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H_2O_2 activity on platelet adhesion to fibrinogen and protein tyrosine phosphorylation

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Abstract

Platelets represent a target of reactive oxygen species produced under oxidative stress conditions. Controversial data on the effect of these species on platelet functions have been reported so far. In this study we evaluated the effect of a wide range of H_2O_2 concentrations on platelet adhesion to immobilized fibrinogen and on $pp72^{syk}$ and $pp125^{FAK}$ tyrosine phosphorylation. Our results demonstrate that: (1) H_2O_2 does not affect the adhesion of unstimulated or apyrase-treated platelets to immobilized fibrinogen; (2) H_2O_2 does not affect $pp72^{syk}$ phosphorylation induced by platelet adhesion to fibrinogen-coated dishes; (3) H_2O_2 reduces, in a dose-dependent fashion, $pp125^{FAK}$ phosphorylation of fibrinogen-adherent platelets; (4) concentrations of H_2O_2 near to physiological values (10–12 μM) are able to strengthen the subthreshold activation of $pp125^{FAK}$ induced by epinephrine in apyrase-treated platelets; (5) H_2O_2 doses higher than 0.1 mM inhibit ADP-induced platelet aggregation and dense granule secretion. The ability of H_2O_2 to modulate $pp125^{FAK}$ phosphorylation suggests a role of this molecule in physiological hemostasis as well as in thrombus generation.

Keywords: H_2O_2; Platelet adhesion; Protein phosphorylation; $pp72^{syk}$; $pp125^{FAK}$

1. Introduction

For many years oxygen radicals and other oxygen-derived species have been regarded as highly reactive molecules which indiscriminately destroy biological components. More recently, oxygen-derived species gained attention as essential molecules that regulate cell functions by modulating tyrosine phosphorylation-dependent signal transduction pathways [1–3]. Platelets are a potential target of reactive oxygen species either produced under oxidative stress conditions, such as inflammation or ischemia–reperfusion [4,5], or released by platelets themselves upon appropriate stimulation [6,7]. The relationship between reactive oxygen species and platelet functions has been extensively investigated [6]. However, the question whether these species act as activators or inhibitors of platelet functions remains unresolved, since largely controversial results have been obtained depending on the oxidant doses or the experimental conditions employed. Several studies report that H_2O_2 does not affect or inhibit platelet aggregation [8,9], whereas some authors found that H_2O_2 may enhance platelet aggregation [10,11].

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Despite the large number of studies on the effect of oxidants on the process of platelet aggregation, few data concerning their role in platelet adhesion have been reported [12]. Platelet adherence to matrix proteins, exposed in the damaged vessel wall or in the ruptured atherosclerotic plaque, is, as well as aggregation, a critical step in hemostasis and thrombosis. Both platelet aggregation and adhesion are mediated by $\alpha_{IIb}\beta_3$, the most abundant and well characterized platelet integrin receptor [13,14]. $\alpha_{IIb}\beta_3$ can exist in different activation states which can be distinguished by the difference in their ligand binding properties [15,16]. The $\alpha_{IIb}\beta_3$ form expressed on unstimulated platelets can bind solid-phase fibrinogen, but does not bind the soluble form of fibrinogen. Agonist stimulation of platelets initiates a signal transduction cascade, the so-called 'inside-out' signalling, which leads to $\alpha_{IIb}\beta_3$ conformational changes. This activated form of the integrin is able to bind both circulating fibrinogen and other adhesive proteins. Furthermore, $\alpha_{IIb}\beta_3$ participates in post-ligand 'outside-in' signalling which leads to platelet functional response, that is aggregation or spreading [17,18]. $pp125^{FAK}$ and $pp72^{syk}$ are non-receptor protein tyrosine kinases involved in $\alpha_{IIb}\beta_3$-mediated signalling [19]. $pp125^{FAK}$ is tyrosine phosphorylated and activated upon association with ligand-occupied $\alpha_{IIb}\beta_3$ and may participate in the formation of focal adhesion complexes, which in turn play a key role in modulating cell adhesion and shape change [17,20,21]. However, the ligation of $\alpha_{IIb}\beta_3$ is not sufficient to stimulate $pp125^{FAK}$ phosphorylation, as co-stimulation of other signalling pathways is required [22]. While $pp125^{FAK}$ has a broad pattern of tissue expression, $pp72^{syk}$ is expressed exclusively in cells of hematopoietic lineage. It has been found to be regulated by both agonist and integrin receptor in a pattern distinct from that of $pp125^{FAK}$ [23]. $pp72^{syk}$ has a more proximal functional position than $pp125^{FAK}$ in the 'outside-in' $\alpha_{IIb}\beta_3$-mediated signalling, because integrin ligation is a necessary and sufficient condition to trigger its phosphorylation.

In this study we have evaluated the effect of H_2O_2 on platelet adhesion to immobilized fibrinogen. We have also evaluated whether H_2O_2 modulates the levels of $pp72^{syk}$ and $pp125^{FAK}$ tyrosine phosphorylation promoted by $\alpha_{IIb}\beta_3$ ligation. Since differences in the methodological approaches appear to be responsible for the conflicting results on the activity of H_2O_2 on platelet functions obtained up to now, we tested a wide range of H_2O_2 concentrations and we used different experimental systems. We also studied the effect of H_2O_2 on ADP-induced aggregation and dense granule secretion.

2. Materials and methods

2.1. Antibodies and chemicals

Rabbit polyclonal (LR) and mouse monoclonal (4D10) human $pp72^{syk}$ antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); rabbit polyclonal anti-$pp125^{FAK}$ serum (BC3) and anti-human $pp125^{FAK}$ polyclonal IgG from Upstate Biotechnology (Lake Placid, NY, USA); phosphotyrosine monoclonal antibody (mAb) PT66, horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG from Sigma Chem. Co. (St. Louis, MO, USA); anti-phosphotyrosine mAb PY20 from Transduction Laboratories (Lexington, KY, USA); human plasma fibrinogen (purity $>$ 98%) from Calbiochem (San Diego, CA, USA); Sepharose 2B from Pharmacia Biotech (Uppsala, Sweden); apyrase, H_2O_2, prostaglandin E_1 (PGE_1), ADP, orthovanadate, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, bovine serum albumin (BSA) from Sigma Chem. Co. All other reagents were of the highest quality available.

2.2. Preparation of platelets

Blood specimens were obtained from healthy volunteers who had not taken any drugs for 9 days before bleeding. Platelet-rich plasma (PRP) was prepared from citrated (0.32% w/v) blood by centrifugation at $200\times g$ for 15 min. To prepare washed platelets, PRP, containing 1 U/ml apyrase and 1 μM PGE_1, was further centrifuged at $1200\times g$ for 10 min and platelet pellet was gentle resuspended in HEPES buffer, pH 7.35, containing 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 3 mM NaH_2PO_4, 5 mM glucose, 3.5 mM HEPES and 3.5 g/l BSA. Then, platelets were gel-filtered on a Sepharose 2B column eluted with HEPES buffer at 1 ml/min flow rate [24]. Platelet concentration in the column eluate (washed

platelets) was assessed spectrophotometrically at 520 nm (1 Abs = 1.27×10^8 platelets/ml). Platelet suspensions were diluted with HEPES buffer to adjust the cell count to the appropriate concentration for aggregation and adhesion assays. All procedures were carried out at room temperature.

2.3. Platelet aggregation assay

Platelet aggregation was monitored at 37°C on a single channel aggregometer (Chrono-Log Aggregometer Mod. 500-CA, Haverton, PA, USA) with continuous stirring at 900 rpm. Gel-filtered platelet suspension (2.5×10^8 platelets/ml) was supplemented with 0.5 mg/ml fibrinogen, and with 5 mM Ca^{2+}. The aggregation mixtures (250 μl) were pre-warmed for 1 min at 37°C before the addition of the agonist ADP. The extent of platelet aggregation was expressed as the percentage of the light transmission, taking as 100% the light transmission of the reference cuvette containing the suspending buffer. Changes in light transmission were recorded for at least 4 min after the addition of the agonist. Irreversible platelet aggregation (about 80% of light transmission) was induced by 50 μM ADP. In the inhibition experiments, platelets were pre-incubated with different concentrations of H_2O_2 for 1 min or for otherwise specified time intervals at 37°C before adding the agonist.

2.4. Platelet adhesion assay

Gel-filtered platelets were allowed to adhere to 6 cm diameter plates (Becton Dickinson, NY, USA) or to 96-well polystyrene flat-bottom plates (Costar Co., MA, USA) pre-coated for 16 h at 4°C with 1 ml/plate or 100 μl/well of fibrinogen solution (100 μg/ml). Unbound sites were blocked with 2 ml/plate or 200 μl/well of heat-denatured BSA (5 mg/ml). After 1 h at 37°C BSA was removed and plates were washed three times with PBS. Then, the platelet suspension (3.5×10^8/ml) was loaded on plates (1 ml suspension/plate) or microtiter wells (50 μl suspension/well). Different concentrations of H_2O_2 or an equal volume of HEPES buffer were added to the platelet suspension just before plating. When specified, platelets were pre-treated with apyrase (10 U/ml platelet suspension) for 10 min before plating. Adhesion assays were performed at room temperature. Plates or wells coated only with BSA were used to monitor aspecific adhesion. At the established times, non-adherent platelets were removed and the plates or wells were washed twice with PBS. The number of adherent platelets was measured by evaluating platelet endogenous acid phosphatase [25]. In details, wells were filled with 130 μl of 50 mM sodium citrate buffer, pH 5.5, containing 10 mM *p*-nitrophenylphosphate and 0.1% Triton X-100. After 1 h at 37°C, 10 μl of 1 N NaOH/well was added to stop the reaction and develop the color. The plates were read at 410/630 nm on an automated plate reader (Bio-Rad Microplate Reader, model 450). For detachment assays, platelets were allowed to adhere to fibrinogen for 1 h. At the end of the attachment period, non-adherent platelets were removed and wells were filled with 25 μl of H_2O_2 or the platelet suspending buffer. After a further incubation period (15 or 30 min), attached platelets were counted as described above.

2.5. Immunoprecipitation and Western blotting

Platelets adherent to 6 cm diameter fibrinogen-coated plates were lysed with 600 μl of ice-cold RIPA buffer, containing 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 158 mM NaCl, 10 mM Tris–HCl, pH 7.2, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 100 kallikrein inactivator units/ml aprotinin, and 1 mM sodium orthovanadate. Plates were then taken for 30 min on ice. Platelet lysates were clarified by centrifugation at 14 000 rpm for 10 min in a refrigerated (+4°C) microfuge. The resulting supernatants were characterized for protein concentration by Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). A spectrophotometrically checked BSA solution in RIPA buffer was taken as standard. Lysates were stored at −20°C until use.

Lysates containing equal amounts of proteins were immunoprecipitated with the ant-$pp125^{FAK}$ BC3 antiserum (2 μg/200 μg proteins) or with the anti-$pp72^{syk}$ LR antiserum (2 μl/300 μg proteins) and protein A/G-agarose overnight at 4°C. Beads were sedimented by brief centrifugation and washed extensively with ice-cold RIPA buffer. Proteins, solubilized in boiling SDS sample buffer (2% SDS, 1% β-mercaptoethanol, 66 mM Tris, pH 7.5, 10 mM EDTA),

were separated on a 7.5% or 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The blots were incubated for 1 h at 42°C in blocking solution (5% BSA, 170 mM NaCl, 0.2% Nonidet P-40, 50 mM Tris, pH 7.5), washed with blocking solution without BSA, and probed for 2 h with anti-phosphotyrosine mAb PT66 (1:10 000). The blots were washed three times, incubated for 2 h with horseradish peroxidase-conjugated secondary antibodies (1:3000 in 3% BSA blocking solution) and the immunoreactive bands were visualized by enhanced chemiluminescence kit (Amersham Corp., Buckinghamshire, UK). To monitor loading of gel lanes, blots were stripped by incubation for 30 min at 70°C with a solution containing 2% SDS, 62.5 mM Tris–HCl, pH 6.7, 100 mM β-mercaptoethanol and then re-probed with the anti-human $pp125^{FAK}$ (1:500) or anti-$pp72^{syk}$ (4D10) (1:200) antibodies; the membranes were blocked with 5% not-fat dry milk at 25°C. Quantitation of proteins was performed by densitometry using a Discover Pharmacia scanner equipped with a sun spark classic densitometric workstation.

2.6. Luminescence experiments

Platelets ($2.5–3\times10^8$ platelets/ml) were pre-incubated for 3 min with the ATP-detecting system luciferin-luciferase (Chrono-lume reagent, Chrono-Log) before adding ADP and/or H_2O_2. ATP secretion was assessed by arbitrarily setting at 0% the luminescence of resting platelets and at 100% that of ADP-stimulated platelets. Chemiluminescence tracings were followed simultaneously with the aggregation ones.

2.7. Cytotoxicity assay

Toxicity of H_2O_2 was evaluated by measuring the extent of lactate dehydrogenase (LDH) released by washed platelets (3×10^8 platelets/ml) after their exposure for 1 h to increasing doses of H_2O_2 (up to 1 mM). Control platelets and H_2O_2-treated platelets were collected by centrifugation at 11 000 rpm for 10 s. LDH activity was expressed as the amount of NADH/min formed after the addition of pyruvate and NAD^+ to supernatants of samples and controls [26]. Total LDH content was measured after platelet lysis by sonication.

3. Results

3.1. H_2O_2 does not affect platelet adhesion to immobilized fibrinogen

The adhesion of platelets to immobilized fibrinogen occurs without the need for any stimulation induced by agonists [16]. Unstimulated platelets have been shown to adhere to fibrinogen-coated plates also in the presence of apyrase, which inactivates the endogenous agonist ADP released from dense granules [22]. Apyrase-treated platelets represent a useful tool to study the effect of H_2O_2 on platelet $\alpha_{IIb}\beta_3$-mediated adhesion, since it allows one to avoid any interfering agonist-induced conformational change of the integrin receptor.

Both control and apyrase-treated platelets were exposed to increasing doses of H_2O_2 during the whole time of adhesion to fibrinogen-coated plates. The concentration of H_2O_2 dilutions, which were prepared daily, was assessed spectrophotometrically using the following extinction coefficient: $\varepsilon_{\lambda254}=43.6$ M^{-1} cm^{-1}. The concentration of H_2O_2 did not significantly change during the adhesion experiments as measured by evaluating the absorption spectrum of H_2O_2 solutions taken on a plate for 1 h: it was qualitatively and quantitatively comparable to that obtained with the same solution before the incubation. The doses of H_2O_2 employed (0.01–1 mM) were not toxic for platelets. In fact, the amount of LDH released in the suspending buffer by platelets exposed for 1 h to H_2O_2 concentrations up to 1 mM was comparable to that produced by control platelets incubated for the same time interval (<18 nmol NADH/3×10^8 platelets/min). The amount of total releasable LDH was 936 ± 41 nmol NADH/3×10^8 platelets/min.

Under these experimental conditions, we found that the presence of H_2O_2 did not affect the number of platelets adhering to fibrinogen-coated dishes. This result was independent of the adhesion time and was observed in the whole range of tested H_2O_2 doses (Table 1). The same results were obtained with apyrase-treated platelets adhering to fibrinogen for 30 min, whereas only a slight decrease (about 20%) of the number of adherent platelets was observed when apyrase-treated platelets were exposed to 1 mM H_2O_2 for 60 min (Table 1).

Table 1
Platelet adhesion to fibrinogen-coated wells in the absence and in the presence of different H_2O_2 concentrations

H_2O_2 concentration (mM)	Adhesion time (min)	Number of adherent platelets ($\times 10^6$)	
		−apyrase	+apyrase
None	30	1.92 ± 0.25	1.76 ± 0.19
	60	2.50 ± 0.36	2.20 ± 0.31
0.01 mM	30	2.09 ± 0.22	1.78 ± 0.23
	60	2.36 ± 0.35	2.17 ± 0.23
0.1 mM	30	1.82 ± 0.26	1.72 ± 0.18
	60	2.52 ± 0.40	2.25 ± 0.36
1 mM	30	1.98 ± 0.29	1.45* ± 0.17
	60	2.38 ± 0.39	1.77* ± 0.19

Gel-filtered control and apyrase-pretreated platelets (3.5×10^8 platelets/ml) were allowed to adhere to 96-well plates for 30 and 60 min either in the absence or in the presence of different amounts of H_2O_2. Non-adherent platelets were removed and the number of adherent platelets was measured by evaluating platelet endogenous phosphatase as described in Section 2. Data reported are the mean values calculated on 10 measures for each of three different experiments.
*$P < 0.05$ as compared with control by Student's two-tailed t-test.

The lack of anti-adhesive activity of H_2O_2 was confirmed by detachment experiments. Control and apyrase-treated platelets were allowed to adhere to fibrinogen for 1 h and then exposed to H_2O_2 or to the suspending buffer for a further 15 or 30 min. At the end of the incubation period, no differences in the number of adherent platelets were observed between samples treated with H_2O_2 doses up to 1 mM or the buffer alone (data not shown).

3.2. H_2O_2 does not affect pp72^{syk} phosphorylation, whereas it modulates pp125^{FAK} phosphorylation in fibrinogen-adherent platelets

The binding of $\alpha_{IIb}\beta_3$ to solid-phase fibrinogen promotes an increase in tyrosine phosphorylation of several proteins [27]. The ability of H_2O_2 to modulate the transduction events induced by integrin ligation was evaluated by measuring the effect of H_2O_2 on the levels of phosphorylation of pp72^{syk} and pp125^{FAK}, two tyrosine kinases activated downstream of $\alpha_{IIb}\beta_3$ engagement [19]. Platelets were allowed to adhere to fibrinogen for different time intervals in the presence of a wide range of H_2O_2 doses (0.01–1 mM). Non-adherent platelets exposed to BSA-coated surfaces were used as controls. Equal amounts of detergent-extracted proteins were then subjected to immunoprecipitation and Western blotting experiments.

Firstly we analyzed the effect of H_2O_2 on the phosphorylation of pp72^{syk}, which is activated in an early phase of integrin signalling. The levels of pp72^{syk} phosphorylation were unchanged after the exposure of platelets to all tested H_2O_2 concentrations as compared to the levels of untreated platelets. Fig. 1A

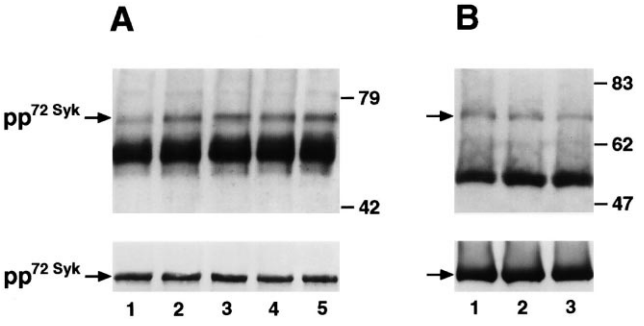

Fig. 1. H_2O_2 activity on pp72^{syk} phosphorylation. Fibrinogen-adherent platelets were lysed and immunoprecipitated with anti-pp72^{syk} antibody (LR). The immunoprecipitates were subjected to 7.5% (A) or 10% (B) SDS electrophoresis. After Western blotting, tyrosine-phosphorylated proteins were visualized by mAb PT66 (A and B, upper blots). Data reported in A and B have been obtained in two separate experiments, each performed at least twice. (A) Control platelets adhering to fibrinogen for 60 min in the absence (lane 2) and in the presence of 0.01 mM H_2O_2 (lane 3), 0.1 mM H_2O_2 (lane 4) and 1 mM H_2O_2 (lane 5). The immunoprecipitate from platelets adhering to BSA-coated plates is in lane 1. (B) Platelets adhering to fibrinogen for 30 min in the absence (lane 1) and in the presence of 0.1 mM H_2O_2 (lane 2) and 1 mM H_2O_2 (lane 3). Positions of molecular markers (in kDa) and pp72^{syk} (arrows) are indicated. The amount of pp72^{syk} loaded was equal in all lanes as was verified on parallel immunoblots probed with the anti-pp72^{syk} antibody (4D10) (A and B, lower blots).

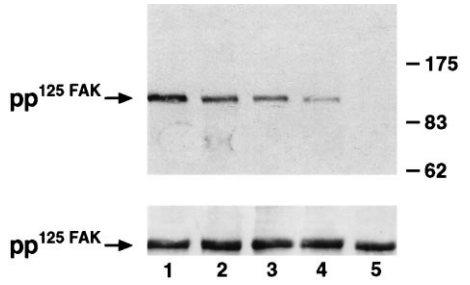

Fig. 2. Dose-dependent inhibition of pp125^{FAK} phosphorylation by H_2O_2. Unstimulated platelets adhering to fibrinogen in the absence (lane 1) and in the presence of 0.1 mM H_2O_2 (lane 2), 0.5 mM H_2O_2 (lane 3) and 1 mM H_2O_2 (lane 4) for 60 min were lysed and immunoprecipitated with anti-pp125^{FAK} antibody (BC3). The immunoprecipitate from platelets adhering to BSA-coated plates is in lane 5. The immunoprecipitates were subjected to 7.5% SDS electrophoresis. After Western blotting, tyrosine-phosphorylated proteins were visualized by mAb PT66 (upper blot). Positions of molecular markers (in kDa) and pp125^{FAK} (arrows) are indicated. The amount of pp125^{FAK} loaded was equal in all lanes as was verified on parallel immunoblots probed with the polyclonal anti-human pp125^{FAK} antibody (lower blot). Data here reported have been confirmed in three separate experiments of equal design.

(upper blot) reports the results obtained with platelets adhering to fibrinogen for 60 min in the absence and in the presence of 0.01, 0.1 and 1 mM H_2O_2. pp72^{syk} phosphorylation also occurred, though to a lesser extent, in platelets attached to BSA-coated plates (Fig. 1A, lane 1). H_2O_2 was ineffective in modulating pp72^{syk} phosphorylation also in experiments performed on platelets adhering to fibrinogen for a time shorter than 60 min. This finding is summarized in Fig. 1B (upper blot), where are reported the levels of pp72^{syk} phosphorylation measured in platelets adhering to fibrinogen for 30 min in the absence and in the presence of 0.1 mM and 1 mM H_2O_2.

Then we evaluated the effect of H_2O_2 on pp125^{FAK} phosphorylation. The levels of pp125^{FAK} phosphorylation measured in platelets adhering to fibrinogen for 60 min were reduced by H_2O_2. The inhibitory effect was detectable at H_2O_2 doses higher than 0.1 mM and was dose-dependent (Fig. 2, upper blot). H_2O_2 concentrations ranging from 0.01 mM to 0.05 mM failed to affect pp125^{FAK} phosphorylation (data not shown). No pp125^{FAK} phosphorylation was observed in BSA-attached platelets (Fig. 2, lane 5). Further experiments were performed to evaluate the effect of H_2O_2 on pp125^{FAK} phosphorylation at adhesion times shorter than 60 min. In agreement with our previous observations [28], pp125^{FAK} phosphorylation increased as a function of the time of platelet adhesion to immobilized fibrinogen, being 1.5-fold higher at 60 min than at 30 min (data not shown). The inhibitory potency of H_2O_2 on pp125^{FAK} activation was independent of the adhesion time. In fact, the ratio between the intensity of the phosphorylated pp125^{FAK} signals obtained with platelets exposed to 1 mM H_2O_2 and that from control platelets was about 0.20 in either 30 or 60 min adhesion experiments.

The results of the experiments described above demonstrate a dose-dependent inhibitory activity of H_2O_2 on one or more of the signal transduction events responsible for pp125^{FAK} phosphorylation. However, using a different experimental model, where pp125^{FAK} phosphorylation is inhibited, we found that H_2O_2 can also induce a 'co-stimulus' for these signal transduction events. pp125^{FAK} phosphorylation is abolished by platelet pre-treatment with apyrase [22]. Apyrase inactivates ADP and thus inhibits the ADP-dependent transduction signals which, co-operating with those triggered by the occupied integrin, lead to pp125^{FAK} activation. However, the addition of 1 mM of the weak agonist epinephrine to apyrase-treated platelets completely restores pp125^{FAK} phosphorylation in this experimental model [22]. We used apyrase-treated platelets to evaluate whether H_2O_2, like epinephrine, might be able to re-establish pp125^{FAK} phosphorylation by activating the same transduction pathways blocked by apyrase treatment or by stimulating alternative ones. Apyrase-treated platelets were allowed to adhere to fibrinogen-coated plates for 60 min in the absence or in the presence of different amounts of H_2O_2 and lysates were subjected to immunoprecipitation and Western blotting experiments. While epinephrine addition to apyrase-treated platelets promoted a strong pp125^{FAK} phosphorylation, H_2O_2 failed to reverse the inhibitory effect of apyrase, since no pp125^{FAK} phosphorylation was detectable in apyrase-treated platelets exposed to H_2O_2 doses ranging from millimolar to micromolar concentrations (Fig. 3, upper blot).

It has been reported that H_2O_2 can act synergistically with agonists in activating platelet response [10]. To test this possibility in our system, apyrase-treated platelets were exposed simultaneously to a

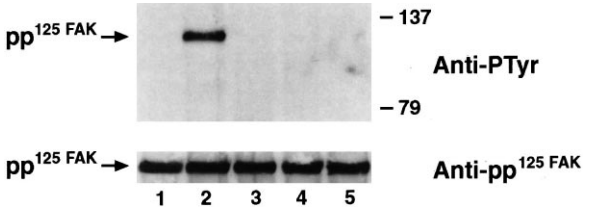

Fig. 3. Effect of H_2O_2 on tyrosine phosphorylation of pp125^{FAK} in apyrase-treated platelets. Apyrase-treated platelets were allowed to adhere for 60 min to fibrinogen-coated plates in the absence and in the presence of different stimuli. After the adhesion period, platelets were lysed and immunoprecipitated with anti-pp125^{FAK} antibody (BC3). The immunoprecipitates were subjected to 7.5% SDS electrophoresis. After Western blotting, tyrosine phosphorylated proteins were visualized by mAb PT66 (upper blot). Lane 1, platelets alone; lane 2, plus 1 mM epinephrine; lane 3, plus 0.01 mM H_2O_2; plus 0.1 mM H_2O_2; lane 4, plus 1 mM H_2O_2. Positions of molecular markers (in kDa) and pp125^{FAK} (arrow) are indicated. The amount of pp125^{FAK} loaded was equal in all lanes as was verified on a parallel immunoblot probed with the antibody against pp125^{FAK} (lower blot). Data reported in this figure have been confirmed in three separate experiments of equal design.

subthreshold dose (0.05 μM) of epinephrine, i.e. a dose unable to completely re-establish pp125^{FAK} phosphorylation, and to different concentrations of H_2O_2 (in the range 1 μM–1 mM). Under these experimental conditions, we found that only H_2O_2 doses near to physiological values (10–20 μM) were able to restore pp125^{FAK} phosphorylation (Fig. 4, upper blot). No pp125^{FAK} phosphorylation was detected in apyrase-treated platelets exposed to 0.05 μM epinephrine and H_2O_2 concentrations either lower than 5 μM or higher than 0.05 mM (data not shown). The lack of the co-agonist activity of H_2O_2 at doses higher than 0.05 mM may be ascribed to an overlapping of inhibitory and stimulatory effects of H_2O_2 on platelet signalling.

3.3. H_2O_2 inhibits platelet aggregation and dense granule secretion

Gel-filtered platelets were pre-incubated for 1 min at 37°C with H_2O_2 before the addition of ADP. No changes in light transmission were recorded during this pre-incubation period, indicating that, under our experimental conditions, H_2O_2 does not show any pro-aggregating activity. Platelet exposure to H_2O_2 doses higher than 0.1 mM reduced the extent of ADP-induced aggregation, whereas lower concentrations did not affect the process. The inhibitory effect of H_2O_2 was dose-dependent and was more pronounced on the second wave than on the first (Fig. 5). In another set of experiments we found that the inhibitory potency of H_2O_2 increased with increasing pre-incubation time with platelets. When platelets were exposed for 1, 3 and 5 min to a fixed dose of H_2O_2 (0.5 mM), the extent of aggregation, measured 2 min after the agonist addition, was reduced by 31, 48 and 62%, respectively.

Since the lack of the second wave of aggregation is indicative of an impairment of the late events of the aggregation process, such as secretion, we evaluated whether H_2O_2 affects this process. The ATP release, in response to platelet stimulation, was evaluated with the luciferin-luciferase system and was followed simultaneously with aggregation. In agreement with data previously reported [11], we found that doses of H_2O_2 which inhibited platelet aggregation also inhibited the nucleotide release. In particular, 0.5 and 1 mM H_2O_2 caused a 39% and 67%, respectively, decrease of the amount of ATP released 2 min after the start of aggregation.

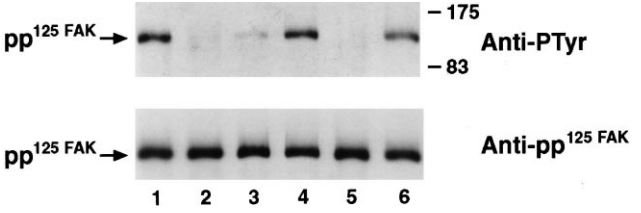

Fig. 4. Induction of pp125^{FAK} tyrosine phosphorylation by H_2O_2 and subthreshold amounts of epinephrine in apyrase-treated platelets. Apyrase-treated platelets were allowed to adhere for 60 min to immobilized fibrinogen in the absence and in the presence of different stimuli. Platelet lysates were immunoprecipitated with anti-pp125^{FAK} antibody (BC3) and the immunoprecipitates were subjected to 7.5% SDS electrophoresis. After Western blotting, tyrosine-phosphorylated proteins were visualized by mAb PT66 (upper blot). Lane 1, platelets plus 1 mM epinephrine; lane 2, plus 0.01 mM H_2O_2; lane 3, plus 0.05 μM epinephrine; lane 4, plus 0.05 μM epinephrine and 0.01 mM H_2O_2; lane 5, plus 0.02 mM H_2O_2; lane 6, plus 0.05 μM epinephrine and 0.02 mM H_2O_2. Positions of molecular markers (in kDa) and pp125^{FAK} (arrows) are indicated. The amount of pp125^{FAK} loaded was equal in all lanes as was verified on a parallel immunoblot probed with the antibody against pp125^{FAK} (lower blot). Data reported in this figure have been confirmed in three separate experiments.

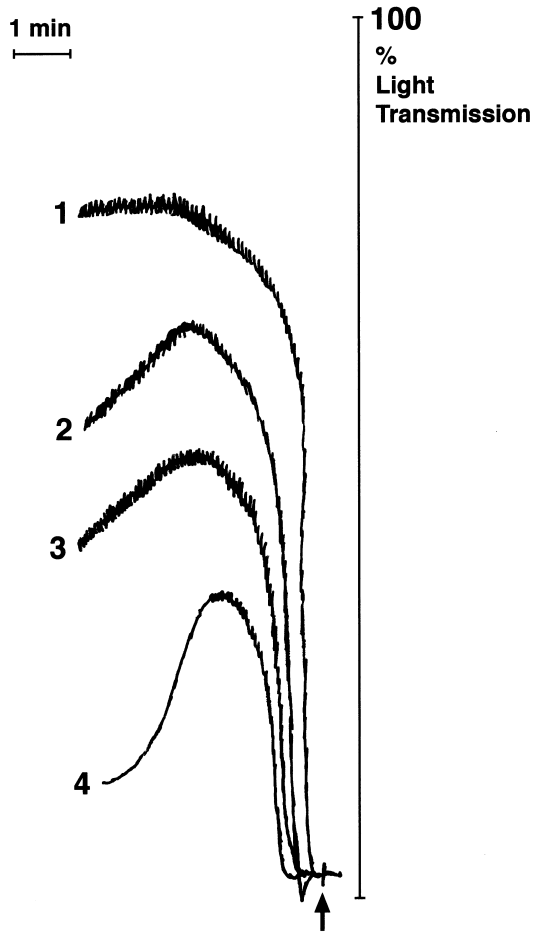

Fig. 5. Inhibition of platelet aggregation by H_2O_2. Platelets were preincubated at 37°C for 1 min with H_2O_2 before the addition of 50 μM ADP (arrow). Aggregation tracings: 1, control platelets; 2, plus 0.3 mM H_2O_2; 3, plus 0.5 mM H_2O_2; 4, plus 1 mM H_2O_2. Chart speed was 1 cm/min. Data reported are from one experiment representative of three experiments performed in duplicate.

4. Discussion

A growing body of evidence demonstrates that oxygen reactive species may act as fundamental signalling molecules in many cellular processes [1–3]. H_2O_2 has been shown to stimulate tyrosine and or serine/threonine protein phosphorylation [29,30], to induce an increase of the intracellular level of Ca^{2+} [31] and to act as a second messenger [32]. In this study we evaluated whether H_2O_2 interferes with the capability of platelets to bind immobilized fibrinogen and with the subsequent post-ligand transduction events, such as $pp72^{syk}$ and $pp125^{FAK}$ phosphorylation.

Fibrinogen in vivo can be immobilized on the surface of activated platelets and also deposited within the vessel wall at the site of vascular injury [33]. $\alpha_{IIb}\beta_3$ is the functional integrin receptor which mediates selective and irreversible platelet adhesion to solid-phase fibrinogen. Under our experimental conditions, a large range of H_2O_2 concentrations does not affect the extent of platelet adhesion to fibrinogen-coated plates (Table 1). The binding of $\alpha_{IIb}\beta_3$ to immobilized fibrinogen does not require agonist-induced activation of the integrin, but it depends only upon the integrity of the complementary recognition sequences present in the molecules of these two proteins [16]. Thus, the lack of any effect of H_2O_2 on the $\alpha_{IIb}\beta_3$-fibrinogen recognition and binding indicates that H_2O_2, at a molecular level, does not cause any structural modifications of either fibrinogen or its receptor. However, a prolonged exposure of platelets to a high dose of H_2O_2 (1 mM) causes a slight decrease of apyrase-treated platelet attachment to immobilized fibrinogen. This result could be ascribed to structural modifications of fibrinogen induced by the oxidative conditions. In a previous study we showed that oxidative modifications reduce the fibrinogen capability to support the adhesion of unstimulated platelets [34].

While integrin-immobilized fibrinogen binding occurs in the absence of any stimulus, being a simple protein–protein interaction, several post-ligand transduction events are required for full platelet activation and spreading. Millimolar concentrations of H_2O_2 inhibit, at least in part, protein tyrosine phosphorylation triggered by $\alpha_{IIb}\beta_3$ engagement. In particular, our results demonstrate that H_2O_2 does not affect the tyrosine phosphorylation of the early activated protein $pp72^{syk}$ (Fig. 1), but it reduces the extent of phosphorylation of $pp125^{FAK}$ (Fig. 2), which is phosphorylated in a later phase of the occupied integrin-induced signalling. These findings confirm that $pp72^{syk}$ and $pp125^{FAK}$ do not depend on each other for their activation and that the signalling pathways required for $pp125^{FAK}$ phosphorylation differ from those needed for $pp72^{syk}$ phosphorylation [23]. A stimulatory activity of H_2O_2 on $pp72^{syk}$ phosphorylation has been previously described [35–38]. However, in these studies the tested doses of H_2O_2 were higher than those used by us [35,37]; further, H_2O_2

was tested in combination with vanadate, a phosphatase inhibitor [36,38].

pp72^{syk} is phosphorylated on tyrosine immediately after $\alpha_{IIb}\beta_3$ ligation to immobilized fibrinogen, whereas co-ordinate signalling induced by the occupied integrin and by endogenous agonists, released during adhesion, must converge within the cell to lead to pp125^{FAK} phosphorylation [22]. H_2O_2 does not show any effect on either $\alpha_{IIb}\beta_3$-fibrinogen binding or the early transduction signals following integrin ligation. Thus, H_2O_2 might reduce the extent of pp125^{FAK} phosphorylation by inhibiting the additional signalling pathways elicited by the ADP binding to its receptor. Actually the inhibition of the post-receptorial cascade might depend on the capability of H_2O_2 to interfere with one or both of the following sequential events: (i) endogenous ADP release reaction; (ii) binding of ADP to its heptahelical receptor.

The results obtained from aggregation and luminescence experiments may be of help to establish the contribution of these two mechanisms to the H_2O_2 inhibitory action. We found that H_2O_2 doses which are effective in reducing pp125^{FAK} phosphorylation are also able to inhibit both aggregation and dense granule secretion in ADP-stimulated platelets (Fig. 5). We used the aggregation system, instead of the adhesion assay, to evaluate the capability of H_2O_2 to impair the release reaction, since it has been reported that in the medium of fibrinogen-adherent platelets there are no detectable amounts of agonist released from dense granules [27]. Aggregation tracings (Fig. 5) show that the inhibitory effect of H_2O_2 is more pronounced on the second wave of the process and that the inhibitory potency of H_2O_2 increases as a function of its pre-incubation time with platelets. These experimental data suggest that H_2O_2 interferes mainly with post-receptorial aggregation events. However, a reduction of ADP receptor binding affinity might also occur as a consequence of H_2O_2 platelet treatment. This supports previous observations by Ohyashiki et al., demonstrating a reduced binding for ADP in platelets exposed to H_2O_2 [9].

Even though most studies demonstrate the ability of H_2O_2 to positively modulate protein phosphorylation in several cellular systems [29,35–38], our data show an inhibitory rather than a stimulatory action of H_2O_2 on this process. However, using a restrictive experimental model in which pp125^{FAK} phosphorylation is completely abolished, we were able to demonstrate a co-stimulatory activity of H_2O_2 on transduction signals involved in pp125^{FAK} phosphorylation. Platelets treated with apyrase, which blocks signals downstream of ADP receptor binding, do not show any pp125^{FAK} phosphorylation [22]. The addition to the apyrase-treated platelet system of agonists able to re-activate these signals, or alternative ones, restores pp125^{FAK} phosphorylation. The weak agonist epinephrine is able to re-establish pp125^{FAK} phosphorylation [22] and the activation of arachidonic acid metabolism has been proposed as the mechanism by which it overcomes the consequences of endogenous ADP inactivation [39]. Several lines of evidence suggest that H_2O_2 can also stimulate arachidonic acid metabolism either by activating phospholipase A2 [40] or by promoting the synthesis of arachidonic acid metabolites, such as thromboxane [11]. Furthermore, H_2O_2 has been shown to activate protein kinase C [41], which plays a central role in the transduction pathway responsible for pp125^{FAK} phosphorylation [42]. Despite the potential agonist-mimetic properties, H_2O_2 fails to restore pp125^{FAK} phosphorylation when added alone to apyrase-treated platelets (Fig. 3). However, phosphorylated pp125^{FAK} can be detected when platelets are exposed simultaneously to H_2O_2 doses near to physiological values and to a subthreshold dose of epinephrine (Fig. 4). This property of H_2O_2 to act as a co-agonist by strengthening the effect of subthreshold concentrations of epinephrine has been already reported in a previous study, where H_2O_2 was able to trigger the aggregation of platelets primed with subthreshold doses of exogenous agonists [43].

The data reported here highlight a dose-dependent discrepancy between inhibitory and stimulatory activity of H_2O_2 on signalling events triggered by platelet adhesion to fibrinogen. The inhibitory effect of H_2O_2 on signals required for pp125^{FAK} phosphorylation does not appear to be of physiological relevance as it occurs at concentrations of the chemical far from those occurring in vivo. Previously a close association has been found between leukocytes and platelets during thrombus generation [4]. Interestingly, the property of H_2O_2 to act synergically with subthreshold doses of epinephrine in inducing pp125^{FAK} phosphorylation is observed with amounts

of H_2O_2 near to those released in vivo by activated leukocytes (10–12 μM) [44]. The initial platelet contact with immobilized fibrinogen, which in vivo can be localized at the site of vascular lesions by absorption from plasma and/or by formation of insoluble fibrin, raises a cascade of transduction signals which lead to platelet spreading. Since this structural and functional modification of platelets is strictly associated with $pp125^{FAK}$ phosphorylation [22], our results on the capability of H_2O_2 to modulate the phosphorylation of this protein suggest that H_2O_2 might play a role in physiological hemostasis and/or in thrombus generation.

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