Activation of *src* tyrosine kinases by peroxynitrite

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Abstract In this study, we demonstrate that the phosphorylation activity of five tyrosine kinases of the *src* family from both human erythrocytes (*lyn*, *hck* and *c-fgr*) and bovine synaptosomes (*lyn* and *fyn*) was stimulated by treatment with 30–250 μM peroxynitrite. This effect was not observed with *syk*, a non-*src* family tyrosine kinase. Treatment of kinase immunoprecipitates with 0.01–10 μM peroxynitrite showed that the interaction of these enzymes with the oxidant also activated the *src* kinases. Higher concentrations of peroxynitrite inhibited the activity of all kinases, indicating enzyme inactivation. The addition of bicarbonate (1.3 mM CO_2) did not modify the upregulation of *src* kinases but significantly protected the kinases against peroxynitrite-mediated inhibition. Upregulation of *src* kinase activity by 1 μM peroxynitrite was 3.5–5-fold in erythrocytes and 1.2–2-fold in synaptosomes, but this could be the result, at least in part, of the higher basal level of *src* kinase activity in synaptosomes. Our results indicate that peroxynitrite can upregulate the tyrosine phosphorylation signal through the activation of *src* kinases.

Key words: Peroxynitrite; Nitric oxide; Carbon dioxide; Tyrosine phosphorylation; Red blood cell; Synaptosome; *src* Tyrosine kinase; *syk* Tyrosine kinase

1. Introduction

Peroxynitrite, formed by the near diffusion-limited reaction of nitric oxide[1] ($^{\bullet}NO$) with superoxide ($O_2^{\bullet-}$) [1–3], is a potent oxidant that is believed to contribute to tissue injury in a wide range of human diseases [4]. This oxidant may be formed by cells generating simultaneously $O_2^{\bullet-}$ and $^{\bullet}NO$ as well as in tissues over-expressing the inducible form of nitric oxide synthase, an enzyme producing both radicals during its catalytic process [5]. In biological samples, peroxynitrite[2] reacts preferentially with carbon dioxide and metal-centres of proteins causing anti-oxidant depletion and oxidation/nitration of lipids, proteins and DNA [4,6,7]. In tissues, where CO_2 is 1–2 mM, the formation of the reactive peroxynitrite/CO_2 adduct ($ONOOCO_2^-$) would be expected to drastically reduce the lifetime of peroxynitrite [8] and to inhibit, at least in part, protein oxidations mediated by $ONOO^-$ and ONOOH [9,10]. However, CO_2 modulates but does not abolish peroxynitrite-dependent oxidations. The current proposal is that, in the presence of substrates, a fraction (about 35%) of the short-lived $ONOOCO_2^-$ adduct can engage one-electron oxidation [11] and aromatic nitration [12,13] probably through homolysis to $^{\bullet}NO_2$ and $CO_3^{\bullet}$ radicals [14–16].

One of the major protein modifications induced by peroxynitrite is the oxidation/nitration of cysteines, tyrosines and tryptophan residues [12,13,17–19]. Although nitration of tyrosine residues at the *ortho*-position (3-nitrotyrosine) and the formation of stable *S*-nitrosothiols has been demonstrated in several pathological tissues [20–22], the possible role of these protein modifications has not been fully explored.

Phosphorylation of key tyrosine residues plays a crucial role in signal transduction and it has been proposed that one of the deleterious effects of peroxynitrite-mediated nitration of tyrosine residues may be the downregulation of phosphotyrosine-dependent signalling [23,24]. However, increasing evidence suggests that reactive oxygen and nitrogen species may interfere with intracellular signalling pathways also by upregulating tyrosine phosphorylation [25–29]. We have previously shown that 10–100 μM peroxynitrite upregulated the erythrocyte band 3 tyrosine phosphorylation, while at higher concentrations, tyrosine phosphorylation was downregulated and, simultaneously, substrate nitration and inhibition of phosphotyrosine kinase (PTKase) activity were observed [26]. Although inhibition by peroxynitrite of phosphotyrosine phosphatases may explain the elevated tyrosine phosphorylation of band 3 [26], the hypothesis that peroxynitrite directly increases PTKase activity cannot be ruled out [27].

In this study, we investigated whether peroxynitrite can increase protein tyrosine phosphorylation by activating PTKases. Our results clearly demonstrated a selective activation of *src* PTKases by μM concentrations of peroxynitrite not observed with $p72^{syk}$ (*syk*)[3], another tyrosine kinase of red blood cells. This effect of peroxynitrite may lead to the upregulation of *src*-dependent tyrosine signalling.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (>3000 Ci/mmol) was obtained from DuPont NEN (Boston, MA, USA). Antibodies were obtained from the following sources: anti-*syk*, anti-*lyn*, anti-*hck*, anti-*c-fgr* and anti-*fyn* from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-*c-src* clone 327 from Calbiochem (Cambridge, MA, USA), peroxidase-conjugated anti-mouse and anti-rabbit antibodies from Amersham (Arlington

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Abbreviations: PTKase, phosphotyrosine kinase; DTPA, diethylenetriaminepenta-acetic acid; PMSF, phenylmethanesulfonyl fluoride; MBP, myelin basic protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; IgG_H, immunoglobulin G heavy chain

[1] The IUPAC-recommended name is nitrogen monoxide.

[2] This term refers to both the anion $ONOO^-$ and the different forms of its conjugate acid ONOOH, peroxynitrous acid. The IUPAC-recommended name for the anion is oxoperoxonitrate (1-) and for peroxynitrous acid, it is hydrogen oxoperoxonitrate.

[3] Hereafter in the text, we indicate the kinases as follows: $p72^{syk}$, *syk*; $p60^{src}$, *c-src*; $p53/56^{lyn}$, *lyn*; $p56/59^{hck}$, *hck*; $p55^{c-fgr}$, *c-fgr*; $p59^{fyn}$, *fyn*.

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Heights, IL, USA). Nitrocellulose was obtained from Shleicher and Schuell (Germany), Chelex 100 from Bio-Rad (Richmond, CA, USA) and Immunopure-protein G or Trysacryl-immobilised protein A from Pierce (Rockford, IL, USA). All other reagents were purchased from Sigma Chemical (St. Louis, MO, USA).

2.2. Peroxynitrite preparation

Peroxynitrite was synthesised from sodium nitrite and hydrogen peroxide and was stabilised by alkali as described by Radi et al. [17]. The peroxynitrite preparation was treated with manganese dioxide (1 mg/ml, 30 min, 4°C) to eliminate excess hydrogen peroxide. The mixture was filtered three times to remove the manganese dioxide. When freeze-fractionated (−80°C), peroxynitrite solution forms a yellow top layer, which was retained for further studies. The top layer typically contained 250–500 mM peroxynitrite. The peroxynitrite concentration was determined spectrophotometrically at 302 nm in 1.5 M NaOH ($\varepsilon_{302} = 1670\ M^{-1}\ cm^{-1}$). The contamination of nitrite of our peroxynitrite preparation was 60–70% with respect to peroxynitrite.

2.3. Peroxynitrite treatment of erythrocytes and synaptosomes

Heparinised fresh human blood was obtained from healthy donors following informed consent. After centrifugation for 10 min at $1000\times g$, plasma and buffy coat were removed and red blood cells were washed three times with isotonic phosphate-buffered saline (PBS), pH 7.4. Peroxynitrite anion is a relatively stable species, but its protonated form is unstable and rapidly decomposes at a neutral to acidic pH ($t_{1/2} \leq 1$ s). For this reason, peroxynitrite was added last and as a bolus to erythrocytes suspended (2% hematocrit) in 30 mM NaCl, 80 mM phosphate buffer, 0.1 mM diethylenetriaminepenta-acetic acid (DTPA), pH 7.2, and immediately mixed. After peroxynitrite treatment, the erythrocytes were washed once in PBS. Synaptosomes were prepared from bovine brain as previously described [30]. To avoid metal-catalysed nitration by peroxynitrite [31], the phosphate buffer was treated extensively with Chelex 100 and all samples contained 0.1 mM DTPA. Peroxynitrite was added as a single bolus directly to synaptosomes suspended 1:10 (final concentration 1 mg/ml) in 150 mM phosphate buffer, 0.1 mM DTPA, pH 7.5, and immediately mixed. After 10 min at room temperature, synaptosomes were washed once with the same phosphate buffer. When indicated, sodium bicarbonate was added to the phosphate buffer before the addition of peroxynitrite. The CO_2 level in the erythrocytes and synaptosomes suspension and in phosphate buffers was measured immediately before peroxynitrite addition with an ABL 330 (Radiometer, Copenhagen, Denmark). Decomposed peroxynitrite was obtained by adding peroxynitrite to the phosphate buffer for 5 min at room temperature before the addition of erythrocytes or synaptosomes (reversed order of addition).

2.4. Western blot immunoassays

For Western blot analysis, proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to nitrocellulose paper at 35 V overnight. Blots were then washed with 50 mM Tris, 150 mM NaCl, pH 7.5 (TBS) and incubated overnight with 5% non-fat dry milk in TBS. Washed nitrocellulose filters were incubated overnight at 4°C with the appropriate antibody. After extensive washes in TBS-0.05% Tween 20, the presence of protein on a blot was revealed by chemiluminescence (ECL kit, Amersham) and exposure to X-ray film.

2.5. Immunoprecipitation and immunocomplex kinase assay

Washed and packed erythrocytes (150 μl) were diluted with 300 μl PBS and then lysed by addition of 150 μl of 4×RIPA buffer (100 mM Tris, pH 7.5, 0.6 M NaCl, 4% Triton X-100, 4% Na-deoxycholate, 0.4% SDS, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 4 mM phenylmethanesulfonyl fluoride (PMSF), 400 μM sodium *o*-vanadate, 40 μM phenylarsine oxide). After 10 min of incubation on ice, the lysate was centrifuged at $12000\times g$ for 10 min at 4°C. The supernatant was incubated for 1 h at 4°C in a rotating wheel with 30 μl of Trysacryl-immobilised protein A and clarified by centrifugation. The supernatants were incubated for 3 h at 4°C in a rotating wheel with 20 μl protein A-Trysacryl pre-adsorbed with the appropriate polyclonal antibodies. The immunoprecipitation of *syk* was performed by adding a monoclonal anti-*syk* antibody (overnight at 4°C) and then incubating with the immobilised protein G for 2 h at room temperature in a rotating wheel. Synaptosomes were solubilised by treatment for 10 min at 0°C with an equal volume of 4×RIPA buffer, then diluted three times with PBS and centrifuged at $12000\times g$ for 10 min at 4°C. After clearing, the supernatants were incubated overnight at 4°C in a rotating wheel with the different antibodies. Immunocomplexes were precipitated by addition of 50 μl protein A-Trysacryl. The immunoprecipitate, obtained from erythrocytes or synaptosomes, was collected by centrifugation in a microfuge, washed twice with 1×RIPA buffer, twice with TBS and once with the kinase buffer (20 mM Tris-HCl, pH 7.4, 10 mM $MnCl_2$, 0.1 mM sodium *o*-vanadate). The immunocomplex kinase assay was performed as previously described [30]. When indicated, the kinase assay was performed in the presence of 1 μg myelin basic protein (MBP) and samples were submitted to 15% SDS-PAGE to obtain a better separation between kinases and MBP. The ^{32}P-labelled proteins were quantified by exposure of dried gels to a Phosphorimager instrument (Packard, Camberra, CO, USA).

3. Results

3.1. Peroxynitrite modulates PTKase activities in intact erythrocytes and synaptosomes

The human erythrocyte contains several tyrosine kinases of the *syk/ZAP70* (*syk*) and *src* (*lyn, hck* and *c-fgr*) families whose function is to phosphorylate tyrosine residues of some cellular proteins including the band 3 cytoplasmic domain [32–34]. To test the effects of peroxynitrite on these kinases, intact erythrocytes were treated with 0–1000 μM peroxynitrite and kinases were immunoprecipitated using the appropriate antibody. Kinase activities were evaluated by an immunocomplex kinase assay performed in the presence of sodium *o*-vanadate to avoid the contribution of phosphotyrosine phosphatases.

As shown in lane 1 of Fig. 1A, the *syk* kinase assay of untreated erythrocytes revealed a phosphorylated band at 72 kDa attributed to *syk* autophosphorylation (top panel)

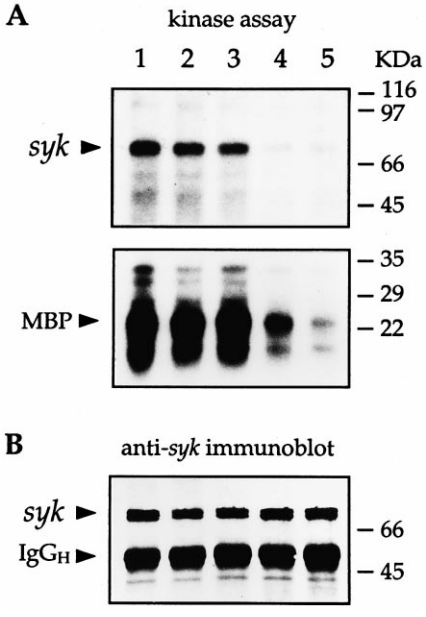

Fig. 1. Peroxynitrite-dependent inhibition of the activity of *syk* kinase. A: Immunocomplex kinase assay of anti-*syk* immunoprecipitates obtained from erythrocytes treated with 0, 30, 100, 500 and 1000 μM peroxynitrite (lanes 1–5, respectively). The catalytic activity of *syk* was measured as autophosphorylation (top panel) or as MBP phosphorylation (bottom panel). Proteins were resolved on 10% (top panel) or 15% (bottom panel) SDS-PAGE. B: Anti-*syk* immunoblot of parallel anti-*syk* immunoprecipitates. The molecular mass markers in kDa are indicated on the right.

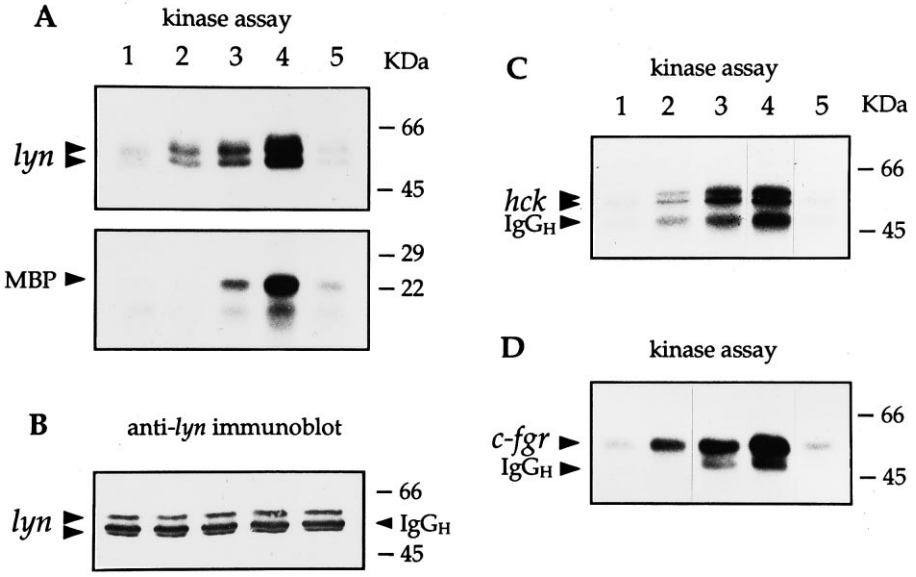

Fig. 2. Peroxynitrite-induced modulation of the activity of *lyn*, *hck* and *c-fgr* kinases. Immunocomplex kinase assay of (A) anti-*lyn* (C) anti-*hck* and (D) anti-*c-fgr* immunoprecipitates obtained from erythrocytes treated with 0, 30, 100, 200 and 500 μM peroxynitrite (lanes 1–5, respectively). The catalytic activity of *lyn* was measured as autophosphorylation (A, top panel) or as MBP phosphorylation (A, bottom panel). Anti-*lyn* immunoblot of the parallel anti-*lyn* immunoprecipitates is shown in (B). The molecular mass markers in kDa are indicated on the right.

and a phosphorylated band at 22 kDa in samples where the exogenous substrate MBP was added (bottom panel). The phosphorylation of *syk* and MBP was slightly decreased by up to 100 μM peroxynitrite and almost completely abolished by 1000 μM (Fig. 1A, lanes 2–5). The identity of the 72 kDa band as *syk* was verified by immunoblotting with anti-*syk* monoclonal antibodies, which also revealed comparable amounts of immunoprecipitated protein in all samples (Fig. 1B). To verify that the inhibitory effect of peroxynitrite was not due to contamination products or due to stable end-products of peroxynitrite decay, peroxynitrite was added to phosphate buffer before the addition of red blood cells. In this experiment, the *syk* autophosphorylation and MBP phosphorylation activity was comparable to that of the control (results not shown).

We next examined whether the treatment of intact erythrocytes with peroxynitrite affected the activity of kinases of the *src* family. Interestingly, the behaviour of these kinases differed from that of *syk*. Both autophosphorylation of the characteristic p53/56 kDa doublet of *lyn* and the phosphorylation of the exogenous substrate MBP were stimulated dose-dependently by treatment with 30–200 μM peroxynitrite (Fig. 2A, lanes 1–4). The maximum activation of *lyn* was achieved at 200 μM peroxynitrite (Fig. 2A, lane 4), while 500 μM peroxynitrite inhibited *lyn* phosphorylation activity (Fig. 2A, lane 5). To detect the amount of *lyn* in the rabbit polyclonal anti-*lyn* immunoprecipitate, we used monoclonal anti-*lyn* antibodies in the immunoblotting because the p53/56 kDa doublet of *lyn* migrated in the gel in the same region as the immunoglobulin G heavy chain (IgG_H) (55 kDa). This minimises cross-reaction between antibodies. As shown in Fig. 2B, peroxynitrite treatment did not change the *lyn* doublet intensity.

The same modulation by peroxynitrite of PTKase activity was also observed for the *hck* and *c-fgr* kinases of red blood cells (Fig. 2C and D, respectively). It should be noted that with this kinase assay, in addition to the autophosphorylation band, a phosphorylated band corresponding to the IgG_H was frequently observed, a finding also reported by others [32]. Thus, if phosphorylated by the kinase, the IgG_H may be seen as an exogenous substrate. Interestingly, phosphorylation of IgG_H by *hck* and *c-fgr* was increased when erythrocytes were treated with peroxynitrite up to 200 μM and decreased at higher concentrations (Fig. 2C and D, lanes 2–5). Immunoblotting analysis of *hck* and *c-fgr* immunoprecipitates revealed that the amount of proteins was identical in all samples (results not shown). Control experiments with peroxynitrite (0.01–1.0 mM) decomposed in phosphate buffer before the

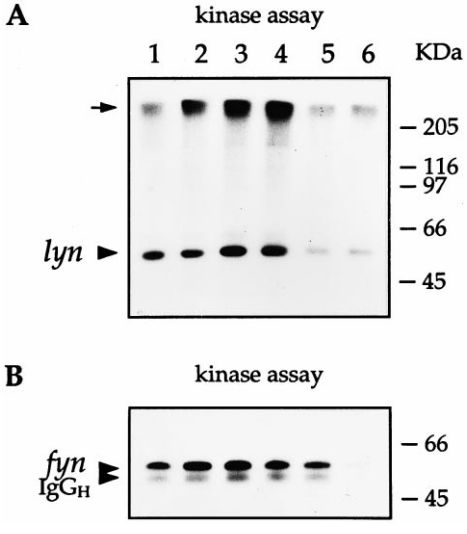

Fig. 3. Peroxynitrite-induced modulation of the activity of neuronal *lyn* and *fyn* kinases. Immunocomplex kinase assay of (A) anti-*lyn* and (B) anti-*fyn* immunoprecipitates obtained from synaptosomes treated with 0, 50, 100, 250, 500 and 1000 μM peroxynitrite (lanes 1–6, respectively). Proteins were resolved on 10% SDS-PAGE. The molecular mass markers in kDa are indicated on the right.

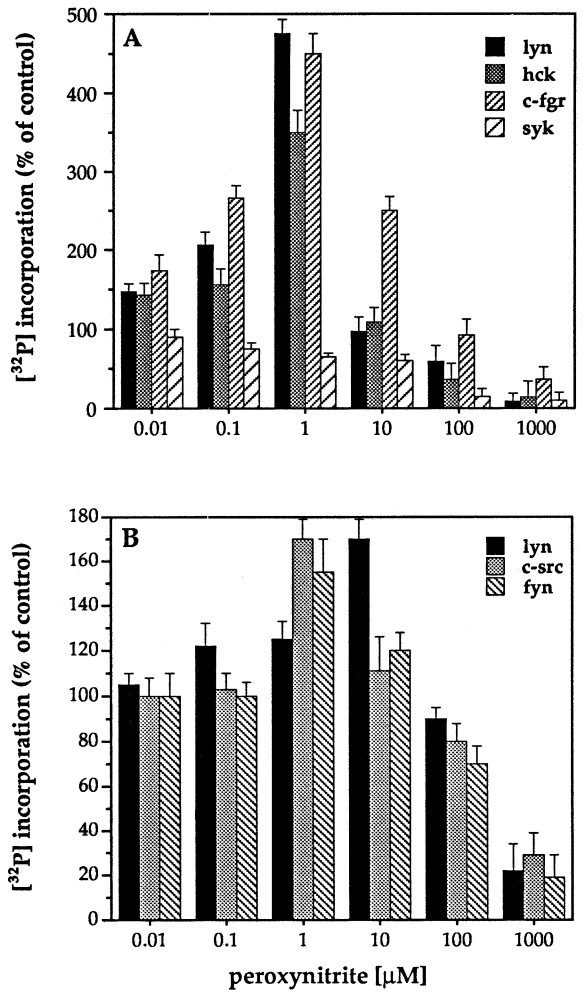

Fig. 4. Effects of peroxynitrite on kinase activity of immunoprecipitates. The kinases from (A) erythrocytes and from (B) synaptosomes were immunoprecipitated with the specific antibody and then treated with 0–1000 μM peroxynitrite in 150 mM phosphate buffer, 0.1 mM DTPA, pH 7.4, at room temperature for 5 min. Proteins were resolved on 10% SDS-PAGE. The extent of ^{32}P incorporation in the kinases was quantified in dried gels using a Phosphorimager instrument and expressed as a percentage of the value in the relative untreated control samples. cpm values of untreated kinases in erythrocytes were: *lyn* 180 ± 85, *hck* 95 ± 40, *c-fgr* 35 ± 22, *syk* 43 ± 20 and in synaptosomes: *lyn* 1130 ± 250, *c-src* 4260 ± 480, *fyn* 4750 ± 360. The data are the mean values ± S.D. from three or four separate experiments.

addition of erythrocytes or treatment of cells with 1 mM nitrite revealed that the modulation of kinases was not due to peroxynitrite decomposition or contaminating products (results not shown).

In a recent study, we observed that the *c-src* kinases of synaptosomes were upregulated by peroxynitrite [30]. This prompted us to investigate whether other kinases of synaptosomes, which contain several kinases of the *src* family, including *lyn* and *fyn*, were upregulated by peroxynitrite. The kinase assay of anti-*lyn* and anti-*fyn* immunoprecipitates obtained from peroxynitrite-treated synaptosomes revealed that the autophosphorylation activity of these kinases was increased by 50–250 μM peroxynitrite and inhibited at higher concentrations (Fig. 3A and B). The extent of *src* kinases upregulation by peroxynitrite in synaptosomes was, however, lower than in erythrocytes (compare Figs. 2 and 3).

It should be noted that only one isoform of the *lyn* kinase was phosphorylated (probably the p53 kDa band). This result agrees with the data reported by Achen et al. [35], who showed that no band corresponding to the phosphorylated p56 *lyn* isoform was observed in several bovine tissues. The absence of the p56 *lyn* band was confirmed by immunoblotting, which did not reveal a *lyn* doublet (results not shown). Moreover, the *lyn* kinase assay showed a peroxynitrite-modulated high molecular weight phosphorylated band at the top of the gel (arrow in Fig. 3A). This band probably was not a *lyn* aggregate because it was not recognised by the anti-*lyn* antibody and thus may be due to co-precipitated high molecular weight protein(s). In contrast to *lyn*, the *fyn* kinase phosphorylated the IgG_H and in addition to the p59 kDa autophosphorylation band of *fyn*, a peroxynitrite-modulated 55 kDa phosphorylated band was found (Fig. 3B). The immunoblotting of anti-*lyn* and anti-*fyn* immunoprecipitates revealed that the amount of both proteins was not changed during peroxynitrite treatment and no effects on tyrosine kinase activities were observed in samples treated with 30–1000 μM decomposed peroxynitrite (results not shown).

3.2. Peroxynitrite modulates immunoprecipitated PTKase activity

The above results suggest that one effect of peroxynitrite is to increase the activity of *src* kinases, but they do not rule out the possibility that peroxynitrite may perform this action through reactions occurring during cell treatment and preceding the kinase immunoprecipitation. To define this question further, we decided to immunoprecipitate the kinases and then treated the immunoprecipitates with 0–1000 μM peroxynitrite.

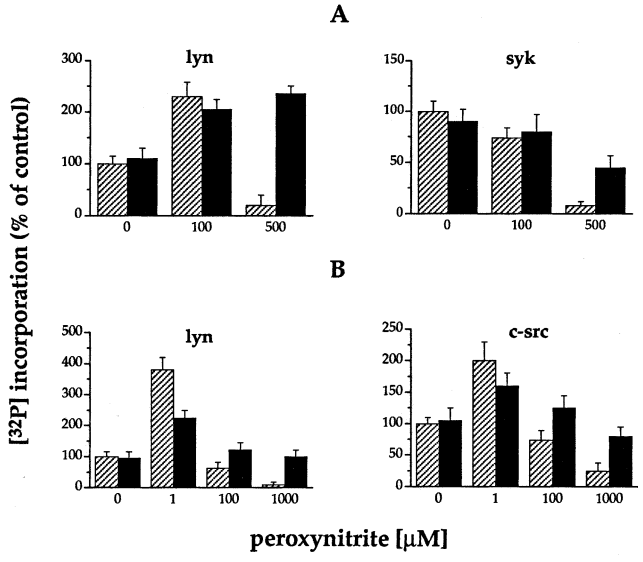

Fig. 5. Effects of CO_2 on peroxynitrite-mediated modulation of kinase activity. (A) immunocomplex assay of kinases (*lyn* and *syk*) precipitated from peroxynitrite-treated erythrocytes. (B) Immunocomplex assay of kinases precipitated from erythrocytes (*lyn*) and synaptosomes (*c-src*) and then treated with peroxynitrite at the concentrations indicated. The hatched and black columns represent the experiments performed in the absence and in the presence of 25 mM bicarbonate, respectively. Data are mean values ± S.D. from three separate experiments. The experimental procedure and cpm values of untreated kinases are as in the legend of Fig. 4.

The immunoprecipitates were submitted to an immunocomplex kinase assay and the extent of ^{32}P incorporation in each kinase was measured after SDS-PAGE separation.

As shown in Fig. 4, peroxynitrite up to a concentration of 1 μM was able to stimulate the autophosphorylation of all *src* kinases obtained either from erythrocytes (*lyn*, *hck* and *c-fgr*) or from synaptosomes (*lyn*, *c-src* and *fyn*). At 10 μM, only the kinase activity of *c-fgr* from erythrocytes and that of *lyn* from synaptosomes was significantly increased. Higher peroxynitrite concentrations induced a dramatic loss of kinase activity. Treatment of the *syk* immunoprecipitate with peroxynitrite only induced a dose-dependent inhibition (Fig. 4A). The autophosphorylation signal in samples treated with decomposed 1–1000 μM peroxynitrite did not differ from that of the control samples.

As observed also in peroxynitrite-treated erythrocytes and synaptosomes, the extent of activation induced by peroxynitrite on the immunoprecipitates was higher in erythrocyte than in neuronal *src* kinases. The difference may be due, at least in part, to a higher basal level of activation of *src* kinases in synaptosomes. This hypothesis was supported by the observation that the basal autophosphorylation activity of *src* kinases was on average 30 times higher (range 6–140) in neuronal than in erythrocyte forms (values in cpm are reported in the legend to Fig. 4). The difference in the extent of *lyn* activation was not due to difference in the amount of immunoprecipitated protein, since densitometric analysis of anti-*lyn* immunoblottings revealed comparable amounts of p53*lyn* from synaptosomes and p53/56*lyn* from erythrocytes (5.0 and 4.8 arbitrary units, respectively).

3.3. Effects of CO_2 on peroxynitrite-mediated modulation of tyrosine kinases

The high concentration of CO_2 in tissues is expected to significantly modify the peroxynitrite reactivity. We have recently reported that the addition of 25 mM bicarbonate to synaptosomes did not inhibit the upregulation of neuronal *c-src* by peroxynitrite, but significantly protected kinases and phosphatases against peroxynitrite inhibition [30]. We investigated the effect of CO_2 in the kinase activity of peroxynitrite-treated erythrocytes and we observed that the addition of 25 mM bicarbonate (1.3 mM CO_2) did not modify the upregulation of *lyn* activity by 100 μM peroxynitrite, but the major effect was a protection against kinase downregulation at higher concentrations (Fig. 5A). CO_2 protected also the peroxynitrite-induced inactivation of *syk* (Fig. 5A).

We further investigated the effect of 25 mM bicarbonate on the modulation by peroxynitrite of the autophosphorylation activity of immunoprecipitated *lyn* and *c-src* (from erythrocytes and synaptosomes, respectively). As shown in Fig. 5B, in the presence of 25 mM bicarbonate, the upregulation of *lyn* and *c-src* induced by 1 μM peroxynitrite was maintained, while the inhibitory effect at 100 and 500 μM peroxynitrite was reduced. The lower protection afforded by CO_2 in these experiments was probably due to the absence of the membrane barrier (see Section 4). However, it should be stressed that a significant concentration of CO_2 was always present in cells and in air-equilibrated buffers even if bicarbonate was not purposely added. The CO_2 measured in red blood cells, synaptosomes and phosphate buffers without addition of bicarbonate was 0.13 ± 0.05, 0.24 ± 0.05 and 0.05 ± 0.02 mM ($n = 4$), respectively. Thus, in experiments performed without addition of bicarbonate, the formation of $ONOOCO_2^-$ adduct cannot be excluded.

4. Discussion

Treatment of cells with peroxynitrite has been reported to induce several processes, including apoptotic cell death [24,36]. However, the biochemical events involved in these processes are still unclear and, since peroxynitrite is a physiological oxidant, these may involve redox signalling pathways. We and others [24–27,29] have shown that one of the effects of peroxynitrite is upregulation of the tyrosine phosphorylation signal. In this study, we have demonstrated that peroxynitrite-induced tyrosine phosphorylation may occur through the activation of some PTKases, in particular some of the *src* family. The activity of *src* kinases was increased in association with tyrosine phosphorylation of exogenous substrates. This effect of peroxynitrite appears to be characteristic of *src*, since the related but structurally different kinase *syk* was not upregulated by peroxynitrite. The activation of *src* kinases by peroxynitrite was not specific to red blood cells but was also observed in *fyn*, *lyn* and *c-src* kinases of synaptosomes. We therefore deduced that the modulating effect of peroxynitrite is probably not tissue-specific.

Results obtained by treatment of immunoprecipitates with peroxynitrite support the hypothesis that this oxidant can activate/inhibit PTKases. The inhibition of PTKases by peroxynitrite may be due to oxidative modification(s) leading to loss of catalytic activity, whereas enzyme activation may be linked to the characteristic and multiple mechanisms of *src* kinase activation. It has been shown, in fact, that both *src* autophosphorylation and the phosphorylation of substrates are increased by a mechanism that probably involves enzyme structural modifications induced by sulfhydryl oxidation [37]. Furthermore, the *src*-type kinases, in contrast to *syk*/*ZAP*, possess a regulatory tyrosine residue (Tyr-527 for *c-src*) whose phosphorylation/dephosphorylation modulates enzyme activity [38]. It is interesting to note that both cysteine and tyrosine residues may be preferential targets of peroxynitrite and thus, modification of one or more of these regulatory residues may well explain the activation/inactivation of kinases. However, it should be stressed that tyrosines, but particularly cysteines, even if not modified directly by an oxidant molecule, may act as a 'sink' for other more easily oxidisable residues [39]. Therefore, we do not rule out that modification of residues critical for the enzymatic activity may be mediated by a 'sink' mechanism. The finding that μM peroxynitrite can affect cysteine- or tyrosine-regulated enzymatic activities is not unprecedented [29,40–44], but whether these effects are due to direct oxidation of cysteine or tyrosine residues or are mediated indirectly by 'sink' reactions requires further investigations.

Notwithstanding the multiple toxic processes attributed to peroxynitrite [4,6,7], this oxidant in biological systems reacts preferentially with a limited number of targets and one of this is carbon dioxide. CO_2 strongly modifies kinetic and toxic properties of peroxynitrite [9,10]. However, the $ONOOCO_2^-$ adduct probably does not fulfil all peroxynitrite reactivity. For example, the rate of peroxynitrite diffusion inside the red blood cell is so fast [45,46] that it travels up to 6 μm and reacts, at least in part, with hemoglobin before reacting extracellularly with CO_2. The $ONOOCO_2^-$ adduct formed extracellularly will decay before crossing the membrane (its

life-time is too short to cross a membrane [8]) and, as a consequence, only the $ONOO^-(H)$ escaped from the extracellular capture or the $ONOOCO_2^-$ formed intracellularly reacts with biological targets. Obviously, peroxynitrite generated inside the cell can react with intracellular targets more easily. However, it should be stressed that crossing of the plasmamembrane by the oxidant molecule is not obligatory for cell signalling, since a stimulus at the membrane surface can induce cytosolic enzyme processes such as thiol redox chemistry [47]. In this work, we found that the upregulation of *src* kinases by μM peroxynitrite was unaffected by the increase of CO_2 from μM up to mM levels, while the inactivation of kinases was protected by mM CO_2. We speculate that the activating effect on *src* kinases may occur also with peroxynitrite generated extracellularly, whereas kinase inactivation probably requires peroxynitrite-kinase closeness and trapping of the oxidant by the enzyme before CO_2. The occurrence of kinase inhibition by peroxynitrite in a CO_2-rich environment, such as in vivo, is a matter of discussion but the resultant inhibition of tyrosine signalling is expected to significantly affect cell functions [24].

Finally, in some cellular processes and in some cell types, the activity of other tyrosine kinases, or even of *src* kinases, may be differently affected by biological oxidants. For example, the *Janus* kinase activity appears to be inhibited by •NO-donors [48] and peroxynitrite induced in epithelial cells the activation of mitogen-activated protein kinases, another important class of tyrosine kinases involved in the cell response to oxidative stress [29]. The effects on *src* kinases described in this study and the relatively low concentration needed for upregulation (μM) suggest that peroxynitrite may regulate *src*-dependent phosphotyrosine signalling, particularly in tissues expressing high levels of the inducible nitric oxide synthase.

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