The nitration of platelet cytosolic proteins during agonist-induced activation of platelets

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Abstract The nitration of protein tyrosine residues by peroxynitrous acid has been associated with pathological conditions. Here it is shown, using a sensitive competitive enzyme-linked immunosorbent assay and immunoblotting for nitrotyrosine, that spontaneous nitration of specific proteins occurs during a physiological process, the activation of platelets by collagen. One of the main proteins nitrated is vasodilator-stimulated phosphoprotein. Endogenous synthesis of nitric oxide and activity of cyclo-oxygenase were required for the nitration of tyrosine. The nitration was mimicked by addition of peroxynitrite to unstimulated platelets, although the level of nitrotyrosine formation was greater and its distribution among the proteins was less specific.

Key words: Platelet; Collagen; Protein nitration; Vasodilator-stimulated phosphoprotein

1. Introduction

Platelet aggregation, induced by specific agonists, leads to both biochemical and morphological changes in the cell. Platelets release nitric oxide (NO) during platelet aggregation, but not at rest [1,2]. NO, which activates soluble guanylyl cyclase leading to the formation of cGMP, inhibits platelet activation through the action of cGMP-dependent kinases [3] and their phosphorylation of vasodilator-stimulated phosphoproteins (VASPs) [4]. The platelet-derived NO limits the activation of neighbouring platelets [5] to avoid excessive recruitment of these cells into thrombi. Differences in the potency of platelet agonists to elicit NO biosynthesis have been reported: collagen and arachidonic acid are the most potent, while thrombin has a much smaller effect [1,6]. During activation, platelets also form superoxide anions ($O_2^{\bullet-}$) and hydrogen peroxide [7,8]: the peroxide strongly enhances the ability of NO to inhibit platelet aggregation [9].

NO reacts with $O_2^{\bullet-}$ to form peroxynitrite ($ONOO^-$), a potent oxidant [10], which has been shown to be released by endothelial cells [11]. Peroxynitrite inhibits platelet aggregation, but less effectively than NO [9,12–14]. The mechanism of this inhibition has been proposed to be both dependent [12,14] and independent [13] of cGMP. Peroxynitrite is extremely reactive and oxidises, hydroxylates and nitrates phenol groups on amino acids [15] and this may account for the presence of nitrated proteins in normal plasma [16]. Furthermore, nitrotyrosine has been found in inflammatory conditions, e.g. atherosclerotic plaques [17] and synovial fluid in rheumatoid arthritis [18], and has been used extensively as a marker of peroxynitrite formation in vivo.

Since both the reactants required for peroxynitrite formation, NO and $O_2^{\bullet-}$, are produced during the platelet activation/aggregation process, we examined the possibility of the nitration of protein tyrosine residues. The spontaneous nitration of proteins was shown to arise as part of a normal cellular process, in this case platelet activation, and that this nitration may target specific proteins in these cells.

2. Materials and methods

Prostacyclin (synthetic sodium salt), thrombin (human), bovine serum albumin (BSA), N^G-nitro-L-arginine methyl ester (L-NAME), acetylsalicylic acid (ASA) and (3-[3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate (CHAPS) buffer were purchased from Sigma (Poole, UK), collagen Type I (equine) from Hormonchemie (Munich, Germany) and oxadiazoloquinoxaline-1-one (ODQ) from Tocris-Cookson Chemicals (Southampton, UK).

Biotinylated goat anti-rabbit IgG antibodies and ECL reagents were obtained from Amersham-Pharmacia (Hertfordshire, UK), avidin–biotin horseradish peroxidase from Dako (Beaconsfield, UK), anti-nitrotyrosine was from TCS Biologicals plc (High Wycombe, Buckinghamshire, UK) and anti-VASP antibodies from Alexis (Nottingham, UK). Pre-cast 10% were prepared in the laboratory and nitrocellulose (pore size 0.45 μm) were purchased from Novex (San Diego, CA, USA).

2.1. Platelet preparations

Venous blood was taken with informed consent from healthy volunteers, who denied taking any medication in the previous 14 days, placed in acid-citrate-dextrose anticoagulant and centrifuged for 20 min at $150\times g$ to yield platelet-rich plasma (PRP). Washed platelets (WP) were prepared from PRP in the presence of prostacyclin as described elsewhere [9,19], suspended in buffer (NaCl 137 mM, NaH_2PO_4 4.2 mM, $NaHCO_3$ 11.9 mM, KCl 2.7 mM, pH 7.4) and diluted to a count of 3×10^8 platelets/ml. The platelets were allowed to rest for 1 h at room temperature and used within the following 2 h.

To prepare cytosolic and membrane fractions, WP were sonicated at 4°C (Soniprep, M.S.E., UK) for 2×15 s bursts separated by a 15 s pause and the cytosol separated from the membrane fractions by centrifugation at $1500\times g$ for 10 min. The pellet, which contained the membrane fraction, was solubilised in 0.1% CHAPS buffer [20].

2.2. Preparation of peroxynitrite

Sodium peroxynitrite was prepared as described previously [9]: con-

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Abbreviations: ODQ, oxadiazoloquinoxaline-1-one; L-NAME, N^G-nitro-L-arginine methyl ester; CHAPS, (3-[3-cholamidopropyl) dimethylammonio]-1-propane-sulphonate; NOS, nitric oxide synthase

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trols were prepared by the same procedure, except that the solutions were passed directly into water instead of NaOH, leading to immediate decomposition of the oxidant. NaOH was then added to restore the pH to 10. The concentration of stock peroxynitrite solutions was determined spectrophotometrically ($\varepsilon_{302\,nm} = 1760\ M^{-1}\ cm^{-1}$) using decomposed peroxynitrite as the blank.

2.3. Measurement of nitrated proteins

The nitrotyrosine content of proteins in the platelet samples was estimated using a competitive enzyme-linked immunosorbent assay (ELISA) developed in this laboratory [16] using nitrated (NT)-BSA as a standard. The results were extrapolated from a semi-log plot of the standard curve and were expressed as nmol BSA equivalents/mg protein. The protein concentrations were analysed using a modified Lowry method [21].

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

After various treatments, the reactions were stopped by addition of an equal volume of Laemmli's buffer [22]. Proteins were separated by SDS–PAGE on 10% gels. The separated proteins were transferred to nitrocellulose membranes using wet transfer techniques. Membranes were blocked with BSA (0.5%) dissolved in phosphate-buffered saline (PBS)/Tween (1% PVP-10, 1% PEG, 0.2% Tween and 10 mM NaF) for 60 min. Membranes were probed with either, anti-nitrotyrosine (1:800) or anti-VASP (1:2000) for 60 min. The membranes were washed several times with PBS/Tween, followed by incubation with horseradish peroxidase-linked goat anti-rabbit IgG for 60 min. The protein bands were visualised using ECL reagents.

In some experiments, membranes were stripped by washing with a stripping buffer (100 mM mercaptoethanol, 2% SDS and 62.5 mM Tris–HCl; pH 6.7) at 50°C for 30 min. The membranes were washed and probed with anti-VASP antibodies. After washing, the membranes were incubated with horseradish peroxidase-linked goat anti-rabbit IgG.

2.5. Statistical analysis

All data are presented as the mean ± S.E.M. of at least three independent experiments, unless otherwise stated. Statistical analysis was performed using Student's unpaired *t*-test.

3. Results

3.1. Nitration of proteins following agonist-induced activation of platelets

Low levels of nitrated proteins were found in most, but not all unstimulated platelets (Fig. 1) as measured by ELISA. In the cytosolic fraction 0.18 ± 0.08 nmol nitrotyrosine/mg protein were found, but the basal level of nitration in the membrane fraction was higher at 0.31 ± 0.08 nmol/mg protein. The CHAPS buffer used to solubilise the membrane fraction was found to have no effect on the reproducibility of the ELISA.

The activation of WP by collagen (0.2–20 μg/ml) for 1 min led to a concentration-dependent increase in the formation of nitrotyrosine residues on platelet proteins in the absence of exogenous peroxynitrite. The greatest effect was found with collagen (20 μg/ml) where nitrotyrosine was increased to 0.75 ± 0.15 nmol nitrotyrosine/mg protein, an almost four-fold increase above basal levels ($P < 0.01$) (Fig. 1). Collagen (2 μg/ml) increased nitrotyrosine levels on cytosolic proteins from a basal level of 0.18 ± 0.08 nmol to 0.56 ± 0.1 nmol nitrotyrosine/mg protein ($P < 0.05$) after 1 min. However, at lower concentrations of collagen (0.2 μg/ml) the elevation in nitrotyrosine did not reach statistical significance. There was some variation between platelet preparations, from a two- to a five-fold increase in nitrotyrosine, which may reflect the varying responsiveness to collagen of platelets taken from different donors. No increase in the levels of nitrated proteins was detected in the membrane fractions at any concentration of collagen. In experiments where thrombin (0.02–0.2 U/ml) was used to stimulate platelets, it was less effective in causing platelet nitration and the apparent increase was not statistically significant (not shown). The presence of EGTA (1 mM) abolished the collagen-induced formation of nitrotyrosine (not shown).

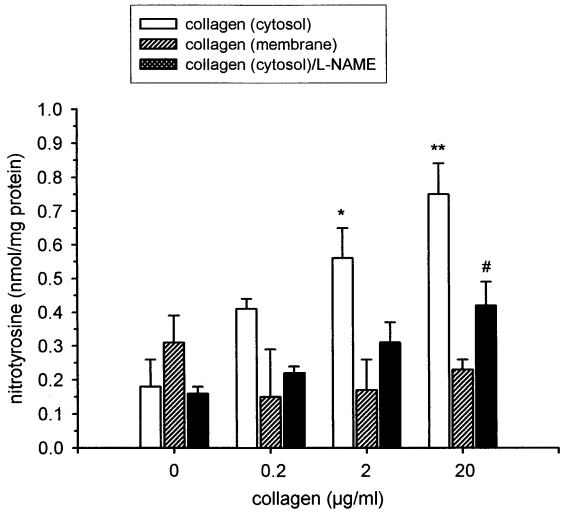

Fig. 1. Protein bound nitrotyrosine formation in collagen-stimulated platelets. WP were incubated with collagen (0.2–20 μg/ml) for 1 min with stirring at 37°C. The membrane and cytosolic fractions were prepared as described in Section 2. Nitrotyrosine was measured by a competitive ELISA. The data shows the nitrotyrosine formed in the cytosol after treatment of collagen (no fill) and membrane (hatched fill). In some experiments platelets were preincubated with L-NAME for 30 min prior to the addition of collagen (black fill). The results are expressed as nmol nitrotyrosine/mg protein and represent the mean ± S.E.M. of four independent experiments: $^{*}P < 0.05$ or $^{**}P < 0.05$ for nitration compared to basal levels, $^{\#}P < 0.05$ for the effects of L-NAME.

The experiments were repeated in the presence of L-NAME, a competitive inhibitor of nitric oxide synthase (NOS), used at submaximal concentrations (100 μM). Collagen (20 μg/ml) induced 0.75 ± 0.15 nmol nitrotyrosine/mg protein, while in the presence of L-NAME this was reduced to 0.42 ± 0.09 nmol ($P < 0.05$) (Fig. 1). The reduction in nitrotyrosine suggests that endogenously synthesised NO was required for the formation of a nitrating species and subsequently nitrotyrosine.

The presence of nitrotyrosine in specific platelet proteins was determined by immunoblotting with an anti-nitrotyrosine antibody. Low levels of nitrotyrosine were detected in the proteins of resting platelets (Fig. 2A, lane 2) particularly those with molecular weights of 46 to 50 kDa. However, stimulation of the platelets with collagen led to a marked increase in the extent of nitration in these proteins. The greatest effect was again observed with 20 μg/ml collagen (Fig. 2A, lane 5), confirming results described above with the ELISA on total platelet proteins. The presence of L-NAME (100 μM) also reduced the signal for nitrotyrosine in the bands of nitrated proteins. Furthermore, the pre-incubation of platelets with ASA (1 mM) returned the extent of protein nitration of VASP to those of resting platelets or lower. ODQ (10 μM), a specific inhibitor of soluble guanylyl cyclase [23], had no effect on the amounts of nitrotyrosine detected.

The proteins which were most strongly nitrated following activation of platelets by collagen, molecular weights of 46

and 50 kDa, were shown to be VASP, representing the phospho (50 kDa) and de-phospho (46 kDa) forms of the protein, by stripping and re-probing the membrane with anti-VASP antibody (Fig. 2B). This was confirmed by immuno-precipitation of VASP with this antibody, re-running on a gel and probing with anti-nitrotyrosine antibody to reveal nitrated protein only at the 46 and 50 kDa bands (not shown).

3.2. Nitration of platelet proteins by exogenous peroxynitrite

The extent of nitration of proteins induced by collagen was compared to that in platelets exposed to solutions of authentic peroxynitrite. This led to a concentration-dependent increase in the formation of nitrotyrosine residues on both membrane and cytosolic proteins (Fig. 3). Nitrotyrosine levels were greater than those previously induced by agonist-stimulated platelet activation. However, as with spontaneous nitration, nitrotyrosine was found to be higher in the proteins from the cytosolic fraction. Peroxynitrite (3 μM) induced 12.6 ± 3.2 nmol nitrotyrosine/mg protein in the cytosolic fraction and 1.6 ± 0.3 nmol/mg protein in the membrane fraction (Fig. 3). The presence of exogenous glutathione (1 mM) protected platelet proteins, causing an almost complete abolition of nitration (not shown). Decomposed peroxynitrite did not raise nitrotyrosine concentrations significantly above the levels found basally (Fig. 3).

Immunoblotting of the peroxynitrite-treated samples showed that the oxidant caused the nitration of a broad spectrum of different proteins of which VASP was only a minor contributor (not shown). This is in contrast to collagen, which induced a more selective process of nitration.

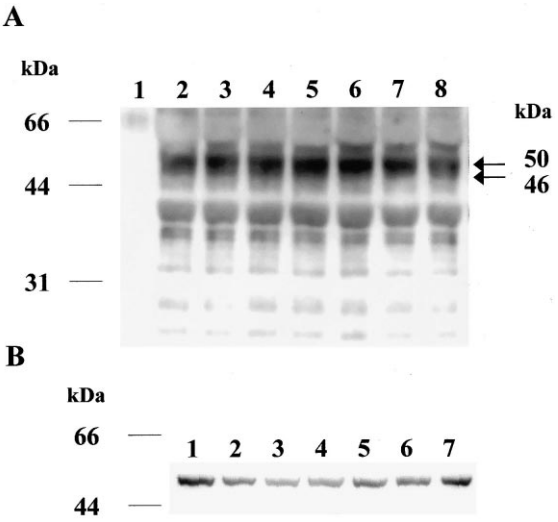

Fig. 2. A: Protein-bound nitrotyrosine formation in collagen-stimulated platelets. WP were incubated with collagen (0.2–20 μg/ml) for 1 min with stirring at 37°C. In the same experiment the platelets were incubated with L-NAME (100 μM), ASA (1 mM) or ODQ (10 μM) for 30 min, prior to addition of collagen. Platelets were lysed with Laemmli's buffer and subjected to SDS–PAGE and immunoblotting as described in Section 2. Lane 1, NT-BSA; lane 2, resting platelets; lane 3, collagen 0.2 μg/ml; lane 4, collagen 2 μg/ml; lane 5, collagen 20 μg/ml; lane 6, collagen 20 μg/ml+ODQ 10 μM; lane 7, collagen 20 μg/ml+L-NAME (100 μM); lane 8, collagen 20 μg/ml after pre-incubation with 1 mM ASA for 30 min. The blot is representative of four independent experiments. B: Identification of VASP as a possible target for endogenous nitration. Experimental protocol was identical to that of (A), except the membranes were stripped and re-probed with anti-VASP antibody. Lane 1, resting platelets; lane 2, +collagen 0.2 μg/ml; lane 3, collagen 2 μg/ml; lane 4, collagen 20 μg/ml; lane 5, collagen 20 μg/ml+ODQ (10 μM); lane 6, collagen 20 μg/ml+L-NAME (100 μM); lane 7, collagen 20 μg/ml after pre-incubation with ASA 1 mM. The blot is representative of four independent experiments.

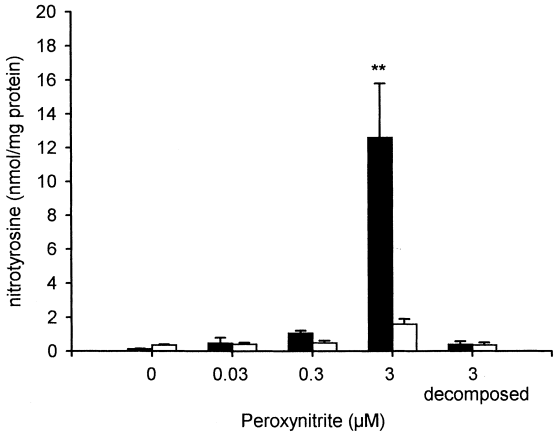

Fig. 3. Peroxynitrite-induced nitration of platelet proteins. Peroxynitrite was added to WP and incubated for 1 min at 37°C with continuous stirring. The platelets were separated into cytosolic and membrane fractions as described in the Section 2. Nitrotyrosine was then measured using a semi-quantitative ELISA. Results are expressed as nmol nitrotyrosine/mg protein and represent the mean ± S.E.M. of five independent experiments. $^{**}P < 0.01$ for nitration compared to basal levels.

4. Discussion

In the present study we investigated whether the nitration of proteins could be formed as part of a normal physiological process. Low levels of nitration were detected in the majority of, but not all, samples of resting platelets and has been observed previously in unstimulated cell lysates of cultured endothelial cells [24] and in human plasma [16]. The basal levels of nitration found in platelets suggested that they were exposed to a nitrating species either in vivo, or possibly by activation of the cells during the isolation process.

The activation of platelets with collagen, but not thrombin, led to an increase in the levels of cytosolic nitration. This is direct evidence that nitration may occur as part of a normal physiological process. The reduction in nitrotyrosine formation in the presence of L-NAME indicates that endogenously synthesised NO is a key requirement for the formation of nitrating species and subsequently nitrotyrosine. The lower rates of synthesis of NO in thrombin-stimulated platelets [1,6] probably accounts for the inability of thrombin to induce significant nitration. ASA was also found to be an inhibitor of the nitration process. Activation of prostaglandin H synthase-2 leads to the formation of a tyrosyl radical within the enzyme complex that may react with NO to form nitrotyrosine leading to autonitration of this enzyme and possibly other proteins [25]. Alternatively, the formation of thromboxane A_2 during platelet activation will be inhibited by ASA. This eicosanoid acts primarily to increase platelet $[Ca^{2+}]_i$ leading to the activation of platelet NOS. Thus, in the presence of ASA no nitrating species would be formed.

The nitration of platelet proteins following activation by collagen is not uniform across all platelet proteins. Firstly, nitration occurred mainly among the cytosolic proteins. Fur-

thermore, specific proteins appear to be targeted for nitration during the activation process. One of these proteins was identified as VASP, a protein involved in cytoskeletal rearrangement during platelet aggregation [26]. VASP is a primary target for both cGMP- and cAMP-dependent kinases leading to the phosphorylation of two serine and one threonine residues [27]. The phosphorylation of serine 157 leads to a change in the apparent molecular weight from 46 to 50 kDa, but both forms appear to be capable of being nitrated. VASP has four tyrosine residues and their functional significance is unknown. The consequences of the nitration of VASP on its function require further investigation. The identity of the other protein bands that appear to be nitrated has not been established.

The exposure of platelets to low concentrations of authentic peroxynitrite, capable of the inhibition of platelet activation [10], caused extensive nitration of platelet proteins again mainly in the cytosolic fraction (Fig. 3), a phenomenon already observed both in platelets [28] and neuroblastoma cells [29]. This suggests that peroxynitrite formed extracellularly, e.g. by the endothelium, may diffuse into platelets (or other cells) and modify their cytosolic proteins [30]. The nitration occurred over a broad spectrum of proteins, appearing to be indiscriminate, but much more intense than after stimulation with collagen. This indicates that when platelets are exposed to collagen, the formation of endogenous NO and peroxynitrite is not sufficient to inhibit their activation. It is possible that the nitration process may be part of a mechanism by which nitric oxide limits the extent of the activation process in platelets by inhibiting the activation of neighbouring platelets. Furthermore, the chronic exposure of platelets to both NO and peroxynitrite, at low levels from endothelial cells, may lead to a more selective action on specific proteins such as VASP. This may prevent the formation of thrombi unless a major stimulus such as the exposure of collagen or the release of thrombin occurs.

It is still an open question whether collagen-induced nitration is a process of physiological significance. The pre-treatment of cells with peroxynitrite can substantially reduce the level of tyrosine phosphorylation [24,28] thereby compromising cell signalling. Conversely, a recent study has shown that c-Jun NH_2-terminal kinase is regulated by tyrosine nitration [31]. This may suggest that endogenous nitration may serve some regulatory function.

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