The role of protein nitration in the inhibition of platelet activation by peroxynitrite

Sylvia Y. Low[a], Mojhgan Sabetkar[a], K. Richard Bruckdorfer[a], Khalid M. Naseem[b,*]

[a] *Departments of Biochemistry and Molecular Biology, Royal Free and University College Medical School (University College London), Rowland Hill Street, London NW3 2PF, UK*

[b] *Department of Biomedical Sciences, University of Bradford, Richmond Road, Bradford BD7 1DP, West Yorkshire, UK*

Received 26 November 2001; accepted 30 November 2001

First published online 19 December 2001

Edited by Barry Halliwell

Abstract Peroxynitrite at low concentrations (3–10 μM) inhibited agonist-induced platelet aggregation by a mechanism not dependent on the formation of cyclic guanosine monophosphate. Platelets recovered completely from peroxynitrite-induced inhibition within 30 min. Peroxynitrite induced nitration of cytosolic proteins, but this diminished to near basal levels within 60 min of exposure to the oxidant. During this period there was a reduction in tyrosine phosphorylation of specific proteins such as syk, but this was not due to direct nitration of these same proteins. The inhibition of phosphorylation was reversible with platelet proteins recovering the ability to be phosphorylated within 15 min of exposure to peroxynitrite. Conversely, peroxynitrite increased phosphorylation of other proteins, but again these events were not directly linked to nitration. Nitration may affect the phosphorylation of tyrosine residues in a number of proteins, but by an indirect route, possibly by acting on proteins upstream in the signalling cascades. We suggest that low concentrations of peroxynitrite reversibly inhibit platelet aggregation by preventing the phosphorylation of key signalling proteins.

Key words: Platelet; Protein nitration; Tyrosine phosphorylation; Cyclic guanosine monophosphate

1. Introduction

Platelet aggregation, induced by specific agonists, is the culmination of several exquisitely integrated signalling pathways, which lead to biochemical and morphological changes in the cell. Excessive platelet activation is limited by the endothelial-derived platelet inhibitors prostacyclin (PGI_2) and nitric oxide (NO) [1]. NO, unlike PGI_2, is able to inhibit both platelet activation and adhesion to the subendothelium. NO, which activates soluble guanylyl cyclase (sGC) leading to the formation of cyclic guanosine monophosphate (cGMP), inhibits platelet activation by reducing the intracellular calcium concentration through the action of cGMP-dependent kinases [2]. The regulation of platelet activity by NO is crucial in preventing excessive platelet aggregation and thrombi.

NO undergoes a diffusion limited reaction with superoxide anion ($O_2^{\bullet-}$) to form peroxynitrous acid (ONOOH), a potent oxidant [3] which has been shown to be released by endothelial cells [4]. This can lead to a reduction in the bioavailability of NO at the endothelial surface, and possibly to a loss of platelet regulation. The influence of peroxynitrite on platelet function has been shown to be concentration-dependent. At high concentrations, in excess of 150 μM, peroxynitrite acts as a platelet agonist by stimulating aggregation [5]. At lower concentrations or in the presence of plasma, peroxynitrite acts as a platelet inhibitor, although much less effectively than NO [5–7]. The mechanism of this inhibition has been proposed to be cGMP-dependent [5,6] since peroxynitrite nitrosates thiol-containing proteins, though inefficiently, to form nitrosothiols [8]. The nitrosothiols then inhibit platelets through the release of NO. However, peroxynitrite also undergoes a range of different reactions at physiological pH, which may affect cell function. Peroxynitrite nitrates phenols such as tyrosine [9], accounting for the presence of nitrated proteins in normal plasma [10]. The presence of nitrated proteins has been used as a marker for peroxynitrite formation in tissues, while the formation of nitrotyrosine intracellularly may have important consequences for cell function. Peroxynitrite-induced nitration of peptides has been shown to reduce their phosphorylation when exposed to specific protein kinases [11,12], suggesting that protein nitration may interfere with protein phosphorylation signalling pathways. Furthermore, we have recently shown that nitrated proteins form spontaneously in platelets undergoing collagen-induced aggregation [13]. The function of these nitrated proteins is unknown.

In the present study, we examined the effects of peroxynitrite on platelet function with the aim of dissecting the relative contributions of the NO-cGMP pathway and protein nitration. Our data indicate that peroxynitrite inhibits platelet aggregation in a cGMP-independent and reversible manner, and that the major action of peroxynitrite is to prevent, reversibly, the agonist-induced phosphorylation of signalling proteins.

2. Materials and methods

Prostacyclin (synthetic sodium salt), thrombin (human), bovine serum albumin (BSA), trichloroacetic acid (TCA) and (3-[3-cholamidopropyl]dimethylammonio)-1-propane-sulphonate (CHAPS) buffer were purchased from Sigma Chemical Co. (Poole, UK), collagen Type I (equine) from Hormonchemie (Munich, Germany) and ODQ from Tocris-Cookson Chemicals (Southampton, UK).

Biotinylated goat anti-rabbit IgG and horseradish peroxidase (HRP)-linked anti-rabbit IgG antibodies and nitrocellulose (pore size 0.45μm) were obtained from Amersham Pharmacia Biotech (Bucks, UK). Avidin–biotin HRP was from DAKO Ltd. (Beaconsfield, UK). Polyclonal anti-nitrotyrosine antibody was produced in

*Corresponding author. Fax: (44)-1274-309742.
E-mail address: k.m.naseem@bradford.ac.uk (K.M. Naseem).

0014-5793/02/$22.00
PII: S0014-5793(01)03279-3

our laboratory, while anti-phosphotyrosine HRP-linked (PY-20) antibody was from Santa Cruz (Insight Biotechnology Ltd., Middlesex, UK), anti-syk monoclonal antibody and rabbit anti-nitrotyrosine polyclonal antibody were from Upstate Biotechnology (TCS Biologicals, Bucks, UK). Pre-cast NuPAGE 10% and 4–12% gradient gels and NuPAGE sample buffer were purchased from Invitrogen Life Technologies (Paisley, UK). Enhanced chemoluminescence (ECL) reagents were obtained from Perbio (Cheshire, UK). The cGMP ELISA kits were purchased form Cayman Chemicals (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 into acid–citrate–dextrose anti-coagulant and centrifuged for 20 min at $150\times g$ to yield platelet-rich plasma (PRP). Washed platelets (WPs) were prepared from PRP in the presence of prostacyclin as described elsewhere [14], 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 rested for 1 h at room temperature and used within the next 2 h.

To prepare cytosolic and membrane fractions, WPs 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 [15].

2.2. Preparation of peroxynitrite

Sodium peroxynitrite was prepared using the acidified nitrite method [16]; controls 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. Residual H_2O_2 was removed by passing the peroxynitrite through a MnO_2 column and checked by absorbance at 220 nm. The concentration of stock peroxynitrite solutions was determined spectrophotometrically (302 nm = 1670 M^{-1} cm^{-1}) [17] using decomposed peroxynitrite as the blank. Working dilutions of both active and decomposed peroxynitrite were prepared in the same manner using 1 mM NaOH.

2.3. Measurement of cGMP concentrations

WPs were pre-incubated with isobutylmethylxanthine (200 μM) for 30 min prior to experimentation. WPs were then incubated with either peroxynitrite or NO and after 1 min the reactions halted by the addition of TCA (5%). The cGMP was extracted with diethylether, with the ether subsequently removed by heating extracted samples to 70°C for 5 min. The cGMP was then analysed using a commercially available ELISA kit.

2.4. Quantification of nitrated proteins

The nitrotyrosine content of proteins in the platelet samples was estimated using a competitive semi-quantitative ELISA developed in this laboratory [10]. The assay used nitrated BSA (NT-BSA) as a standard and a commercially available rabbit anti-human polyclonal anti-nitrotyrosine antibody. The amount of nitrotyrosine present after peroxynitrite treatment of BSA was determined by absorbance at 438 nm (pH 9) using the molar extinction coefficient of 4300 M^{-1} cm^{-1}. The molar ratio of nitrotyrosine to albumin was within 3–6 mol nitrotyrosine/mol protein. The results were extrapolated from a semi-log plot of the standard curve and were expressed as nmol of NT-BSA equivalents/mg of protein. The protein concentrations were established using a modified Lowry method [18].

2.5. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting

After various treatments, the reactions were stopped by addition of NuPAGE sample buffer. Proteins were separated by SDS–PAGE on either NuPAGE Bis–Tris 10% or 4–12% gradient gels. The separated proteins were transferred to nitrocellulose membranes using wet transfer techniques. Membranes were blocked with BSA (0.5%), dissolved in PBS containing 1% PVP-10, 1% PEG, 0.2% Tween and 10 mM NaF, for 60 min. Membranes were probed with anti-nitrotyrosine (1:500), anti-phosphotyrosine (1:5000) or anti-syk (1:2500) for 60 min. The membranes were washed several times with PBS/Tween 0.05%, followed by incubation with HRP-linked goat anti-rabbit IgG for 60 min. The protein bands were visualised using ECL reagents.

The in house polyclonal anti-nitrotyrosine antibody was raised against nitrated Keyhole Limpet Hemocyanin and purified using standard affinity purification techniques. Extensive characterisation was performed using nitrated peptides and a variety of tyrosine derivatives. The performance of this antibody in our ELISA was very similar to the commercial polyclonal antibody, but gave clearer resolution on immunoblots. The selectivity of the antibody was checked by treatment of nitrated proteins with the reductant dithionite and by its differential recognition of peptides containing tyrosine, aminotyrosine and nitrotyrosine.

2.6. 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. The influence of peroxynitrite on platelet aggregation

Incubation of WPs with peroxynitrite for 1 min prior to the addition of thrombin (0.02 U/ml) led to the inhibition of platelet aggregation compared to agonist alone (Table 1). The effect of peroxynitrite was concentration-dependent, with the IC_{50} value established at 4.6 ± 0.9 μM. In control experiments, decomposed peroxynitrite failed to inhibit thrombin-induced platelet activation.

In order to ascertain whether peroxynitrite-mediated inhibition of platelet aggregation was NO and cGMP-dependent, the experiments were repeated in the presence of ODQ (10 μM), an inhibitor of sGC [19] and, therefore, of the conventional NO-mediated pathway. The presence of ODQ did not influence the inhibition of thrombin-induced platelet aggregation by peroxynitrite (Table 1a). It was also established that

Table 1
The influence of peroxynitrite on platelet aggregation and cGMP formation

a: Peroxynitrite (μM)	Inhibition of aggregation (%)	
	without ODQ	with ODQ
0.01	4.5 ± 2.5	6.5 ± 6.5
0.1	17.5 ± 6.7	23.4 ± 8.9
1	ND	37.3 ± 7.2
10	67.6 ± 9.3	68.2 ± 17.2
100	98.2 ± 0.2	98.6 ± 1.4
Decomposed (10 μM)	0	0
b: Peroxynitrite (μM)	cGMP (pmol 1×10^8 platelets)	
	without ODQ	with ODQ
0	0.31 ± 0.12	0.09 ± 0.06
0.3	0.34 ± 0.17	0.06 ± 0.02
3	0.62 ± 0.29	0.21 ± 0.13
30	1.18 ± 0.27**	0.34 ± 0.11
Decomposed (10 μM)	0.27 ± 0.09	0.12 ± 0.07

Peroxynitrite (0–100 μM) was incubated with platelets for 1 min before the addition of thrombin (0.02 U/ml) and aggregation measured 3 min later. For cGMP measurement, platelets were pre-incubated with isobutylmethylxanthine (200 μM) for 30 min prior to addition of peroxynitrite. The reaction was halted after a 1 min incubation with peroxynitrite by the addition of ice-cold TCA (5%). The cGMP was extracted using diethyl and measured using a competitive enzyme immunoassay. The results are expressed as pmol cGMP/1×10^8 platelets. In some experiments the platelets were pre-incubated with ODQ (10 μM) for 15 min before the addition of peroxynitrite. Results for both inhibition of aggregation ($n = 5$) and cGMP ($n = 4$) are expressed as mean ± S.E.M. $^{**}P > 0.01$ compared to basal.

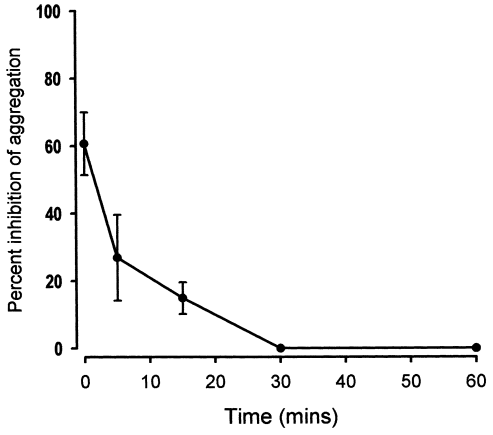

Fig. 1. The influence of peroxynitrite on platelet aggregation as a function of time. Peroxynitrite (5 μM) was added to platelets and incubated for up to 1 h at 37°C without stirring, before the addition of thrombin (0.02 U/ml). Platelet aggregation was measured 3 min after the addition of thrombin. Results are expressed as percent inhibition of aggregation and represent the mean ± S.E.M. of four independent experiments.

the inhibitory action of ODQ on GC activity was not changed by exposure to the peroxynitrite. ODQ was exposed to peroxynitrite (10 μM) prior to addition to platelets and still shown to inhibit the action of NO on platelets (results not shown). These results indicate that peroxynitrite acts primarily through a mechanism independent of cGMP.

In a second series of experiments, we investigated whether the effects of peroxynitrite on platelets were reversible. Platelets were incubated with peroxynitrite for up to 1 h before stimulation with thrombin (0.02 U/ml). Incubation of peroxynitrite (5 μM) for 1 min resulted in 60.7 ± 9.3% inhibition of thrombin-stimulated aggregation. After a 15 min incubation, the level of inhibition had declined to 14.9 ± 4.7% and at 30 min, no inhibition was evident (Fig. 1). These data suggest that the actions of peroxynitrite are transient and non-toxic, since it does not permanently impair the ability of platelets to respond to thrombin. Similar data were obtained using collagen as an agonist (data not shown).

3.2. cGMP synthesis in response to peroxynitrite and NO

The results of the aggregation experiments suggested that the effects of peroxynitrite were independent of both NO and cGMP. To confirm this, the ability of peroxynitrite to induce cGMP formation was assessed, and compared with that of authentic NO solutions. When this oxidant was added to platelets as a bolus and incubated at 37°C for 1 min, peroxynitrite caused a concentration-dependent increase in the formation of cGMP from the basal level. However, this increase was only significant at the highest concentration of peroxynitrite used (30 μM) which elevated cGMP from a basal level of 0.31 ± 0.12 to 1.18 ± 0.27 pmol/1 × 10^8 platelets ($P < 0.01$) (Table 1); 0.3 and 3 μM peroxynitrite had no significant effect on basal levels. The presence of ODQ (10 μM) prevented the rise of cGMP above basal levels with all concentrations of peroxynitrite (Table 1b). The rates of cGMP synthesis were much lower than those measured for NO (1 μM NO produces 4.1 ± 0.6 pmol cGMP/10^8 platelets after 1 min). The inhibition of platelet aggregation by peroxynitrite at 3–5 μM (the approximate IC_{50}) does not appear to be due to increases in cGMP formation.

3.3. Peroxynitrite-induced nitration of platelet proteins

Measurement of nitrotyrosine levels using a semi-quantitative ELISA demonstrated the presence of low amounts of nitrated proteins in both cytosolic and membrane fractions of unstimulated platelets. The subsequent addition of peroxynitrite (3 μM) led to a significant increase in nitrated platelet proteins, but only of cytosolic proteins. In the cytosolic fraction, peroxynitrite increased nitrotyrosine levels from a basal level of 0.3 ± 0.1 to 6.4 ± 2.8 nmol NT-BSA equivalents/mg protein ($P < 0.01$), whereas in the membrane fraction the increase in nitration was not significant.

The inhibition of platelet aggregation by peroxynitrite (Fig. 1) was reversible, and therefore if protein nitration was a regulator of platelet function, this too would be reversible. After a 1 min incubation, peroxynitrite (3 μM) formed 6.4 ± 2.8 NT-BSA equivalents/mg protein in the cytosolic fraction. The amount of nitrotyrosine fell, by 60 min, to 3.1 ± 0.9 NT-BSA equivalents/mg protein ($P > 0.05$ compared to levels after 1 min) and, after 90 min, the concentration was only 1.2 ± 0.4 NT-BSA equivalents/mg protein ($P < 0.01$; Fig. 2). We also observed considerable variations between individual platelet preparations in both the initial levels of nitration and in the speed of the loss of nitration. In these experiments, the low levels of nitrotyrosine in the membrane fraction did not increase and remained constant over 90 min.

3.4. Influence of peroxynitrite on thrombin-stimulated protein phosphorylation

Previously, it had been shown that peroxynitrite (at high concentrations above 150 μM) caused both nitration and phosphorylation of proteins in platelets [20]. WPs were incubated with 10 μM peroxynitrite, for time intervals up to 60 min and the extent of protein nitration was determined by Western blotting. The incubation of platelets with a range of peroxynitrite concentrations resulted in increased nitration of many different protein bands (Fig. 3A), whereas thrombin

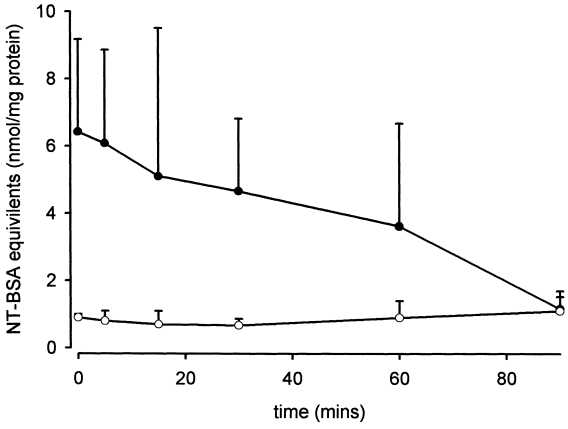

Fig. 2. The influence of time on peroxynitrite-induced nitration of platelet proteins. Peroxynitrite (3 μM) was incubated with WPs for up to 90 min at 37°C without stirring. Aliquots were taken at various time points before being cooled and sonicated. The platelets were subsequently centrifuged at 1500 × *g* for 5 min to separate the membrane (○) and cytosolic fractions (●). The membrane fraction was solubilised using CHAPS buffer (0.1%). Nitrotyrosine was then measured in the membrane and cytosolic samples using a semi-quantitative ELISA. Results are expressed as nmol NT-BSA equivalents/mg protein and represent the mean ± S.E.M. of five independent experiments.

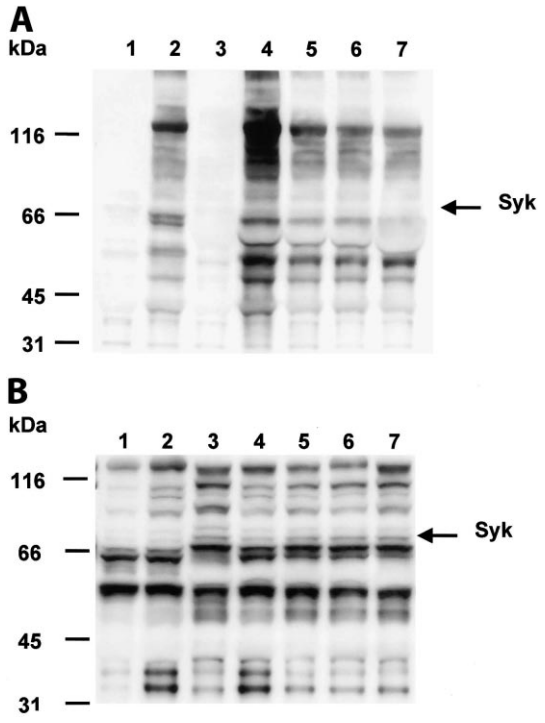

Fig. 3. A: The effect of peroxynitrite on the nitration of platelet proteins. WPs were treated with peroxynitrite (10 μM) for up to 60 min before the addition of thrombin (0.02 U/ml), and then incubated for 1 min with stirring at 37°C. Platelets were lysed with NuPAGE sample buffer and subjected to SDS–PAGE using a NuPAGE 4–12% gradient gel. The separated proteins were transferred onto nitrocellulose membrane and then probed with anti-nitrotyrosine antibody (1:500). The antigen was visualised using the ECL system. Lane 1: basal, 2: peroxynitrite 10 μM (1 min), 3: thrombin 0.02 U/ml, 4: peroxynitrite (1 min)+thrombin 0.02 U/ml, 5: peroxynitrite (15 min)+thrombin 0.02 U/ml, 6: peroxynitrite (30 min)+thrombin 0.02 U/ml, 7: peroxynitrite (60 min)+thrombin 0.02 U/ml. Each well contained 20 μg platelet protein. The blot is a representative of four separate experiments. B: The influence of peroxynitrite on the phosphorylation of platelet proteins. WPs were treated with peroxynitrite (10 μM) for up to 60 min before the addition of thrombin (0.02 U/ml), and then incubated for 1 min with stirring at 37°C. Platelets were lysed with NuPAGE sample buffer and subjected to SDS–PAGE using a NuPAGE 4–12% gradient gel. The separated proteins were transferred onto nitrocellulose membrane and then probed with anti-phosphotyrosine antibody (1:5000). The antigen was visualised using the ECL system. Lane 1: basal, 2: peroxynitrite 10 μM (1 min), 3: thrombin 0.02 U/ml, 4: peroxynitrite (1 min)+thrombin 0.02 U/ml, 5: peroxynitrite (15 min)+thrombin 0.02 U/ml, 6: peroxynitrite (30 min)+thrombin 0.02 U/ml, 7: peroxynitrite (60 min)+thrombin 0.02 U/ml. Each well contained 20 μg platelet protein. The blot is a representative of four separate experiments.

alone produced little nitration. Interestingly, the combination of peroxynitrite, followed by thrombin 1 min later, appeared to enhance the nitration; again the extent of nitration of most of the protein bands decreased markedly after 60 min, even after addition of thrombin at each interval.

To assess the influence on platelet-signalling proteins, WPs were incubated with peroxynitrite for time intervals up to 60 min before stimulation with thrombin (0.02 U/ml), and protein phosphorylation assessed by Western blotting. Thrombin stimulation of platelets increased phosphorylation over a broad range of protein sizes compared to unstimulated cells (Fig. 3B). Incubation of platelets with peroxynitrite (10 μM) for 1 min prior to thrombin stimulation led to a decrease in phosphorylation of proteins with apparent molecular weights of 72, 94 and 112 kDa. However, this inhibitory effect of peroxynitrite on the phosphorylation of some proteins was reversible. The thrombin-induced phosphorylation of the 72 kDa protein, subsequently identified as syk (results not shown), was strongly diminished by a 1 min pre-incubation of the platelets with peroxynitrite. However, if the cells were left to recover for 15 min after peroxynitrite, thrombin-stimulated phosphorylation returned to control levels. Similarly, phosphorylation of the 112 kDa began to recover within 15 min and was fully phosphorylated after 60 min, while the thrombin-stimulated phosphorylation of the 94 kDa was still only partial 60 min after peroxynitrite treatment. These data suggest that peroxynitrite at low concentrations inhibits protein phosphorylation of specific proteins, and that the mechanism is reversible in some cases. Immunoblotting experiments did not reveal any nitrated proteins with the same molecular weight as syk, indicating that syk may not be nitrated by peroxynitrite or thrombin (Fig. 3A).

In contrast, peroxynitrite was able to increase the tyrosine phosphorylation of certain proteins. After 1 min of peroxynitrite treatment, increased levels of protein phosphorylation were observed in the 35 and 38 kDa proteins in the presence or absence of thrombin, and to a greater extent than with thrombin alone (Fig. 3B). However, these effects were transient, since they returned to levels seen with thrombin alone 15 min after addition of peroxynitrite. As shown many times before, the src group of proteins was phosphorylated even in the resting platelets and this was not changed by addition of peroxynitrite. The level of phosphorylation of a 64 kDa protein was reduced by thrombin; the significance of this is unknown.

4. Discussion

The influence of peroxynitrite on platelet function has been studied previously, but the results have been contradictory. In earlier studies, peroxynitrite at concentrations above 150 μM was shown to stimulate platelet aggregation in plasma-free platelet preparations [5,6], while only in the presence of plasma did it act as a platelet inhibitor [5,6]. The inhibitory effect of peroxynitrite was attributed to the formation of nitrosothiols, which act as NO donors and therefore inhibit platelets via the stimulation of sGC. Indeed, in the presence of glutathione, peroxynitrite does activate sGC [21]. We tested the role of sGC in peroxynitrite-mediated inhibition using much lower concentrations of the oxidant. At 1–10 μM, peroxynitrite was a potent inhibitor of platelets, but only a modest activator of sGC compared to authentic NO (1 μM). Furthermore, peroxynitrite still inhibited platelets that had been exposed to the sGC inhibitor ODQ, which was shown to be active even after addition of low concentrations of peroxynitrite. Therefore, the data indicate that the inhibition of platelets by peroxynitrite occurs by a process in which cGMP plays only a minor part. This is reminiscent of our recent work in which a synergistic inhibition of very low concentrations of NO and hydrogen peroxide did not appear to require elevated concentrations of cGMP [22]. However, in this case, a basal concentration of cGMP was required because the inhibition was blocked by ODQ [22].

The inhibition of platelets by peroxynitrite was fully reversible, indicating that this was not simply a destructive effect of

the oxidant on platelet proteins and lipids. The recovery occurred within 30 min and was 70% recovered after 15 min after addition of 5 μM peroxynitrite (Fig. 1). An alternative explanation of a role for cGMP is that the inhibition of platelets is due to the nitration of platelet proteins by the formation of nitrotyrosine. Nitration did occur, even at low concentrations of peroxynitrite and the extent of nitration increased significantly at concentrations immediately above the IC_{50}, as detected by the ELISA. This increase occurred primarily in the soluble fraction, suggesting that the nitrating species passed through the membrane into the cytosol; this has been reported in red blood cells [23]. The extent of the cytosolic nitration also diminished after a period of 1 h, which was slightly longer than the recovery of the platelets in terms of their response to thrombin. There was a significant variation in the rate of loss of nitrated proteins in platelets from individual donors. Nitrotyrosine is stable and its direct reversion to tyrosine seems unlikely, although an intermediate non-enzymatic reduction to amines in tissues has been proposed [24]. The alternative would be proteolysis of the modified proteins or further modification of the nitro group. Certainly, a recent study has demonstrated that protein nitration enhances the susceptibility to proteolytic cleavage by proteasomes [25]. However, there appear to be no reports of proteasomal activity in platelets although they do contain lysosomes. Further work is required to establish the mechanism of nitrotyrosine disappearance.

The stimulation of platelets results in the activation of several key tyrosine kinases and phosphatases, leading to the phosphorylation of several key cellular proteins [26]. The events occur very early in the activation response and are critical to further cellular activation and aggregation. Therefore, it is possible that peroxynitrite inhibited platelet aggregation through the nitration of key signalling proteins which are normally phosphorylated during activation. We have shown that pre-treatment of platelets with peroxynitrite inhibited thrombin-induced phosphorylation of several proteins, but not of others. These results may indicate that peroxynitrite inhibits platelet activation through the temporary impairment of tyrosine phosphorylation of specific signalling proteins. Implicit in this mechanism is the question of whether the reversibility of platelet inhibition and protein nitration, following exposure to peroxynitrite, is associated with a recovery of platelet responsiveness to thrombin-induced tyrosine phosphorylation of specific proteins. Thrombin-induced phosphorylation of several proteins was lost, including syk, after a 1 min pre-incubation with peroxynitrite. However, the phosphorylation returned within 15–30 min of exposure to the oxidant; this correlated temporally very well with the recovery of aggregation response. The rate of recovery for phosphorylation was different for individual proteins, and not all proteins fully recovered. This is new evidence that the effect of peroxynitrite on tyrosine phosphorylation is reversible. In other studies, the recovery may be blocked due to damage caused by excessive nitration and oxidation of sulphydryl groups by the use of peroxynitrite at concentrations of up to 1 mM peroxynitrite. In one study in platelets, accumulative concentrations up to 800 μM peroxynitrite were used and no reversal of the high levels of nitration were observed over 60 min [20].

An obvious mechanism for this inhibition may be that nitration of the same tyrosine residue blocks its phosphorylation. However, no correlation existed between the inhibition of tyrosine phosphorylation and the increase in nitration, and subsequent changes occurring during the hour period. We have been unable to demonstrate that proteins such as syk are nitrated by peroxynitrite and, therefore, the reduction in phosphorylation is likely to be due to the inhibition of another protein kinase upstream from syk. In neuroblastoma cells, the inhibition of tyrosine phosphorylation is associated with compromised phosphoinositide signalling [27], an event crucial for platelet function. The loss of nitro groups from specific proteins, in a short time frame, may be a key event in restoring platelet sensitivity to agonists. Non-specific nitration of proteins may not be important, except when exposed to excess peroxynitrite. Therefore, partial loss of nitrated proteins, or selective loss of nitro groups may be sufficient to reverse the inhibition of platelet function.

Peroxynitrite enhanced the phosphorylation of proteins to a greater extent than did thrombin, in particular those at 35 and 38 kDa; the latter co-migrates with p38 MAP kinase, which his known to be activated by peroxynitrite [28]. Conversely, some proteins are phosphorylated only after addition of thrombin and not by peroxynitrite (e.g. 68 kDa protein). The peroxynitrite-induced protein phosphorylation was transient and diminished after 15 min. There is no evidence that they are directly related to protein nitration and again may be due to activation of kinase upstream of these signalling proteins. There are several mechanisms by which peroxynitrite has been proposed to increase tyrosine phosphorylation. These include the inhibition of specific phosphatases [29], direct activation of proteins kinases [30] or via the activation of specific tyrosine kinase receptors [31]. The precise molecular relationship between protein nitration and phosphorylation in platelets remains to be determined, but it is clearly indirect.

Acknowledgements: The authors wish to thank The Wellcome Trust for their support of this work.

References

[1] Moncada, S., Palmer, R.M.J. and Higgs, E.A. (1990) Thromb. Res. XI (Suppl.), 3–13.
[2] Haynes, D.H. (1993) Platelets 4, 231–242.
[3] Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A. and Freeman, B.A. (1990) Proc. Natl. Acad. Sci. USA 87, 1620–1624.
[4] Kooy, N.W. and Royall, J.A. (1994) Arch. Biochem. Biophys. 310, 352–359.
[5] Moro, M.A., Darley-Usmar, V., Goodwin, D.A., Read, R.G., Zamora-Pino, R., Feelisch, M., Radomski, M.W. and Moncada, S. (1994) Proc. Natl. Acad. Sci. USA 91, 6702–6706.
[6] Brown, A.S., Moro, M.A., Masse, J.M., Cramer, E.M.., Radomski, M. and Darley-Usmar, V. (1998) Cardiovasc. Res. 40, 380–388.
[7] Yin, K., Lai, P.S., Rodrigues, A., Spur, B.W. and Wong, P.Y.K. (1995) Prostaglandins 50, 169–178.
[8] Williams, D.L.H. (1997) Nitric Oxide 1, 522–527.
[9] Beckman, J.S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C.D., Chen, J., Harrison, J., Martin, J.S. and Tsai, H. (1992) Arch. Biochem. Biophys. 298, 438–445.
[10] Khan, J., Brennan, D.M., Bradley, N.J., Gao, B., Bruckdorfer, K.R. and Jacobs, M. (1998) Biochem. J. 330, 795–801.
[11] Kong, S.K., Yim, M.B., Stadtman, E.R. and Chock, P.B. (1996) Proc. Natl. Acad. Sci. USA 93, 3377–3382.
[12] Gow, A., Duran, D., Malcolm, S. and Ischiropoulos, H. (1996) FEBS Lett. 385, 63–66.
[13] Naseem, K.M., Low, S.Y., Sabetkar, M., Bradley, N.J., Khan, J., Jacobs, M. and Bruckdorfer, K.R. (2000) FEBS Lett. 473, 119–122.

[14] Vargas, J.R., Radomski, M. and Moncada, S. (1983) Prostaglandins 23, 929–945.
[15] Hjelmeland, L.H. (1980) Proc. Natl. Acad. Sci. USA 77, 6268–6271.
[16] Naseem, K.M. and Bruckdorfer, K.R. (1995) Biochem. J. 110, 149–153.
[17] Hughes, M.N. and Nicklin, H.G. (1970) Biochem. Biophys. Acta 222, 660–661.
[18] Markwell, M.A., Haas, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
[19] Moro, M.A., Russel, R.J., Cellek, S., Lizasoain, I., Su, Y., Darley-Usmar, V.M., Radomski, M.W. and Moncada, S. (1996) Proc. Natl. Acad. Sci. USA 93, 1480–1485.
[20] Mondoro, T.H., Shafer, B.C. and Vostal, J.G. (1997) Free Rad. Med. Biol. 22, 1055–1063.
[21] Mayer, B., Schrammel, A., Klatt, P., Koesling, D. and Schmidt, K. (1995) J. Biol. Chem. 270, 17355–17360.
[22] Sabetkar, M., Naseem, K.M., Tullet, J.M., Friebe, A., Koesling, D. and Bruckdorfer, K.R. (2001) Nitric Oxide 3, 233–242.
[23] Denicola, A., Souza, J.M. and Radi, R.Y. (1998) Proc. Natl. Acad. Sci. USA 95, 3566–3571.
[24] Balabani, B., Kamisaki, Y., Martin, E. and Murad, F. (1999) Proc. Natl. Acad. Sci. USA 96, 13136–13141.
[25] Souza, J.M., Choi, I., Weisse, M., Daikin, E., Yudkoff, M., Obin, M., Ara, J., Horwitz, J. and Ischiropoulus, H. (2000) Arch. Biochem. Biophys. 380, 360–366.
[26] Jackson, S.P., Schoenwaelder, S.M., Yuan, Y., Salem, H.H. and Cooray, P. (1996) Thromb. Haemost. 76, 640–650.
[27] Li, X., De Sarno, P., Song, L., Beckman, J.S. and Jope, R.S. (1998) Biochem. J. 331, 599–606.
[28] Schieke, S.M., Briviba, K., Klotz, L.O. and Sies, H. (1999) FEBS Lett. 448, 301–303.
[29] Takakura, K., Beckman, J.S., Macmillan-Crow, L.A. and Crow, J.P. (1999) Arch. Biochem. Biophys. 15, 197–207.
[30] Klotz, L., Schieke, S.M., Sies, H. and Holbrock, N.J. (2000) Biochem. J. 352, 219–225.
[31] Zhang, P., Wang, Y., Kagen, E. and Bonner, J.C. (2000) J. Biol. Chem. 275, 22479–22486.