Short Communication

The effects of nitronium ion on nitration, carbonylation and coagulation of human fibrinogen

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Abstract. The effect of nitronium ion on nitration, carbonylation and coagulation of human fibrinogen (Fg) *in vitro* was investigated. We observed that nitration of tyrosine, induced by NO_2BF_4 (0.01 mmol/l), was increased. No changes in carbonylation by NO_2BF_4 (0.01 mmol/l) were noticed. Mentioned alterations were associated with amplified coagulation of Fg. Higher concentrations of NO_2BF_4 (1 and 0.1 mmol/l) triggered growth of nitration and carbonylation of Fg, but led to inhibition of polymerization. Slight nitration may be responsible for increase, whereas sizable nitration and oxidation may lead to inhibition of Fg coagulation.

Key words: Fibrinogen — Nitrotyrosine — Carbonyl groups — Nitronium ion — Polymerization — Coagulation

Fibrinogen (Fg) is the circulating precursor of fibrin, converted by thrombin to fibrin monomers, which aggregate spontaneously to form fibrin fibers (Standeven et al. 2005).

This complex protein molecule is composed of two sets of three non-identical polypeptide chains Aα, Bβ and γ. All six amino-terminals meet together in a small central domain E connected with two terminal domains D by long coiled coils. Chains Bβ and γ form respective βC and γC subunits in D domain, whereas Aα chain folds back and by connector region goes to αC subunit, which interacts close to E domain (Doolittle and Kollman 2006).

Fg is highly susceptible to reactive oxygen and nitrogen species which can induce structural changes and lead to the impairment of their biological functions (Shacter et al. 1995). The rapid reaction between nitric oxide and superoxide forms peroxynitrite (PN) (Beckman et al. 1990) which may induce nitration of tyrosine (Ischiropoulos and al-Mehdi 1995). Oxidized and nitrated Fg was found in plasma of patients with coronary disease, lung cancer, rheumatoid arthritis, chronic renal failure, septic shock, diabetes and lung injuries (Kaur and Halliwell 1994). Nitrotyrosine (Ntyr) may accelerate Fg clotting, whereas oxidation of Fg seems to decrease that activity (Vadseth et al. 2004). Altered Fg shows reduced ability to mediate platelet adhesion and aggregation *in vitro* (Nowak and Wachowicz 2002). There is little known about nitration of Fg by nitronium ion (NI). The aim of our study was to determine the effect of NI, derived from nitronium tetrafluoroborate (NO_2BF_4), on nitration, carbonylation and coagulation of human Fg *in vitro*.

Fg was prepared from plasma, purchased from Lodz Blood Bank, according to Doolitlle et al. (1967). Its concentration was determined spectrophotometrically at 280 nm (extinction coefficient 1.55 for 1 mg/ml). NO_2BF_4 was obtained from Fluka (USA). 2,4-dinitrophenylhydrazine and anhydrous acetic acid were purchased from POCh S.A. (Poland). Goat anti-Ntyr polyclonal antibodies were from Abcam (USA). Biotynylated antigoat/mouse/rabbit antibodies and streptavidin-biotynylated horseradish peroxidase were from DAKO (USA). All other reagents were purchased from Sigma (USA).

Preparations of human Fg (2 mg/ml) in 100 mmol/l potassium phosphate buffer (pH 7.5) were exposed to NO_2BF_4 at final concentrations of 1, 0.1 and 0.01 mmol/l. 1 mol/l NO_2BF_4 stock was prepared in anhydrous acetic acid immediately before incubation with Fg samples. The final concentrations were received by addition of following substances to the samples in an ice bath: the stock, the stock 10 times dissolved in anhydrous acetic acid, and the stock 100 × dissolved in anhydrous acetic acid, directly to Fg in a ratio 1 : 1000 (1 μl to 1 ml of Fg solution). The samples were vigorously mixed and incubated 1 h at 37°C. Control samples of Fg (0 mmol/l NO_2BF_4) were prepared by adding anhydrous acetic acid in the same ratio to Fg solution (pH dropped only from 7.5 to 7.0).

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The level of Ntyr in Fg (control or NO_2BF_4-treated) was estimated by the competition ELISA (c-ELISA) method (Khan et al. 1998). The nitrated Fg at concentration 0.5 μg/ml (3–6 mol Ntyr/mol proteins) was prepared for use in the standard curve. The concentrations of nitrated proteins that inhibited anti-Ntyr antibodies binding were estimated from the standard curve and were expressed as nitro-Fg equivalents (Ntyr-Fg) as described previously (Olas et al. 2004). The amount of Ntyr in standard Fg was determined spectrophotometrically (at pH 11.5, ε_{430nm} = 4400 mol/l^{-1}·cm^{-1}) (Ischiropoulos and al-Mehdi 1995). The amount of Ntyr in NO_2BF_4-nitrated Fg was estimated from the standard curve. Carbonyl groups (C=O groups) in Fg (control or NO_2BF_4-treated protein) were measured by ELISA method (Buss et al. 1997).

Clotting activity of Fg was experimentally monitored by measurement of turbidity as a function of time as described previously (Nowak et al. 2007). Turbidity curves were characterized by 3 parameters: a lag time – when soluble fibrin monomers and protofibrils do not change the absorbance; a growth rate (maximum polymerization velocity, V_{max}) – when the slope depends primarily on the rapid lateral protofibrils association to fibers; and the maximum turbidity (T_{max}) – when the final network of fiber bundles is formed (Weisel and Nagaswami 1992).

The significance was analyzed by one-way ANOVA followed by Dunnet's and Tukey's post hoc tests. A level $p < 0.05$ was accepted as statistically significant.

Exposure of Fg to nitronium fluoroborate resulted in increase in Ntyr as determined by c-ELISA method (Fig. 1A). We observed statistically significant increase in Ntyr level ($p < 0.005$) in Fg treated with the lowest concentrations (0.01 mmol/l) of NO_2BF_4 in relation to control (0 mmol/l). The higher concentrations of NO_2BF_4 (0.01, 1 mmol/l) also caused increase in Ntyr amount in Fg (Fig. 1A). Increase in C=O groups caused by NO_2BF_4 at the lowest concentration (0.01 mmol/l) was statistically insignificant (Fig. 1B), whereas the incubation of Fg with higher concentrations of NO_2BF_4 (0.1 and 1 mmol/l) resulted in significant augmentation of C=O groups ($p < 0.01$, $p < 0.05$, respectively). NO_2BF_4 induced changes of Fg coagulation properties. NO_2BF_4 at the lowest dose (0.01 mmol/l) caused statistically insignificant decrease in lag time (Fig. 2A), but statistically significant increase in V_{max} and T_{max} (Fig. 2B,C). The higher concentrations of NO_2BF_4 (0.1 and 1 mmol/l) led to increase in lag time and decrease in V_{max} and T_{max} (Fig. 2B,C).

Oxidative stress may lead to oxidative/nitrative modifications of Fg associated with alterations of its biological properties. Oxidative factors may inhibit polymerization of fibrin monomers (Shacter et al. 1995). PN has been found to enhance, or diminish the haemostatic activity of Fg. It was described that 1 mmol/l PN inhibited clotting properties of Fg (Lupidi et al. 1999), whereas other scientists showed that the same relatively high concentration of PN increased polymerization rate (Gole et al. 2000). Depend-

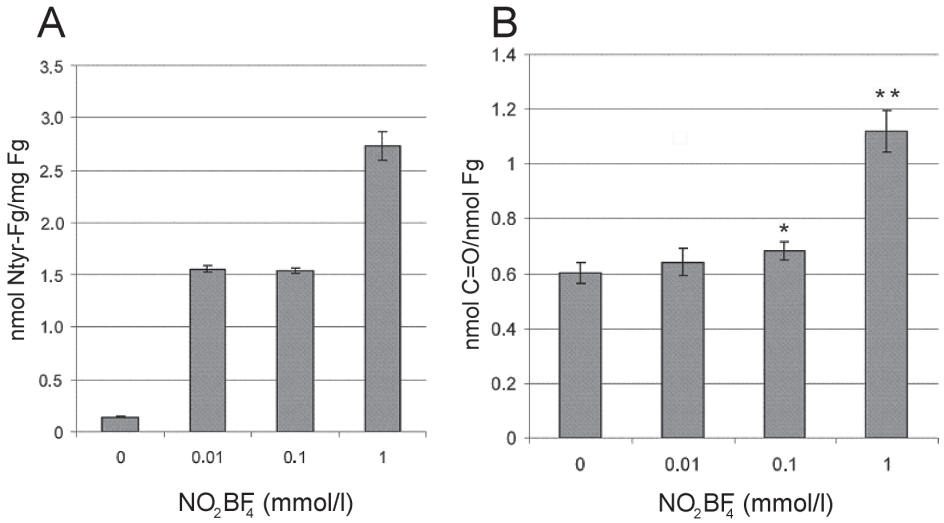

Figure 1. A. Effect of nitronium tetrafluoroborate (NO_2BF_4, 0.01–1 mmol/l) on nitration of tyrosine residues in human fibrinogen (Fg). The tyrosine nitration was measured with c-ELISA. The results are representative of 9 independent experiments, and are expressed as means ± SEM of NO_2BF_4-treated Fg *versus* control Fg (0 mmol/l NO_2BF_4), ($p < 0.001$). **B.** 2,4-dinitrophenylhydrazine-reactive carbonyl formation following treatment of human Fg with NO_2BF_4 (0.01–1 mmol/l). The protein carbonylation was measured using ELISA method. The results are representative of 7 independent experiments, and are expressed as means ± SEM of NO_2BF_4-treated Fg *versus* control Fg (0 mmol/l NO_2BF_4). Ntyr, nitrotyrosine; * $p < 0.05$; ** $p < 0.001$.

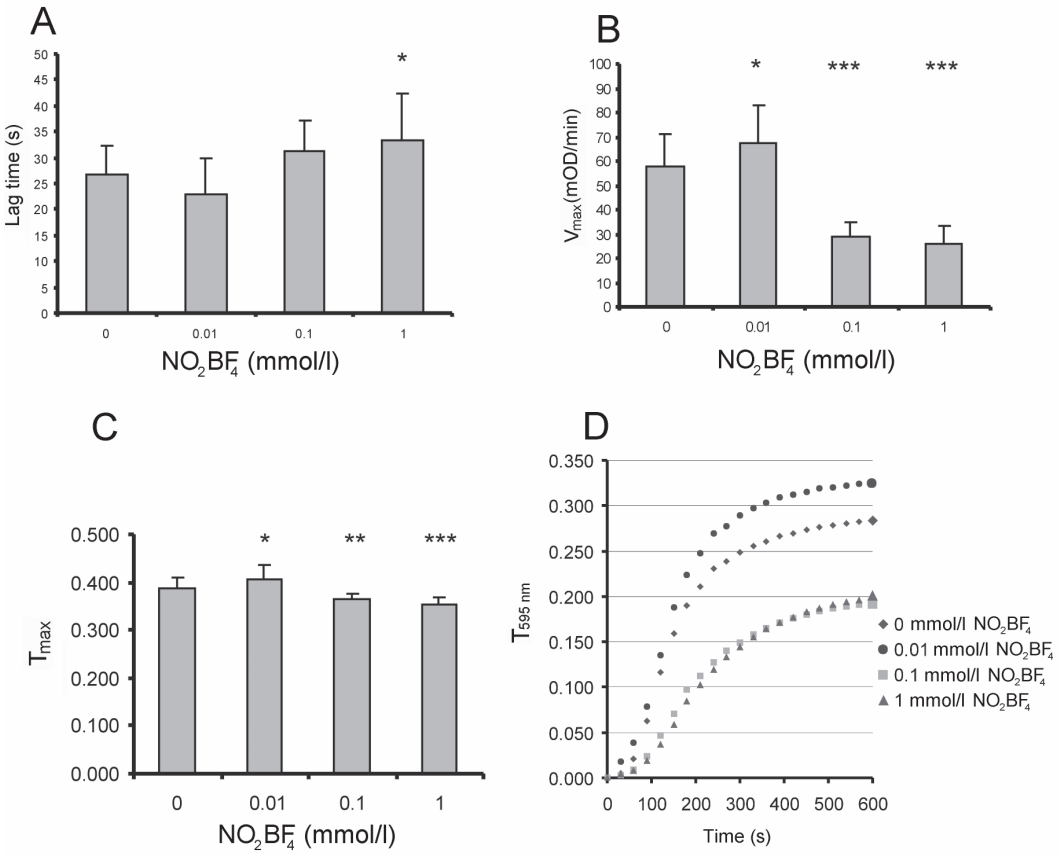

Figure 2. Effect of nitronium tetrafluoroborate (NO_2BF_4, 0.01–1 mmol/l) on thrombin-catalyzed fibrin polymerization. **A.** Lag time expressed in seconds. **B.** Maximum polymerization velocity (V_{max}) expressed as change of turbidity *per* minute × 10^3 (mOD/min) at 595 nm filter. **C.** Maximum turbidity (T_{max}) after 1 h of incubation. **D.** Kinetic plots. The results are representative of 21 independent experiments, and are expressed as means ± SEM of NO_2BF_4-treated Fg *versus* control Fg (0 mmol/l NO_2BF_4). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

ing on different conditions, PN could be oxidative as well as nitration agent.

Nitration of tyrosine in proteins can be triggered in many ways; by free radical mechanism of NO_2 derived from dissolution of $ONOO^-$, by action of its intermediate compound formed in reaction with CO_2, by action of NO_2Cl formed by mieloperoxydase-hypochlorous acid-nitrite (MPO/HOCl/NO_2^-) and by NI formed by MPO/Me^{n+}/NO_2^- (Radi 2004). Transition metal centers of enzymes such as superoxide dismutase or mieloperoxydase additionally may catalyze PN to NI *in vivo* (Quijano et al. 2001). NO_2^+ possesses the abilities to nitrate aromatic ring by electrophilic substitution (Olah et al. 1981). This solid chemical dissolves in aprotic solvents and dissociates into NI that is converted to extra active form in the presence of H^+ (Olah et al. 1992, 1997). This compound nitrates not only unsaturated fatty acids (O'Donnell et al. 1999), but also tyrosine of peptides in physiological pH (Kong et al. 1996).

In our study we selected NO_2BF_4 as a source of NI to examine nitration and oxidation in a system independent from PN formation and to test its effect on Fg coagulation. We showed that low concentration of NO_2BF_4 caused increased polymerization of fibrin monomers. These polymerization changes were observed when the level of Ntyr in Fg was enlarged, but C=O groups were not formed. High concentrations of NO_2BF_4 induced increase in nitration and carbonylation of Fg molecule, associated with inhibition of fibrin monomer polymerization. Our results confirmed earlier observations of Vadseth and co-workers that slight nitration might be responsible for increase in Fg coagulation properties. By the other hand it seems that sizable nitration and oxidation may lead to inhibition of Fg coagulation.

Human dimeric molecule of Fg contains precisely 100 tyrosine residues (9, 21, 20 in one Aα, Bβ and γ chain, respectively), but only 20–30 are exposed to the solvent phase (York and Blomback 1979). It is possible that nitration of some mentioned tyrosine residues may have effect on polymerization of fibrin monomers. A knowledge which of them are responsible for alterations described in this work

could be important for explanation of the role of nitrated Fg in atherosclerosis and needs more research in the future.

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