Modification of tissue factor by peroxynitrite influences its procoagulant activity

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Abstract Peroxynitrite, a reactive oxidising species resulting from a reaction between nitric oxide and the superoxide anion, modifies proteins by nitration of certain amino acids such as tyrosine. Tissue factor (TF), a transmembrane protein, is expressed on cells under inflammatory conditions and initiates the coagulation cascade. The extracellular domain of TF is rich in tyrosine. Exposure of recombinant TF and cellular TF to peroxynitrite was associated with a reduction in procoagulant activity. This was accompanied by an elevated level of nitrotyrosine residues. Peroxynitrite may have a protective role by attenuation of the thrombotic properties of TF.

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Key words: Atherosclerosis; Peroxynitrite; Tissue factor; Procoagulant activity; Nitration

1. Introduction

Nitric oxide reacts with the superoxide anion to form the potent oxidising species peroxynitrite [1]. Peroxynitrite modifies proteins by nitration of specific amino acid residues, in particular the ortho positions of tyrosine residues to form nitrotyrosine [2]. Phenylalanine may also be nitrated and tryptophan to a limited extent whereas sulphydryl groups are readily oxidised to sulphoximes. These events may, in turn, influence the biological activity of proteins [3]. Nitrotyrosine has been detected in a number of human pathologies including Alzheimer's disease [4] and rheumatoid arthritis [5] and thus could be a useful marker for the presence of peroxynitrite in vivo. Nitrotyrosine residues have also been detected in atherosclerotic plaques, but not in normal blood vessels, suggesting peroxynitrite plays a role in atherosclerosis [6]. Peroxynitrite oxidises low density lipoprotein, making it recognisable to the macrophage scavenger receptor leading to foam cell formation [7]. There is evidence that NO and peroxynitrite are formed by bovine aortic endothelial cells [8], by rat macrophages [9] and more recently by human macrophages [10]. Stimulated human neutrophils are reported to release NO and $O_2^{-\cdot}$ at rates that favour the formation of peroxynitrite [11] and may also be a source of this oxidant in vivo. Protein bound nitrotyrosine has been detected using an ELISA technique in normal human plasma, in the range of 120 nM, at least half of which is bound to albumin [12].

Tissue factor (TF), a transmembrane glycoprotein (47 kDa) which initiates the blood coagulation by acting as a receptor for coagulation Factor VII, may have a separate role in angiogenesis and neointima formation [13,14]. TF is expressed on monocytes following exposure to bacterial lipopolysaccharide [14,15], and from endothelial cells and macrophages exposed to oxidised LDL [16,17]. It has an extracellular (active) domain of 219 amino acids, which initiates the coagulation cascade, a short membrane spanning domain and an intracellular domain of 21 amino acids [18,19]. The extracellular domain of TF has 12 tyrosine residues, eight of which are exposed and thus susceptible to attack by peroxynitrite. The importance of specific tyrosine residues in this domain of TF and the coagulation response has been reported as well as phenylalanine and possibly tryptophan [19].

In this study, peroxynitrite was shown to attenuate the procoagulant activity of TF in parallel with the increased nitration of its tyrosine residues measured by ELISA.

2. Materials and methods

Materials and cells were obtained from the following sources: recombinant TF (rTF) (Baxter Diagnostics Inc., Deerfield, Illinois, USA), polyclonal anti-nitrotyrosine IgG (TCS Biologicals Ltd, Botolph Claydon, Bucks, UK), THP-1 monocytes (European Collection of Animal Cell Culture, Salisbury, Wilts, UK), cell culture reagents, phosphate buffered saline (PBS) and Versene (Gibco, Paisley, Scotland), phorbol-12-myristate-13 acetate (PMA) (Sigma Chemical Co., Poole, Dorset, UK), Nutridoma HU (Boehringer Mannheim UK, Lewes, Sussex, UK). All solutions were prepared with de-ionised H_2O.

2.1. Preparation of peroxynitrite

Peroxynitrite was prepared by mixing $NaNO_2$, H_2O_2 and HNO_3 and passing the solution into NaOH: excess H_2O_2 removed by filtering through a manganese oxide column, as described by others although traces may remain [20]. Authentic peroxynitrite was maintained at pH 10, but on dilution at pH 7.4 and addition to recombinant TF (rTF), large losses would have occurred in the amount of peroxynitrite delivered. Decomposed peroxynitrite was prepared by neutralisation at pH 7.4: the pH was then adjusted back to pH 10 by addition of NaOH and this was used as a control. The concentration of peroxynitrite in the stock solutions was determined by measuring its absorption at 302 nm using the ε of 1670 $mol/l^1 \cdot cm^1$.

2.2. Exposure of rTF to peroxynitrite

rTF was diluted in H_2O to give a stock solution of 100 μg protein/ml and exposed to peroxynitrite or decomposed peroxynitrite at concentrations between 0–3 mM, diluted from a stock solution into phosphate buffered saline (PBS) and added within the space of 5 s by rapid vortexing. The controls were rTF treated with PBS pH 7.4.

2.3. Spectrophotometric analysis of tissue factor exposed to peroxynitrite

The UV and visible absorption spectra of rTF exposed to peroxynitrite and decomposed peroxynitrite, were scanned between 320–620 nm on a Beckman DU70 spectrophotometer, against a blank of H_2O, following neutralisation to pH 7. This was compared to the changes in absorbance when peroxynitrite was added to free L-tyrosine and L-tryptophan.

The fluorescence spectra of L-tryptophan and rTF were also measured with emission wavelength of 340 nm [21] and scanning from 320–

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410 on a Perkin Elmer MPF44B fluorimeter and repeated on rTF before and after exposure to peroxynitrite or its decomposed control. In all cases, the effects of hydrogen peroxide were determined on these spectra, in the event that traces remained in the peroxynitrite. Similar traces of the peroxide would also be found in the decomposed peroxynitrite.

2.4. Measurement of nitrotyrosine residues

Immuno-reactive nitrotyrosine residues in rTF following exposure to various concentrations of peroxynitrite were quantified by competitive ELISA assay as recently described [22]. A standard curve was constructed using nitrated bovine serum albumin (NO_2-BSA) at a series of dilutions. Competition assays were performed by addition of appropriate samples of peroxynitrite treated rTF instead of NO_2-BSA and inhibition of the antibody binding determined from the standard curve.

Measurement of nitrotyrosine was also made on the cytosolic and membrane proteins of human THP-1 monocytes following exposure to peroxynitrite (0–100 μM). The cells were sonicated by exposure to two intervals of ultrasound in a one-minute period, followed by centrifugation at $2000\times g$ for 10 min to separate the cytosol and the total membrane fraction. The membrane fraction was washed $3\times$ with PBS prior to solubilisation in 1% CHAPS buffer [23].

2.5. Measurement of procoagulant activity

The procoagulant activity of rTF which had been exposed to peroxynitrite and decomposed peroxynitrite was measured using the one-stage prothrombin time assay [24]. rTF samples were diluted (final concentration of 2 μg protein/ml) and the procoagulant activity quantified by reference to a rTF standard curve constructed by plotting log TF (units/ml) versus log t (clotting time in s). These data were compared to the control values (rTF exposed to PBS) and the degree of inhibition of procoagulant activity calculated from the equation:

$$\%\ \text{inhibition} = \frac{\text{initial activity} - \text{residual activity}}{\text{initial activity}}.$$

All the assays were completed within 10 min of addition of peroxynitrite to the rTF.

2.6. Induction of tissue factor activity in THP-1 monocytes

THP-1 monocytes were cultured as described previously [25]. Cells were seeded 24 h prior to experiment in serum free media supplemented with Nutridoma HU (1% v/v), at 0.5×10^6 cells/ml in 25 cm^2 flasks. Cells were stimulated with 10 nM PMA for 4 h and adherent cells removed by the addition of Versene. The cells were washed once in PBS and resuspended to 10^7 cells/ml and were exposed to 0–100 μM peroxynitrite and decomposed peroxynitrite, which had been diluted from stock solution in PBS. The procoagulant activity was determined by the one-stage prothrombin time assay and compared to control cells (PBS addition only). The percentage inhibition was determined as described previously.

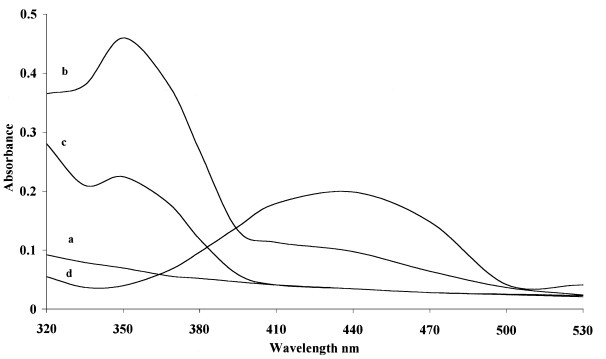

Fig. 1. Identification of modification of rTF by peroxynitrite using visible spectroscopy. Absorption spectra of a: unmodified rTF at 100 μg protein/ml; b: rTF modified by addition of 3 mM peroxynitrite; c: rTF with addition of 3 mM decomposed peroxynitrite; d: 100 μM 3-nitrotyrosine. The results are typical of at least six independent experiments.

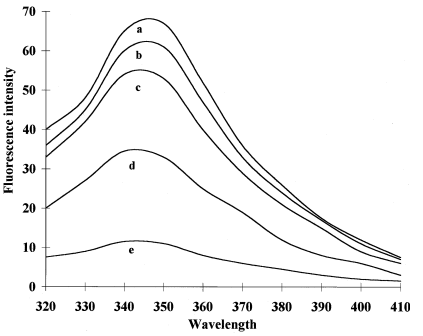

Fig. 2. The influence of peroxynitrite on tryptophan fluorescence of rTF. rTF fluorescence was measured with an excitation wavelength of 340 nm. The fluorescence over a range of wavelengths was measured before (a) and after addition of peroxynitrite (final concentration) at 300 μM (c) and 3 mM (e) or decomposed peroxynitrite at the same initial concentrations, (b) and (d) respectively. These results are from one experiment typical of three independent experiments.

2.7. Measurement of lipid peroxides

rTF is dispersed in a small amount of lipid which is necessary for the biological activity of this protein. Lipid peroxidation was estimated by the FOX assay [26] within 10 min of addition of the peroxynitrite.

3. Results

3.1. Spectrophotometric measurements after nitration of rTF with peroxynitrite

The UV absorption spectrum of rTF shows two major peaks at 230 and 278 nm which are reminiscent of those of tryptophan, but may include peaks of tyrosine at neutral pH: these did not change significantly on addition of peroxynitrite up to 3 mM (not shown). In the visible region, rTF did not show strong absorbance (Fig. 1a), but on addition of 3 mM peroxynitrite a peak appeared at 350 nm with a smaller peak at 410–440 nm region (Fig. 1b), the latter corresponding to the visible absorption spectra of nitrotyrosine (Fig. 1c). Addition of 3 mM decomposed peroxynitrite to rTF (Fig. 1d) led to the appearance of the peak at 350 nm which may be attributable in part to a contaminant in peroxynitrite: there was no peak at 410–440 nm. Lower concentrations of peroxynitrite (30–300 μM) showed little change in absorption at any of these wavelengths.

The fluorometric spectra of rTF suggest that tryptophan was modified to a very limited extent by the addition of 300 μM peroxynitrite (Fig. 2) but was largely eliminated by the addition of 3 mM peroxynitrite. Decomposed peroxynitrite decreased fluorescence to some extent, but less than for active peroxynitrite. No change in fluorescence was noted in the presence of H_2O_2 (not shown) at concentrations which were the maximum assuming no steps were taken to remove it during peroxynitrite synthesis, 0–12 mM.

3.2. Elevated levels of nitrotyrosine residues in TF exposed to peroxynitrite

The spectrophotometric determinations were insufficiently sensitive for quantification of the extent of nitration, but gave an indication that modifications of tyrosine and tryptophan may occur with addition of peroxynitrite. The sensitive competitive ELISA procedure for nitrotyrosine was used,

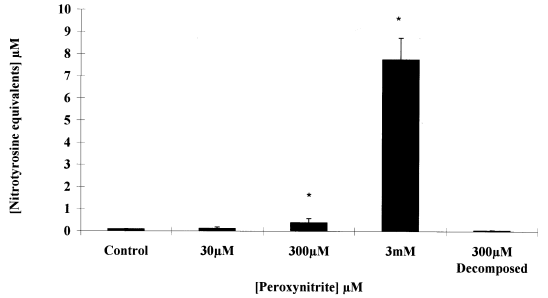

Fig. 3. The nitration of the tyrosine residues of rTF by peroxynitrite. rTF (100 μg protein/ml) was incubated with peroxynitrite (0, 30, 300 and 3000 μM) or decomposed peroxynitrite (300 μM) for a period of 10 min after which the extent of tyrosine nitration was determined by an ELISA procedure using nitrated bovine serum albumin as the standards. The values are expressed as the means ± S.E.M. of at least four independent experiments.

therefore, as a convenient procedure for detection of protein modification by peroxynitrite at low concentrations. Only low levels of nitration of tyrosine in rTF were observed at 300 μM peroxynitrite, suggesting a high proportion of this nitrating species was lost during mixing (Fig. 3). Exposure of rTF to 3 mM peroxynitrite showed a large increase in nitrotyrosine. Decomposed peroxynitrite did not cause nitration tyrosine residues in rTF compared to controls where only buffer was used. There were no increases in the oxidation of the lipid associated with the rTF as detected by the assay for lipid peroxides 10 min after addition of the peroxynitrite (not shown).

Nitration of proteins was determined in THP-1 cells to which 100 μM peroxynitrite was added, but not with decomposed peroxynitrite. Low levels of nitration were detected in the membranes of these cells (100 nmol nitrotyrosine/mg protein) which was increase 200-fold by the addition of the peroxynitrite. Levels of nitrotyrosine were below the limits of detection in the cytosolic proteins and were not increased to a significant extent. At lower concentrations of peroxynitrite (30 μM), small rises in nitration of the membrane fraction were detectable, but did not reach statistical significance.

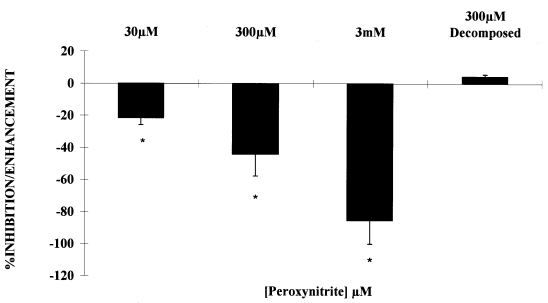

Fig. 4. The influence of the modification of rTF by peroxynitrite on its procoagulant activity. rTF (100 μg/ml) was exposed to a range of concentrations of peroxynitrite or decomposed peroxynitrite (300 μM) for a period of 10 min after which the procoagulant activity was measured using the one-stage prothrombin time assay. The extent of inhibition or activation of the rTF following modification was measured against the control where only buffer was added. The results are expressed as the means ± S.E.M. of four independent experiments.

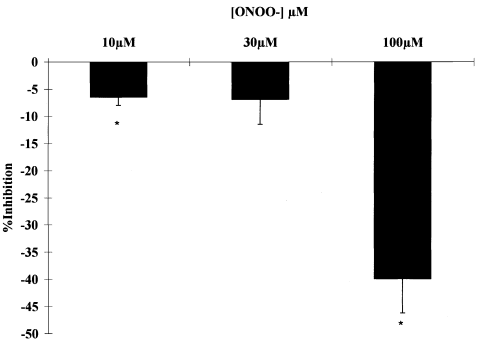

Fig. 5. The influence of the modification of TF activity expressed on the cell surface of a human monocytic cell line by peroxynitrite. Human monocytic THP-1 cells in suspension culture were treated with peroxynitrite (0–100 μM) or decomposed peroxynitrite for 10 min and the cell surface activity of the intact cells measured using the one-stage prothrombin test. The results are the means ± S.E.M. of at least three independent experiments.

3.3. *Reduction in the procoagulant activity of rTF exposed to peroxynitrite*

The exposure of rTF (100 μg protein/ml) to 0–3 mM peroxynitrite resulted in a concentration dependent inhibition of procoagulant activity compared to that of the control sample, almost complete inhibition attained at 3 mM peroxynitrite and significant inhibition at 30 μM and above (Fig. 4). Exposure to decomposed peroxynitrite resulted in a small enhancement in procoagulant activity (Fig. 4). This enhancement is not caused by nitrate or nitrite, or indeed a mixture of the two, when these were tested separately (not shown), nor was it an effect of H_2O_2 0–12 mM (not shown).

3.4. *Reduction in cell surface tissue factor activity following exposure to peroxynitrite*

Monocytic THP-1 cells were pre-treated with 10 nM PMA for 4 h to induce an increased expression of the tissue factor activity on the cell surface. These cells exhibit some procoagulant activity even when unstimulated. This activity was absent if Factor VII deficient plasma was used in the coagulation assay, showing that the procoagulant activity was due to tissue factor (Fig. 5). Inhibition of PCA of cell surface TF was attained at concentrations as low as 10 μM peroxynitrite and an increase in inhibition accompanied higher concentrations of peroxynitrite. The addition of peroxynitrite at concentrations above 100 μM resulted in some cell lysis and therefore measurements at higher concentrations were not made.

4. Discussion

The nitration of proteins has been associated extensively with pathological conditions related to inflammation such as atherosclerosis and arthritis [5,6]. However, this may only be appropriate when there is an excess of nitration. Excessive formation of peroxynitrite may give rise to extensive modifications of proteins and lipids, and ultimately become toxic to the cells. Nitration of tyrosine residues may arise as the result of the release of peroxynitrite from normal cells at low concentrations, but other sources of nitration are possible, e.g. from prostaglandin H synthase [27] and myeloperoxidase [28]. Indeed, low levels of nitration were detected in this study in

the membranes and cytosol of monocytes which were not exposed to exogenous peroxynitrite: this was also observed in resting platelets [29]. The fact that the nitrated proteins are present in normal plasma and other biological fluids [12], strongly suggests that low levels of peroxynitrite may be released from cells in vivo or that nitration arises from the enzymic processes mentioned above. Increased nitration of cellular proteins may occur during normal physiological process, e.g. the activation of blood platelets by collagen or thrombin [29]. A physiological role of nitration has not been established.

In the current study, the nitration of tyrosine residues was associated with the reduction in the procoagulant activity of TF. However, at lower concentrations of peroxynitrite there was some inhibition of procoagulant activity with only very modest increases to the nitration of tyrosine. This may be because only certain key tyrosine residues are important to the procoagulant activity, e.g. tyrosine 71 [19] and that the additional nitrations are superfluous. Peroxynitrite has other actions on proteins, such as nitration or oxidation of tryptophan, phenylalanine and cysteine. The modification of tryptophan was suggested by the spectrofluorometric data, at least at higher concentrations of peroxynitrite. The observed effects on tissue factor may not, therefore, be exclusively related to nitration of tyrosine. There are no free thiols in the extracellular domain of tissue factor [19], but tryptophan residues may contribute to the interactions of tissue factor with Factor VII as part of the WKS motif [19]: nitration of these residues may lead to the partial loss of the procoagulant activity of tissue factor. Phenylalanine is also important in the interactions of Factor VII with tissue factor but at present their nitration cannot be detected by the spectrophotometric assay or the ELISA. Free nitrophenylalanine competes with nitrated bovine serum albumin to a lesser extent than free 3-nitrotyrosine in the ELISA which in term is weak related to protein bound nitrotyrosine [22]. A contribution from nitrated phenylalanine residues in tissue factor cannot be excluded and deserves further investigation.

Peroxynitrite also oxidises lipids and sugars [1], but the lipids associated with recombinant tissue factor were not oxidised when recombinant tissue factor was exposed briefly to peroxynitrite. As there are no carbohydrate residues in recombinant tissue factor, this was not relevant to the impairment of procoagulant activity, but it may influence the glycosylated cellular TF. None of the observed effects of peroxynitrite could be attributed to the presence of residual H_2O_2, probably because of the brief duration of the experiments.

It can be concluded that modification of TF by peroxynitrite can bring about inhibition of its activity not only of the pure protein, but also when located in the cell membrane. This modification may occur through actions on different amino acids, but the nitration of tyrosine appears to be representative of the changes.

Modification of tissue factor protein may afford a self-regulatory process in which unwanted surface procoagulant activity may be limited by the release of NO and formation of peroxynitrite: human monocytes are also capable of NO synthesis in substantial quantities [10]. This modification may not be a permanent in vivo as the nitration of tyrosine residues in platelet cytosolic proteins has been shown to decrease after the initial action of peroxynitrite [29]. Ultimately the effects of endogenous peroxynitrite from macrophages or other cell types on TF function will need to be demonstrated.

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