Protein nitration is predominantly mediated by a peroxynitrite-dependent pathway in cultured human leucocytes

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Protein nitration is a common characteristic of oxidative injury caused by the invasion of leucocytes into inflammatory lesions. Two distinct pathways of nitration of protein tyrosine residues, namely the peroxynitrite (ONOO⁻)-mediated pathway and another catalysed by the haem-containing peroxidases, have been reported under experimental conditions. However, the contribution of these two pathways in human leucocytes is still controversial. The present study demonstrates that the process of phenolic nitration of proteins in cultured human leucocytes is mainly ONOO⁻-mediated and that it differs between granulocytes and mononuclear cells, depending on the cell compartment and the stimuli. We have also shown that NO induces protein nitration via a ONOO⁻-dependent pathway, whereas NO₂⁻, the

NO metabolite, does not increase but decreases nitration in PMA-stimulated leucocytes. The inhibition of myeloperoxidase activity did not reduce protein nitration; on the other hand, the myeloperoxidase inhibitor aminobenzoic hydrazide caused increased nitration, which was mediated by ONOO⁻. These results suggest that protein nitration is predominantly mediated by a ONOO⁻-dependent pathway in cultured human leucocytes and that the myeloperoxidase-catalysed pathway does not play a significant role in protein nitration.

Key words: leucocyte, myeloperoxidase, nitrite, 3-nitrotyrosine, reactive nitrogen species.

INTRODUCTION

The formation of 3-nitrotyrosine in specific tyrosine residues can alter protein structure and function [1], implying that in certain pathological states, nitration is part of the disease process. In this connection, there are studies [1,2] reporting that oxidative stress, commonly associated with inflammatory conditions, leads to the *in vivo* modification of proteins through the nitration of tyrosine residues by peroxynitrite (ONOO⁻). Recently, we have shown that patients undergoing cardiac surgery exhibited increased levels of plasma protein nitration [3,4] and that nitroglycerin significantly reduces this increase [4]. A possible interpretation of these results is that nitroglycerin inhibits NO synthase activities, resulting in a reduction in endogenously produced NO, which in turn decreases protein nitration by a reduction in the formation of ONOO⁻. However, ONOO⁻ is extremely inefficient in nitrating tyrosine when formed from physiological fluxes of O₂⁻ and NO [5], and it is conceivable that the myeloperoxidase pathway may be a major contributor to the production of 3-nitrotyrosine *in vivo* [6–8]. Previous studies have reported that nitrite (NO₂⁻), the primary end-product of NO metabolism, can be oxidized by haem peroxidases such as horseradish peroxidase, lactoperoxidase and myeloperoxidase to form the reactive nitrogen species NO₂, which is highly reactive towards tyrosine residues [9]. Furthermore, NO₂ can react with HOCl, produced by the myeloperoxidase-catalysed reaction between H₂O₂ and Cl⁻ [10, 11], to form nitryl chloride (NO₂Cl), which also nitrates tyrosine residues [8]. However, the importance of these pathways in the *in vivo* nitration of proteins is unclear.

The main aim of the present study was to investigate the role of ONOO⁻ and myeloperoxidase in protein nitration using human leucocytes cultured in media enriched with plasma proteins to simulate the *in vivo* milieu. Previous studies [12–14]

have investigated the myeloperoxidase-catalysed nitration in cell-free preparations or in cultured cells by using tyrosine, small peptides with tyrosine or peptides containing tyrosyl residues, which cannot be metabolized by myeloperoxidase as the nitration target. The present study used 0.25% BSA as the target of extracellular nitration, and cells were treated with various agents and incubated for 6 h. Our results show that in both granulocytes and mononuclear cells, protein nitration is inhibited by scavengers of ONOO⁻ but not by myeloperoxidase inhibition. Protein nitration significantly increased with PMA stimulation of leucocytes, although the magnitude varied between extra- and intracellular compartments, with ONOO⁻ and PMA stimulation having no effect on extracellular protein nitration in mononuclear cells. The results indicate that protein nitration is mainly mediated by ONOO⁻ and not by myeloperoxidase in human leucocytes.

MATERIALS AND METHODS

Materials

Histopaque®-1077 and -1119 media, serum and Phenol Red-free Hanks balanced salt solution (HBSS), nutrient F-12 Ham, PMA, sodium azide (NaN₃) and BSA were purchased from Sigma. Stocks of the ONOO⁻ scavenger 5,10,15,20-tetrakis-(4-sulphonatophenyl)-porphyrinato-iron[III] (FeTPPS) were purchased from CN Biosciences (Nottingham, U.K.). Sodium nitrite and spermine NONOate [N-(2-aminoethyl)-N-(2-hydroxy-2-nitrohydrazino)-1,2-ethylenediamine] were purchased from Alexis Corporation (Nottingham, U.K.) and nitrated BSA was purchased from TCS Cellworks (Botolph Claydon, Buckinghamshire, U.K.). Rabbit anti-nitrotyrosine polyclonal IgG was purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Donkey anti-rabbit horseradish peroxidase conjugate was purchased from Amer-

Abbreviations used: ABAH, aminobenzoic hydrazide; FeTPPS, 5,10,15,20-tetrakis-(4-sulphonatophenyl)-porphyrinato-iron[III]; HBSS, Hanks balanced salt solution; ONOO⁻, peroxynitrite.

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sham Pharmacia Biotech. Anti-rabbit IgG–FITC conjugates were purchased from Sigma Biochemicals.

Cell culture

Human granulocytes and mononuclear cells were isolated from blood by density-gradient centrifugation. Briefly, 3 ml of Histopaque®-1017 was layered on top of 3 ml Histopaque®-1119 medium in conical centrifuge tubes of 15 ml capacity. Whole blood (6 ml) was carefully taken from healthy volunteers (8 or 12 different volunteers for each study group) and layered on to the upper gradient of the media. After centrifugation at 700 *g* for 30 min at room temperature (18–22 °C), distinct opaque layers of granulocytes and mononuclear cells were separated and washed twice in Tris-buffered saline (pH 7.4). The resulting cell pellets were resuspended in HBSS containing equal volumes of nutrient F-12 Ham and 0.25% BSA at pH 7.4. After this, 1 ml aliquots of the HBSS containing 1×10^6 cells were transferred into sterile Eppendorf tubes and cells were equilibrated for 30 min at 37 °C in air/5% CO₂ incubator before being subjected to various treatments. Some of the cell suspensions were stimulated with 100 ng/ml PMA for 10 min before the initiation of treatments.

Treatments

To test the contribution of the ONOO[−]-dependent pathway of protein nitration, the ONOO[−] decomposition catalyst FeTPPS and scavenger uric acid were added to some of the preparations. Stock FeTPPS was prepared by dissolving in autoclaved double-distilled water and stored at −20 °C in 1 mM aliquots. Uric acid was solubilized in 1 M NaOH and further diluted to a 10 mM stock solution in PBS. Single boluses of FeTPPS and uric acid were taken in each tube containing cell suspension, and the tubes were rapidly swirled to give a final concentration of 100 μM. Other preparations were incubated with the peroxidase inhibitors, azide and aminobenzoic hydrazide (ABAH), to investigate the contribution of myeloperoxidase in protein nitration. Azide stock solution (10 mM) was prepared in autoclaved double-distilled water, and 10 μl was added to each 1 ml cell suspension as a bolus. ABAH was prepared as a 10 mM stock solution in 10 mM HCl with 100 μM diethylenetriaminepentaacetic acid, and 10 μl was added as a bolus to 1 ml of cell suspension for a final concentration of 100 μM. Further studies were conducted to investigate the roles of NO radical and the metabolite NO₂[−] on protein nitration. NO₂[−] (100 μM) and the NO-radical donor spermine NONOate (1000 μM) were added to leucocyte suspensions. In other leucocyte preparations (with or without PMA stimulation), FeTPPS was added in combination with NO₂[−] (100 μM), ABAH (1 mM) and spermine NONOate (1000 μM) to elucidate the role played by ONOO[−]. In all the experiments, cells were incubated for 6 h at 37 °C in air/5% CO₂, conditions that were found to induce optimum nitration. After incubation, the culture medium was separated from the cells by centrifugation at 700 *g* for 5 min at 4 °C. The isolated cell pellet and cell-culture medium in separate cryovials were snap-frozen in liquid nitrogen and then stored at −80 °C until assayed.

Protein 3-nitrotyrosine assay

A modification of a sensitive competitive ELISA described previously [14a] was used for the determination of protein 3-nitrotyrosine levels in isolated culture medium (extracellular nitration) and supernatant prepared from lysed cells (intracellular nitration). For the assay, frozen cell pellets were thawed on ice and immediately lysed in 0.3 ml of RIPA buffer (9.1 mM di-

basic sodium phosphate/1.7 mM monobasic sodium phosphate/1% Igepal CA-630/0.5% sodium deoxycholate/0.1% SDS). The lysate was homogenized using an Ultra-Turrax T25 homogenizer with a dispersing tool (IKA Laboratories, Staufen, Germany) at 20000 rev./min for 1 min. After centrifugation at 1000 *g* for 5 min at 4 °C, cell lysate supernatants were transferred on to precooled tubes, snap-frozen in liquid nitrogen and stored at −80 °C until analysis. Quantification of 3-nitrotyrosine-modified proteins was performed by a competitive ELISA using 96-well plates coated with nitrated BSA at 5 μg/ml (immobilized antigen) in coating buffer (50 mM Na₂CO₃ and 50 mM NaHCO₃ in the ratio 1:9). Blocking of plate wells was achieved by adding 250 μl of 0.5% ovalbumin/PBS/Tween 20 to prevent non-specific binding. A standard curve was constructed by incubating serial dilutions of nitrated BSA in PBS with immunoaffinity-purified polyclonal anti-nitrotyrosine rabbit IgG (1:10000) for 2 h at 37 °C in microtitre plates. The competitive inhibition of antibody binding in the rest of the plate wells was used to determine antigen levels in the cell lysates. The plates were then washed three times with PBS composed of 8.3 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 154 mM NaCl, 2.7 mM KCl and 0.05% Tween 20. Sequential incubations were then performed with biotinylated donkey anti-rabbit IgG and avidin–horseradish peroxidase conjugate (1:1000 dilution). After further washing, colour development was initiated by the addition of substrate *o*-phenylenediamine in a phosphate/citrate/perborate buffer (Sigma) to each well and the reaction continued for 30 min at room temperature. The reaction was terminated by the addition of 0.18 M sulphuric acid. Absorbance was read at 490 nm using a benchmark microplate reader (Bio-Rad Laboratories). Plots of the standard curve for *A*₄₉₀ versus concentration of nitrated BSA (nmol/ml) were constructed. The nitrotyrosine concentrations measured in serial dilutions of the standard nitrated BSA were accurate to ±10%. Regression coefficients for the standard curve for each assay were greater than 0.99. The intra- and inter-assay precision was 9.4 and 9.0% respectively.

Immunofluorescence staining for intracellular nitration

We cultured unstimulated and PMA-stimulated leucocytes on glass coverslips inside 6-well plates for 6 h alongside cells treated with a bolus of ONOO[−], FeTPPS, uric acid, azide and ABAH. At the end of the incubation, cells were fixed in 1% (w/v) paraformaldehyde in Dulbecco's PBS for 5 min at room temperature and then in cold 100% ethanol for 5 min. Non-specific staining was blocked with 3% normal goat serum in 0.5 M Tris/HCl (pH 7.4) containing 0.5% Triton X-100 for 1 h at room temperature. Cells were washed twice in PBS each time for 5 min without shaking. For the detection of nitrotyrosine immunoreactivity, cells were incubated with 1:200 dilution of anti-nitrotyrosine rabbit polyclonal serum generated to nitrated key-hole limpet haemocyanin (Sigma) for 16 h at 4 °C, followed by anti-rabbit IgG–FITC conjugate (1:50 dilution). After immunostaining, cells on coverslips were mounted on to glass slides for microscopic examination. Images were obtained using an Axio-plan II fluorescence microscope fitted with a ×40 objective and filter settings for FITC. Images were captured using a SPOT cooled colour digital camera and were imported into Adobe Photoshop software to create merged images.

Protein assay

A standard Bradford assay was used to determine the protein concentration in cell culture media and supernatants of cell homogenates according to the manufacturer's instructions (Bio-Rad Laboratories).

Statistical analysis

Data are expressed as means $\pm$ S.E.M. Analysis of results was by one-way ANOVA and statistical significance was taken to be $P < 0.05$.

Figure 1 Role of ONOO^- in the intracellular (A,C), and extracellular (B,D) protein nitration induced by myeloperoxidase

Unstimulated and PMA-stimulated granulocytes (A,B) and mononuclear cells (C,D) were treated with the ONOO^- scavenger FeTPPS ($100 \mu\text{M}$), independently and in combination with the myeloperoxidase inhibitor ABAH ($100 \mu\text{M}$) for 6 h. Data are presented as means $\pm$ S.E.M. of six experiments performed in duplicate. * $P < 0.05$ versus the corresponding control.

RESULTS

Role of ONOO^- and myeloperoxidase on protein nitration

To investigate the role of ONOO^- and myeloperoxidase on protein nitration, unstimulated and PMA-stimulated granulocytes and mononuclear cells were incubated for 6 h with the myeloperoxidase inhibitor ABAH and the ONOO^- -decomposition catalyst FeTPPS, independently and in combination. As shown in Figures 1(A)–1(D), the mean nitrotyrosine values were significantly decreased by FeTPPS but increased by ABAH in both unstimulated and PMA-stimulated cells. The Figures also

Figure 2 Immunofluorescent detection in unstimulated and PMA-stimulated granulocytes

The effect of ONOO^- ($200 \mu\text{M}$), FeTPPS ($100 \mu\text{M}$), uric acid ($100 \mu\text{M}$), azide ($100 \mu\text{M}$) and ABAH ($100 \mu\text{M}$) on the induction of nitrated intracellular proteins in unstimulated and PMA-stimulated granulocytes has been studied. Images were obtained by using a $\times 40$ objective lens.

Figure 3 Immunofluorescent detection in unstimulated and PMA-stimulated mononuclear cells

The effect of ONOO^- (200 μM), FeTPPS (100 μM), uric acid (100 μM), azide (100 μM) and ABAH (100 μM) on the induction of nitrated intracellular proteins in unstimulated and PMA-stimulated mononuclear cells has been studied. Images were obtained by using a $\times 40$ objective lens.

show that the incubation of cells with FeTPPS and ABAH in combination resulted in a significant decrease in nitrotyrosine levels, suggesting that ONOO^- is the mediator of protein nitration caused by inhibition of myeloperoxidase. It is worth noting that the responses to protein nitration were similar in the intra- and extracellular compartments of granulocytes and also in the intracellular space of mononuclear cells; however, no significant change was detected in the extracellular space of the latter. Identical changes were seen with immunofluorescent microscopy, as shown in Figures 2 and 3.

Figure 4 Effect of NO on intracellular (A,C) and extracellular (B,D) protein nitration

Whether protein nitration is mediated by ONOO^- , unstimulated and PMA-stimulated granulocytes (A, B) and mononuclear cells (C, D) treated for 6 h with spermine NONOate (1000 μM) and FeTPPS (100 μM), independently and in combination was investigated. Data are presented as means $\pm$ S.E.M. of six experiments performed in duplicate. * $P < 0.05$ versus the corresponding control.

Figure 5 Role of NO₂⁻ in intracellular (A,C) and extracellular (B,D) protein nitration

Unstimulated and PMA-stimulated granulocytes (A,B) and mononuclear cells (C,D) were treated with 100 μ M NO₂⁻ for 6 h. Data are presented as means $\pm$ S.E.M. of six experiments performed in duplicate. * P < 0.05 versus the corresponding control; † P < 0.05 versus the PMA-treated group.

Role of NO and its metabolite NO₂⁻ on protein nitration

To investigate whether NO is involved in protein nitration, unstimulated and PMA-stimulated cells were treated for 6 h with the NO-radical donor spermine NONOate (1000 μ M). The results shown in Figures 4(A–D) demonstrate that NO significantly increases nitrotyrosine mean values in the extracellular compartment of granulocytes and to a greater extent in stimulated cells than in unstimulated ones. A lesser but still significant increase in protein nitration was observed in the extracellular space of mononuclear cells. In contrast, protein nitration was decreased in the extracellular space of both cell types. Importantly, FeTPPS (100 μ M) completely abolished the rise in extracellular nitrotyrosine levels caused by spermine NONOate in both cell types, suggesting that ONOO⁻ is the mediator of the protein nitration induced by NO.

To establish the role of NO₂⁻, the NO metabolite, on protein nitration, unstimulated and PMA-stimulated leucocytes incubated for 6 h were treated with 100 μ M NO₂⁻. This concentration induces the formation of nitrating species in the presence of HOCl [6] and myeloperoxidase [8]. Figures 5(A–D) show that NO₂⁻ does not affect the intra- and extracellular levels of protein nitration in unstimulated leucocytes. In contrast, NO₂⁻ abolished the intracellular nitration seen in PMA-stimulated cells and significantly reduced extracellular nitration in PMA-stimulated granulocytes.

DISCUSSION

The present study has demonstrated that ONOO⁻ is the main mediator of protein nitration in cultured human leucocytes and that the activation of myeloperoxidase does not play a significant role. In contrast, inhibition of myeloperoxidase activity resulted in increased protein nitration, an effect that is abolished by the ONOO⁻ scavenger FeTPPS, indicating that this is a ONOO⁻-mediated process. We have also shown that the degree of protein nitration induced by ONOO⁻ depends on the cell type, the stimulation state and the cell compartment (e.g. intracellular versus extracellular). These are novel findings with potentially important implications in cell biochemistry, and therefore warrant further discussion.

The significant reduction of nitration by the ONOO⁻ scavengers, namely uric acid and FeTPPS, suggests that ONOO⁻ is a major mediator of nitration in human leucocytes. The nitration induced by NO is also ONOO⁻-mediated, since the effect was abolished by FeTPPS. The absence of a significant decrease in protein nitration after the inhibition of myeloperoxidase activity by ABAH also suggests that this enzyme does not play a significant role in nitration in our experimental preparation. Interestingly, inhibition of myeloperoxidase activity had the opposite effect, with an increase in protein nitration that was also ONOO⁻-mediated (e.g. FeTPPS abolished the increase). The absence of a significant myeloperoxidase-mediated protein nitration in our studies may be explained by the rapid depletion of 3-nitrotyrosine induced by HOCl that is produced by a reaction catalysed by myeloperoxidase [15]. Our results are in agreement with those in the literature reporting absence of nitration via a myeloperoxidase pathway in *in vitro* and *in vivo* experimental models. Thus Evans et al. [16], using cytokine-treated human neutrophils, demonstrated that the activity of inducible NO synthase was required during nitration of ingested bacteria through the production of ONOO⁻ and that only the inhibition of this enzyme blocks nitrotyrosine formation. More recently, Takizawa et al. [17] have shown in mice with and without myeloperoxidase deficiency that the myeloperoxidase-mediated

pathway is not responsible for the nitration reaction commonly associated with ischaemic brain injury.

In contrast, previous studies have reported a myeloperoxidase-mediated pathway of nitrotyrosine formation in various experimental models. Using an *in vitro* preparation containing proteins but without cells, Sampson et al. [18] have shown that myeloperoxidase and other peroxidases catalyse the nitration of proteins. Eiserich et al. [8] have also reported nitration by myeloperoxidase in cultured neutrophils with synthetic peptides as targets, although it is unclear if myeloperoxidase is free or remains within the vesicles. In this connection, Baldus et al. [19] have shown in *in vivo* studies that a myeloperoxidase-dependent pathway of nitration occurs only when granulocytes release the contents of their vesicles and, therefore, myeloperoxidase. They have also demonstrated in a model of vascular inflammation in mice that myeloperoxidase released by granulocytes and monocytes concentrate in the sub-endothelial matrix of vascular tissues [19]. This occurs by a transcytotic mechanism of translocation where myeloperoxidase may serve as a catalyst for extracellular matrix protein nitration. The authors identified a novel process of myeloperoxidase extravasation that occurs independently of neutrophil diapedesis, leading to its deposition within the vascular extracellular matrix. However, the absence of myeloperoxidase-mediated protein nitration in our model suggests that oxidants generated by myeloperoxidase are inefficient at nitrating proteins, possibly because they remain within vesicle compartments. Our results also contrast with other studies [11,13] that have shown that myeloperoxidase can convert tyrosine to tyrosyl radicals which in turn cause nitration. This process may not be possible with proteins where oxidants generated by myeloperoxidase are required for the oxidation and the subsequent nitration of the tyrosine residues.

Recently, Gaut et al. [20] have reported, using two *in vivo* models of acute peritoneal inflammation in mice with or without functional myeloperoxidase activity, that myeloperoxidase generates nitrotyrosine only under conditions of increased NO_2^- and NO_3^- . The increase in NO_2^- and NO_3^- occurred only after the infection of mice with *Klebsiella pneumoniae* bacteria. These findings support the view that the role of myeloperoxidase in protein nitration may depend on the experimental conditions. This theory is further supported by the work of Brennan et al. [21] who have demonstrated, using myeloperoxidase and eosinophil peroxidase knockout mice in a number of models of inflammation, that in some models peroxidases played a dominant role in accounting for the majority of nitrotyrosine generated, but in others (e.g. leucocyte-rich acute inflammation model) myeloperoxidase and eosinophil peroxidase did not contribute to nitrotyrosine formation. Brennan et al. [21] also showed that the major reactive nitrogen species formed by leucocyte peroxidase-catalysed oxidation of NO_2^- is the one-electron oxidation product $\cdot\text{NO}_2$ and that peroxidases also catalyse a two-electron oxidation of NO_2^- to produce a ONOO^- -like product. They speculated that the ONOO^- -like product was a labile Fe-ONOO^- complex, which may be released under acidic conditions, such as the one existing at sites of inflammation. The formation of ONOO^- -like product may be enhanced by the increased expression of inducible NO synthase [16], which further emphasizes the importance of the local conditions for the mechanism of protein nitration.

An important finding of the present study is that protein nitration induced by the exposure to ONOO^- varied quantitatively and qualitatively in granulocytes and mononuclear cells in their intra- and extracellular compartments. Our studies do not provide an answer for these results but it is possible that they are due to differences in the concentrations and availability of the various elements participating in nitration. This hypothesis

finds support in the observation that spermine NONOate, a NO-radical donor, promotes extracellular nitration but inhibits intracellular nitration in PMA-stimulated leucocytes (Figures 4A–4D) and that NO_2^- variably reduces nitration in the two cell compartments of both granulocytes and mononuclear cells (Figures 5A–5D). These results may suggest that NO from various sources could diffuse to contribute towards the nitration of proteins.

It is of interest to note that although the ONOO^- scavengers FeTPPS and uric acid significantly reduced protein nitration in granulocytes and mononuclear cells, complete elimination did not occur. These results may explain the existence of different reaction kinetics between nitration and the formation and decomposition of ONOO^- . Previously, it has been observed that a faster reaction occurs between ONOO^- and biological targets, particularly enzymes with metal centres (e.g. 10^4 – $10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$) than between ONOO^- and antioxidants such as ascorbate and glutathione (e.g. $10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$) [22]. In contrast, the formation of ONOO^- from NO and O_2^- occurs at nearly diffusion-controlled rates of 4.3 – $6.7 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ [23,24].

It is clear that the full elucidation of the mechanisms involved in protein nitration requires further investigation. The significance and necessity of this investigation is undermined by the important role of protein nitration in physiological and pathological processes, particularly in signal transduction [25]. The recent demonstration by our laboratory of an association between the inflammatory process and the level of 3-nitrotyrosine formation in patients undergoing cardiac surgery [3], and of a significant reduction in plasma levels of 3-nitrotyrosine in patients treated with infusion of nitroglycerin [4], reinforce the case for the biological and potentially clinical importance of protein nitration.

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