIII with NO-donors. Fig. 2C shows that the titrable -SH per monomer of activated FXIII decreases from 1 to 0. This parallels the activity data, suggesting that the inhibition involves one -SH group.

NO has been shown to critically regulate the hemostatic system, as well as to exert other pleiotropic effects. The action of this free-radical in the vascular system is mainly mediated by the activation of guanylate cyclase and the subsequent increase of intracellular concentrations of cGMP which inhibit platelet aggregation [14], thus inhibiting blood clotting. Numerous studies on the mechanism by which several NO-containing compounds block platelet adhesion and relax vascular smooth muscle suggest that these compounds may react with certain cellular thiols to form S-nitrosothiols, which are potent activators of guanylate cyclase. NO, in addition to having inhibitory effects on platelet aggregation, has been shown to counteract other thrombogenic processes, such as local vasoconstriction, coagulation cascade by antagonizing fibrin formation, and interaction between fibrin and platelets [15]. Protective effects of NO against arterial thrombosis were also identified since calreticulin, a Ca^{2+}-binding protein with antithrombotic properties, was observed to interact with endothelial cells to stimulate NO production and inhibit clot formation [16]. NO enhances fibrinolysis by inhibiting the release of plasminogen activator inhibitor from platelets [17] and also S-nitrosylates tissue type plasminogen activator, which incorporates the vasodilatory and anti-platelet properties characteristic of NO [18]. Here, we have shown that NO can inhibit blood coagulation FXIII both *in vitro* and *ex vivo*, through S-nitrosylation of a crucial titrable cysteine residue. This may represent an additional regulatory mechanism to control the delicate equilibrium between coagulation and fibrinolysis, with possible therapeutical applications.

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