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Peroxynitrite-mediated oxidation of fibrinogen inhibits clot formation

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Abstract The clotting activity of human fibrinogen was fully inhibited in vitro by peroxynitrite. The decrease of activity followed an exponential function and the concentration of peroxynitrite needed to inhibit 50% of fibrinogen clotting was 22 μM at 25°C. The oxidative modification(s) induced by the peroxynitrite system (i.e. $ONOO^-$, ONOOH and ONOOH*) appeared specifically to affect fibrin clot formation (through the inhibition of fibrinogen polymerization) since the interaction of peroxynitrite-modified fibrinogen with thrombin appeared to be unaffected. The addition of $NaHCO_3$ decreased the peroxynitrite effect on fibrinogen clotting, suggesting that the reactive species formed by the reaction of CO_2 with peroxynitrite are less efficient oxidants of peroxynitrite itself. Similar effects were observed after addition of bilirubin, which also exerted a significant protection against peroxynitrite-mediated modification of fibrinogen.

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Key words: Fibrinogen; Protein oxidation; Peroxynitrite; Clotting

1. Introduction

Nitric oxide ($NO^\bullet$) is a free radical messenger molecule which plays a key role in several physiological processes, such as neurotransmission, inhibition of platelet adherence and aggregation, blood pressure control, neutrophil aggregation, blood clotting and oxidative modification of low density lipoprotein in plasma [1–4]. On the other hand, many pathophysiological processes, such as reperfusion of ischemic tissues [5] and acute inflammation [6], may be trigger events, which stimulate $NO^\bullet$ production. Owing to its oxidative power, an excessive amount of $NO^\bullet$ can induce, on biological systems, undesirable effects which are strongly increased by the reaction with transition metals and oxygen active species [7–9]. In this context, particular attention has been focused on the reaction of $NO^\bullet$ with superoxide anion O_2^-, which is released by a variety of cells such as endothelial cells, activated neutrophils and macrophages [10–13]. The combination of $NO^\bullet$ with superoxide radicals (O_2^-) produces several active species, which are able to oxidize many cellular components. The main end-product of this reaction is the peroxynitrite anion ($ONOO^-$), which, under physiological conditions, is in equilibrium with the peroxynitrous acid (ONOOH), and rapidly decays to nitrate through the formation of a very active secondary species (i.e. ONOOH*). The peroxynitrite system (i.e. $ONOO^-$, ONOOH and ONOOH*) leads to a covalent modification of several amino acid residues in proteins, such as cysteine, methionine, tryptophan and tyrosine residues [8,14–16]. Therefore, since the rate of peroxynitrite formation depends on the product of O_2^- and $NO^\bullet$ concentrations, even a relatively small increase of one or both species may result in a remarkable increase of peroxynitrite formation up to potentially cytotoxic levels. Evidence for a role of $ONOO^-$ in vivo is largely based upon detection of modified tyrosine residues (as 3-nitrotyrosine) in the biological system, even though the presence of anti-oxidizing agents, such as ascorbic acid, thiols and uric acid, inhibits peroxynitrite-induced oxidation [17]. The levels of nitrated proteins in plasma of healthy subjects are normally low, but they drastically increase in patients with acute lung injury and atherosclerosis [18–21], and even low concentrations of peroxynitrite (of about 1–1.5 mM) have been shown to induce in vitro a large tyrosine nitration of bovine serum albumin and fibrinogen [22] with possible relevance for their biological properties. These effects appear to be related to either a direct interaction of peroxynitrite (and/or its derivatives) with these plasma components and/or to a synergistic action with the highly unstable nitrosoperoxycarbonate ($ONO_2CO_2^-$). This product which originates from the reaction of peroxynitrite with CO_2 (see below, reaction 1) seems to protect protein molecules from various oxidative damage induced by peroxynitrite (in particular from the –SH oxidation), but, at the same time, it stimulates, in a concentration-dependent manner, nitration of tryptophan and tyrosine residues on some plasma proteins, mainly on serum albumin and, with lesser effects, on fibrinogen [22,23]. The latter protein, which represents about 4% of the total plasma proteins and plays a key role in the clotting cascade, appears particularly susceptible to the effect of oxidative compounds; in fact, exposure of fibrinogen to the Fe^{2+}/ascorbate system leads to a significant decrease of clotting activity [24].

In this paper, we show that a similar effect can be obtained when fibrinogen interacts with physiological concentrations of peroxynitrite and that this inhibitory effect can be antagonized by CO_2 and bilirubin.

2. Materials and methods

2.1. Materials

Human fibrinogen was purified by human plasma as described by Bharat et al. [25]. Its concentration was determined spectrophotometrically at 280 nm using an extinction coefficient of 1.5 for 1 mg/ml solution. Human thrombin (3000 U/mg), was purchased from Sigma (USA). Bilirubin was obtained from Aldrich (USA). All other re-

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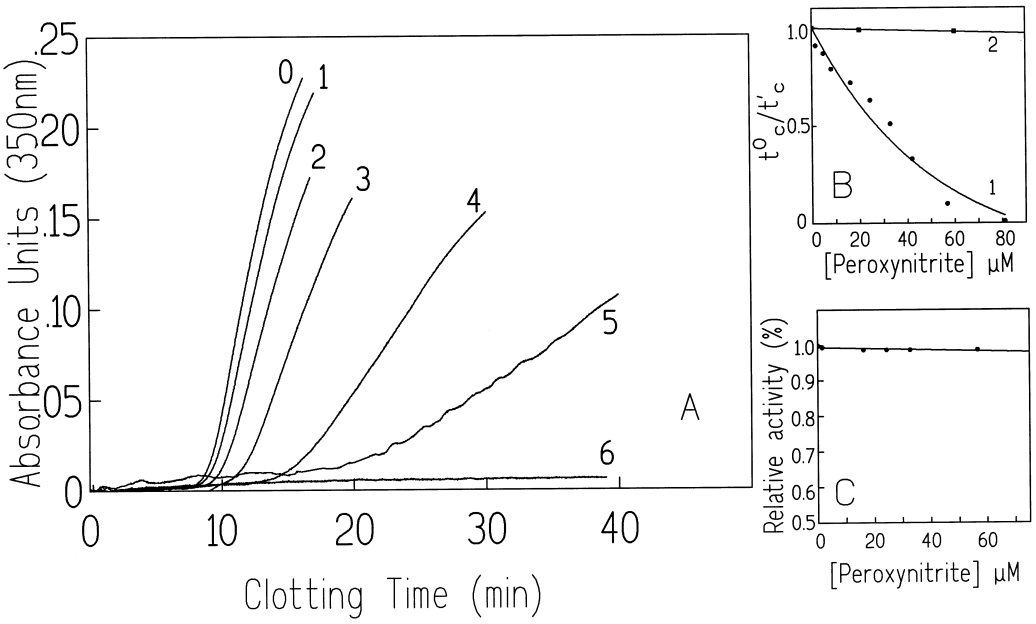

Fig. 1. Effect of peroxynitrite on fibrinogen clotting induced by human thrombin. A: Effect of increasing amounts of peroxynitrite (0, (0); (1) 2 μM; (2) 8 μM; (3) 16 μM; (4) 21 μM; (5) 32 μM; (6) 56 μM) on fibrinogen clotting time. Fibrinogen (0.5 μM) was incubated at 25°C for 1 min in 50 mM phosphate buffer pH 7.1 after addition of peroxynitrite. Clotting was followed at 350 nm after addition of 1.2 units of human thrombin. B: Line 1, inhibition of fibrinogen clotting (●) (expressed as the t^0_c/t^i_c ratio) as function of peroxynitrite concentration. The experiments were performed as reported in (A). For the t^0_c and t^i_c significance, see text. Line 2 refers to the effect of the peroxynitrite decomposition products on fibrinogen clotting (○). At this end, different amounts of peroxynitrite were incubated for 1 min in 50 mM phosphate buffer pH 7.1 before addition of fibrinogen (0.5 μM). After another minute, human thrombin (1.2 U), was added and clotting activity measured as reported in (A). C: Effect of the peroxynitrite decomposition products on the amidolytic activity of human thrombin. Increasing amounts of peroxynitrite were incubated for 1 min in 50 mM phosphate buffer pH 7.1. Human thrombin (0.12 U) was added to the solution, incubated for 1 min and the amidolytic activity of the enzyme was measured at 405 nm following the hydrolysis of 13 μM of Tos-Gly-Pro-Arg-pNA (Chromozym-TH) [31].

agents were of the highest purity grade and used without further purification.

2.2. Synthesis of peroxynitrite

Peroxynitrite was prepared from the reaction between H_2O_2 and $NaNO_2$, as described elsewhere [22]. The final solution was treated with manganese dioxide to remove the excess of H_2O_2 and then filtered twice. The concentration of peroxynitrite was determined measuring the absorbance of the solution at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$) [26]. The frozen stock solution was stored for 3–4 weeks at −40°C with negligible changes in its concentration. Immediately before use, the stock solution was diluted in sodium hydroxide 100 mM and, during the experiments, was maintained in an ice bath.

2.3. Clotting experiments

The thrombin-induced clotting of fibrinogen was measured spectrophotometrically, following the increase in absorbance at 350 nm as a function of time, in a Cary 1 dual-beam spectrophotometer, thermostated at 25°C. Fibrinogen was incubated for 1 min in 100 mM phosphate buffer pH 7.1, 0.1 mM diethylenetriamine penta-acetic acid (DPTC) with different amounts of peroxynitrite and clot formation was triggered by addition of 1.2 units of human thrombin. Clotting curves were analyzed as described in [27]. The effect of CO_2 on the peroxynitrite-mediated fibrinogen oxidation was studied by addition of increasing amounts of $NaHCO_3$ to the fibrinogen sample 2 min before addition of peroxynitrite (in order to account for the CO_2 hydration and dehydration equilibration, which has a half time of 25 s over the pH range 7–8 [28]). Similarly, the effect of bilirubin was evaluated by incubating increasing amounts of bilirubin with fibrinogen for 2 min before addition of peroxynitrite and the clotting activity was evaluated as described above. Stock solutions of bilirubin were kept in NaOH 0.1 M and their concentration was determined spectrophotometrically at 435 nm ($\varepsilon_{435} = 40.9 \pm 0.4\ mM^{-1}\ cm^{-1}$). The eventual presence of biliverdin was determined by the absorbance at 670 nm ($\varepsilon_{670} = 0.17 \pm 0.01\ mM^{-1}\ cm^{-1}$) [22].

3. Results and discussion

The effect of peroxynitrite on fibrinogen clotting induced by human thrombin is shown in Fig. 1. Increasing amounts of peroxynitrite decreased the rate of fibrinogen polymerization

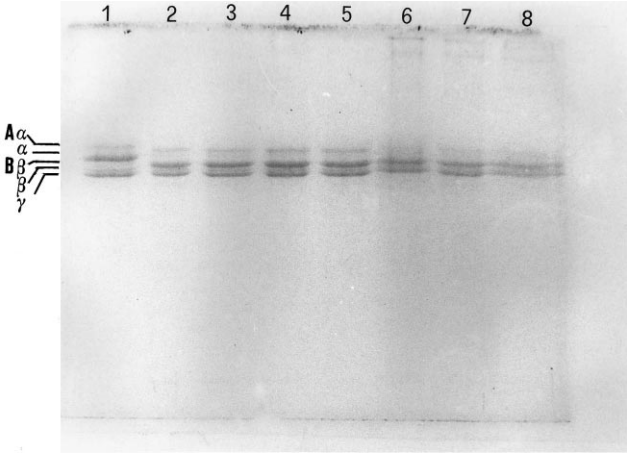

Fig. 2. SDS-PAGE (10%) of fibrinopeptides generated by the action of human thrombin on native (lanes 2–5) and peroxynitrite-treated (lanes 6–8) fibrinogen at different incubation times. Lane 1 refers to time point zero (urea added at fibrinogen prior to thrombin). Aliquots of the incubation mixture were withdrawn at 2 min (lanes 2), 5 min (lanes 3 and 6), 10 min (lanes 4 and 7) and 20 min (lanes 5 and 8), the reaction was stopped by addition of 5 M urea.

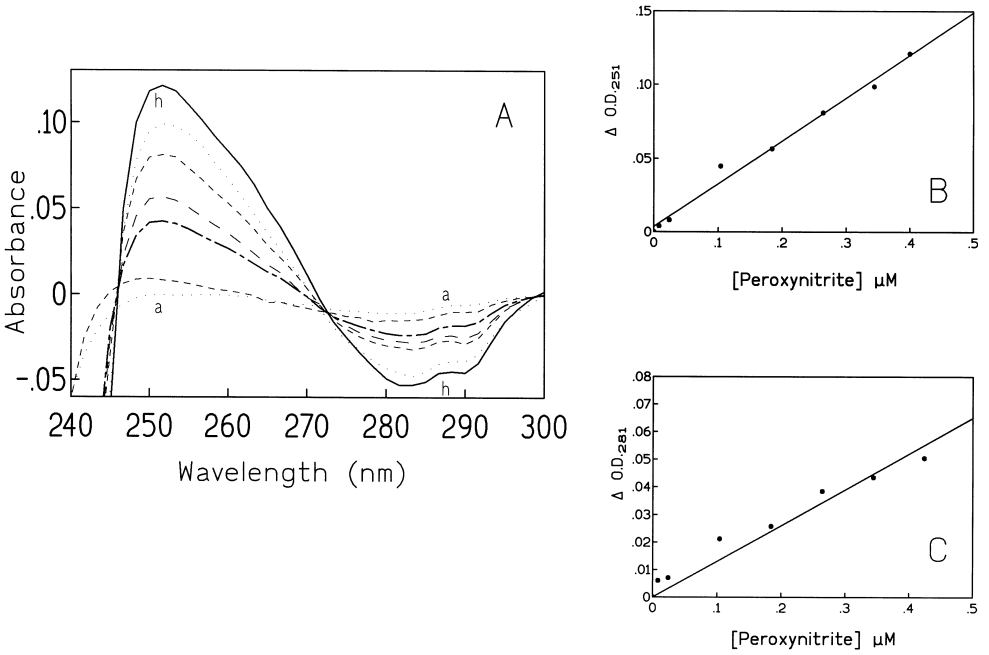

Fig. 3. Effect of increasing amounts (from 0 to 0.4 mM) of peroxynitrite on the UV difference spectra of fibrinogen (A). Measurements were performed at 25°C using 1.5 μM fibrinogen in 50 mM potassium phosphate buffer at pH 7.1. B,C: The absorbance change at 251 nm and 280 nm, respectively, determined by the presence of increasing (from 0 to 0.4 mM) amounts of peroxynitrite.

(expressed as increase of absorbance at 350 nm, see Fig. 1A), this lead to an increase of the clotting time (t_c), that is the interval required for the formation of the polymer (see Fig. 1A,B). In fact, the clotting inhibition (represented as the ratio t_c^0/t_c^i, where t_c^0 is the clotting time in the absence of peroxynitrite and t_c^i is the clotting time in the presence of the concentration i of peroxynitrite) appeared to be correlated to the concentration of peroxynitrite, reaching $\approx 100\%$ inhibition at 60 μM peroxynitrite (see Fig. 1B). As expected, because of the peroxynitrite instability the inhibitory effect was not affected by an increase in the incubation time of peroxynitrite with fibrinogen, and it reached its maximum value within 1 min of incubation time.

The inhibitory effect appeared specifically related to the oxidative action of peroxynitrite since it occurred also when 0.1 mM DTPC was present in the reaction buffer, this allowed us to rule out the possibility that the clotting inhibition was due to oxidative phenomena induced on fibrinogen by metal-catalyzed reactions. Furthermore, no effect of the decomposition products of peroxynitrite on the enzymatic activity of thrombin could be detected (see Fig. 1B,C), this eliminated also the possibility that the observed differences in clotting activity were linked to an unspecific effect on the thrombin function. Finally, the peroxynitrite effect on fibrinogen clotting activity did not seem to be derived from an impairment of its interaction with thrombin and the production of fibrino-

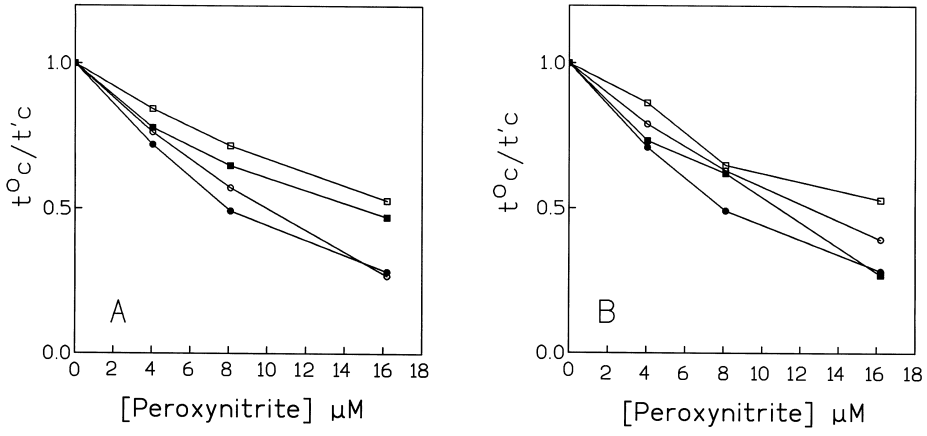

Fig. 4. Effect of increasing amounts of $NaHCO_3$ (A) and bilirubin (B) on the inhibition of fibrinogen clotting induced by peroxynitrite. A: The experiments were performed in absence (●) and in presence of 10 μM (○) and 15 μM (■) $NaHCO_3$, respectively. The curve (□) was obtained in the contemporary presence of 20 mM $NaHCO_3$ and 15 μM bilirubin. B: the experiments were performed in absence (●) and in presence of 5 (○) and 15 μM (■) bilirubin, respectively. The curve (□) was obtained in the contemporary presence of 20 mM $NaHCO_3$ and 15 μM bilirubin. 0.5 μM Fibrinogen was incubated with $NaHCO_3$, bilirubin, or both the reagents, for 2 min before addition of peroxynitrite. Clotting activity was measured as described in the legend of Fig. 1.

peptides, as results from the SDS gel-electrophoresis, displayed in Fig. 2, where the behavior of untreated and peroxynitrite-treated fibrinogen is compared. In fact, the exposure of fibrinogen to peroxynitrite did not alter the production of fibrinopeptides (Aα and Bβ) by thrombin, suggesting that the observed effect was most likely ascribable to a defective polymerization of fibrinopeptides rather than to a defective proteolysis by thrombin. This was further confirmed by the observation that the clotting time ratio t_c^0/t_c^i was independent of the thrombin concentration used in the assay (data not shown).

Therefore, it appeared evident that the peroxynitrite-induced alteration of clotting time (see Fig. 1) was related to a specific effect on the fibrinogen molecule. Thus, increasing concentrations of peroxynitrite brought about modifications of the fibrinogen UV spectra (Fig. 3A), which were characterized by an increase of the absorbance at 280 nm and a decrease at 251 nm. These spectral changes were linearly dependent on the peroxynitrite concentration (see Fig. 3B,C), this clearly indicated an interaction between fibrinogen and $ONOO^-$, which brought about some structural alteration of the protein (the slight increase of pH by $\approx$0.1 pH unit upon addition of peroxynitrite was not responsible for the spectral changes observed). Such a spectroscopic alteration in the 250–270 nm range should be attributed to a specific reaction with an aromatic amino acid residue, most likely a tyrosine. Therefore, this observation suggested that a peroxynitrite-linked alteration of a tyrosyl residue(s) caused a drastic functional modification of fibrinogen, such that polymerization of fibrinopeptides was severely impaired.

In order to have more insight into the physiological significance of the peroxynitrite-induced effect, and possibly into the mechanism of protein modification, we have investigated the effect of blood components, such as CO_2 and bilirubin, on the phenomenon described. In fact, CO_2 (whose concentration in the plasma ranges around 1.3 mM) was in equilibrium with HCO_3^- (at a concentration of about 25 mM) and it may react in blood plasma with peroxynitrite through a fast reaction, leading to the formation of a highly reactive oxidizing nitrosoperoxycarbonate

$$ONOO^- + CO_2 \leftrightarrow ONO_2CO_2^- \qquad \text{(reaction 1)}$$

that rapidly decomposes. This secondary oxidizing agent may either increase (acting synergistically) or inhibit (consuming $ONOO^-$) the oxidative damage induced by peroxynitrite on plasma proteins [29]. The addition of 10 mM $NaHCO_3$ to the incubation mixture reduced by $\approx$41% the peroxynitrite-mediated inhibition of fibrinogen's clotting activity (Fig. 4A) and this in spite of the fact that HCO_3^- itself partially inhibits the clotting activity [30]. Therefore, the results reported in Fig. 4A clearly indicated that the occurrence of reaction 1 (which is favored by the addition of HCO_3^-) resulted in a partial removal of the agent specifically responsible for the inactivation of fibrinogen (i.e. $ONOO^-$), further strengthening the hypothesis that peroxynitrite is by far and large the major factor affecting the clotting activity of fibrinogen.

In addition, the effect of bilirubin on peroxynitrite-induced inhibition of clotting was investigated. Bilirubin is a pigment, derived from heme catabolism (its concentration in plasma ranges around 15–20 μM), which is preferentially bound to albumin and is reported to play an important role as a physiological secondary anti-oxidizing agent against the reactive oxygen species [22]. We observed that addition of bilirubin to the reaction mixture protected fibrinogen from the peroxynitrite-induced oxidation with a recovery of clotting activity of $\approx$29% (see Fig. 4B). Finally, the simultaneous addition of $NaHCO_3$ (20 mM) and bilirubin (15 μM) resulted in a synergistic action enhancing the protection of fibrinogen against peroxynitrite damage and brought about a 47% recovery of clotting activity (expressed as clotting time; see Fig. 4B).

In conclusion, a direct interaction of fibrinogen with peroxynitrite could be observed, such that a drastic inhibition of clotting activity occurred even at very low $ONOO^-$ concentrations (i.e. 1–25 μM). This effect, which is mostly due to a chemical modification of tyrosyl residue(s), impairing the polymerization of fibrinopeptides, is significantly reduced by the presence of anti-oxidizing agents, such as CO_2 and bilirubin, which act synergistically. These results taken together indicate that, under particular pathological conditions, the coagulative process could be impaired by an increase of the peroxynitrite levels through a rapid and irreversible chemical modification of fibrinogen, and that the use of antioxidants, also in association with more specific drugs, could be an important therapeutic tool for the control of peroxynitrite endogenous toxicity.

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