Nitric Oxide Inhibits Thrombin Receptor-activating Peptide-induced Phosphoinositide 3-Kinase Activity in Human Platelets*

Alessio Pigazzi‡, Stanley Heydrick‡‡, Franco Foll‡§, Stephen Benoit**, Alan Michelson**, and Joseph Loscalzo‡ ‡‡

From the ‡Whitaker Cardiovascular Institute and Evans Department of Medicine, Boston University School of Medicine, Boston, Massachusetts 02118, the **Department of Pediatrics, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and ‡Unit for Metabolic Diseases, Istituto Raffaele, via Olgettina 60, Milano, Italy

Although nitric oxide (NO) has potent antiplatelet actions, the signaling pathways affected by NO in the platelet are poorly understood. Since NO can induce platelet disaggregation and phosphoinositide 3-kinase (PI3-kinase) activation renders aggregation irreversible, we tested the hypothesis that NO exerts its antiplatelet effects at least in part by inhibiting PI3-kinase. The results demonstrate that the NO donor S-nitrosoglutathione (S-NO-glutathione) inhibits the stimulation of PI3-kinase associated with tyrosine-phosphorylated proteins and of p85/PI3-kinase associated with the SRC family kinase member LYN following the exposure of platelets to thrombin receptor-activating peptide. The activation of LYN-associated PI3-kinase was unrelated to changes in the amount of PI3-kinase physically associated with LYN signaling complexes but did require the activation of LYN and other tyrosine kinases. The cyclic GMP-dependent kinase activator 8-bromo-cyclic GMP had similar effects on PI3-kinase activity, consistent with a model in which the cyclic nucleotide mediates the effects of NO. Additional studies showed that wortmannin and S-NO-glutathione have additive inhibitory effects on thrombin receptor-activating peptide-induced platelet aggregation and the surface expression of platelet activation markers. These data provide evidence of a distinct and novel mechanism for the inhibitory effects of NO on platelet function.

By inhibiting platelet function, nitric oxide (NO) is believed to play a role in regulating thrombosis and hemostasis. However, the signaling pathways underlying the inhibitory action of NO on the platelet are poorly understood. One signaling event that appears to be critical to thrombin-induced platelet activation is the stimulation of phosphoinositide 3-kinase (PI3-kinase). Thus, the fungal metabolite wortmannin, an irreversible inhibitor of PI3-kinase, mimics some of the physiological and biochemical effects of NO on the platelet, such as inhibition of thrombin receptor-activating peptide (TRAP)-induced platelet aggregation and the surface expression of activated integrin glycoprotein (GP) IIb-IIIa, the principal fibrinogen receptor (1).

PI3-kinase represents a family of ubiquitous enzymes involved in the regulation of mitogenesis, vesicular trafficking, glucose transport, cytoskeletal rearrangements, and other cellular functions. PI3-kinase catalyzes the phosphorylation of the inositol ring at the D3 position in a variety of phosphoinositide substrates forming 3-phosphorylated phosphoinositides (2, 3); some isoforms can also catalyze the serine phosphorylation of themselves and other proteins (4, 5).

The p85/PI3-kinase, the first to be described in the platelet, is a heterodimer composed of a p85 regulatory subunit containing two SRC homology (SH) 2 domains and one SH3 domain, and a catalytic p110 subunit. Its “classical” mechanism of activation, originally described as the mechanism by which it is stimulated in lymphocytes by large T antigen and activation of receptor tyrosine kinases, involves the interaction of one or both SH2 domains with phosphotyrosine moieties on other proteins (6–8). The p85/PI3-kinase is also activated by a number of G protein-linked receptors, including the thrombin receptor in platelets (9, 10). This type of activation may involve either a non-receptor tyrosine-phosphorylated intermediate (11) or the recently described direct activation of some isoforms by G protein βγ subunits (12, 13). A second class of PI3-kinase, PI3-kinase-γ, has also been recently identified in platelets. It appears to consist of unique p110 catalytic and p101 regulatory subunits (14–17) and is activated only by G protein βγ subunits.

Exposure of platelets to thrombin or the thrombin receptor-activating peptide (TRAP) results in the activation and translocation of one or both types of PI3-kinase to the cytoskeleton at sites of integrin-dependent focal adhesions, where the enzyme is thought to have an important function in the cytoskeletal reorganization that accompanies irreversible platelet aggregation and clot retraction (1, 18–19). More recent studies indicate that the p85/PI3-kinase, rather than the PI3-kinase-γ, is involved in the activation of the integrin GPIIb-IIIa in platelets (17). We have previously shown that nitric oxide donors can inhibit GPIIb-IIIa activation and fibrinogen binding (20, 21). Accordingly, we investigated the effects of NO on components of the p85/PI3-kinase pathway in an effort to identify a new mechanism by which NO can inhibit platelet function.

EXPERIMENTAL PROCEDURES

* This work was supported in part by National Institutes of Health Grants HL48976, HL53919, HL55993, and HL48743 and by a Merit Review Award from the Dept. of Veterans Affairs. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Supported by grants from H. S. Raffaele, Milano, Italy and Ministero della Sanita’, Rome, Italy.
‡‡ To whom correspondence should be addressed: Boston University School of Medicine, Whitaker Cardiovascular Institute, 700 Albany St., Boston, MA 02118. Tel.: 617-638-4890; Fax: 617-638-4066; E-mail: jloscalzo@bu.edu.

The abbreviations used are: NO, nitric oxide; PI3-kinase, phosphoinositide 3-kinase; S-NO-glutathione, S-nitrosoglutathione; TRAP, thrombin receptor-activating peptide; FTC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; ANOVA, analysis of variance; PI, phosphatidylinositol; GP, glycoprotein; SH, SRC homology.
fluoride, benzamidine, 8-Brcyclic GMP, glutathione, and all other common reagents were obtained from Sigma. Sodium orthovanadate was purchased from Aldrich. TRAP was obtained from Bachem (King of Prussia, PA). Protein A-Sepharose was purchased from Amersham Pharmacia Biotech. [γ-32P]ATP was obtained from NEN Life Science Products. Lysophophatididylinositol was purchased from Avanti Polar Technology (Alabaster, AL). Enhanced chemiluminescence peroxidase detection kits were obtained from Amersham Pharmacia Biotech or Pierce.

Antibodies—Polyclonal antibodies against LYN and p85 and monoclonal antiphosphotyrosine antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-LYN antibody was obtained from Transduction Laboratories (Milwaukee, WI). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody PAC1 was purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA). Phycoerythrin-conjugated monoclonal antibody P2 was purchased from Immunotech (Westbrook, ME). Biotinylated monoclonal antibody S12 was obtained from Centocor (Malvern, PA). FITC-conjugated monoclonal antibody Y2/51 was purchased from Dako Corp. (Carpinteria, CA). Monoclonal antibody raised against GLUT4 (clone IF8) was a gift from Dr. Paul Pilch of Boston University.

Platelet Preparation—Using standard phlebotomy techniques, human blood was obtained from healthy volunteers who had not taken any antiplatelet medication for at least 10 days. The blood was centrifuged (150 g, 11 min, 22 °C), and the upper one-half to two-thirds of the platelet-rich plasma supernatant was collected for experimentation. The remaining blood was further centrifuged at 3,000 g for 10 min; the platelet-rich plasma was passed over a Sepharose 2B column, as described previously (22). Platelet counts were determined using a Coulter counter (Coulter Electronics, Miami, FL).

Immunoprecipitation and Western Blotting—Gel-filtered platelets or platelet-rich plasma were kept at room temperature for 30 min prior to any experimental manipulation; subsequently, platelets were counted, and the number of cells in each tube was adjusted with platelet-poor plasma or buffer as necessary. Platelets were stimulated under stirring conditions in an agglomerator for the indicated times. Aggregation was stopped by adding 2× ice-cold lysis buffer containing 2% Triton X-100, 20% glycerol, 50 mM HEPES, pH 7.4, 150 mM NaCl, 4 mM EDTA, 10 mM sodium pyrophosphate, 100 mM NaF, 0.1 mg/ml aprotinin, 1 mg/ml leupeptin, 0.05 mg/ml phenylmethylsulfonyl fluoride, 10 mM benzamidine, and 2 mM sodium orthovanadate. The tubes were kept on ice for 15 min and mixed in a rotor for 10 min at 4 °C, the Triton-soluble fraction was then subjected to immunoprecipitation by adding 20 µl of the third washing buffer containing 50 µg/ml histone type III-S (Sigma) and [γ-32P]ATP (6,000 mCi/mmol) to the bead-bound immunoprecipitates. The beads were washed as described for the protein kinase assay. The substrate for PI3-kinase, liver phosphatidylinositol (PI), was prepared as follows. Twenty micrograms per ml of PI in chloroform were dissolved under a gentle stream of argon. The lipids were then resuspended in 10 µl of 10 mM Tris, pH 7.5, and 1 mM EDTA. This PI mix was added to each tube together with 50 µl of the third washing buffer described above and 10 µl of 100 mM MgCl₂, 0.88 mM ATP, and 2 µCi of [γ-32P]ATP. Incubations were carried out for 10 min and stopped with the addition of 20 µl of 8 M HCl and 160 µl of chloroform/methanol (1:1). The tubes were then centrifuged to separate the aqueous and organic phases, and the lipid products were resolved by thin layer chromatography with a running solvent composed of 120 ml of chloroform, 94 ml of methanol, 23 ml of water, and 4 ml of ammonium hydroxide. The radioactive PI3-P product was visualized by autoradiography and quantitated by Cerenkov counting.

Flow Cytometry—Two-color flow cytometric analysis was used to determine the activation of platelet surface glycoprotein IIb-IIIa (the fibrinogen receptor integrin II₃b₃α) or the platelet surface expression of P-selectin. Platelet-rich plasma was incubated with buffer or 100 mM S-NO glutathione for 30 min or 10 µM S-NO-glutathione for 10 min followed by S-NO-glutathione and wortmannin. Subsequently, 15 µl of platelet-rich plasma diluted 1:20 in modified HEPES-Tyrode’s buffer, pH 7.4 (HT), were incubated, in the presence of appropriate antibody combinations, with 2.5 µg glycyl-l-prolyl-l-arginyl-l-proline (Calbiochem) to prevent fibrin polymerization, and either thrombin or TRAP at the concentrations specified in the figure legends. FITC-conjugated monoclonal antibody PAC1 was used to measure activation of the GPIIb-IIIa complex, with the CD41-specific monoclonal antibody P2 conjugated to phycoerythrin used as the specific platelet label. Platelet surface P-selectin exposure was measured by biotin-conjugated monoclonal monoclonal antibody S12, with the CD61-specific monoclonal antibody Y2/51-FITC used as the specific platelet label. Incubations were carried out for 10 min. After adding 30 µg/ml phycoerythrin-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) to samples containing S12-biotin, samples were fixed with 1% formaldehyde for 20 min at room temperature and diluted with 9 volumes of HT. PAC1-FITC samples, in contrast, were fixed directly and diluted with 9 volumes of HT.

Flow cytometry was performed in an EPICS Profile II flow cytometer (Coulter, Miami, FL). FITC and phycoerythrin fluorescence were determined with 525- and 575-nm band pass filters, respectively. Platelets were identified by gating on their characteristic light scatter and positive FITC fluorescence for P-selectin analysis and phycoerythrin fluorescence for GPIIb-IIIa analysis, as described previously (25); subsequently, platelets were analyzed for binding of either the biotinylated S12 or FITC-conjugated PAC1 antibodies. P-selectin is expressed only after platelet degranulation, and PAC1 recognizes only the fibrinogenbinding site in activated GPIIb-IIIa complexes and, therefore, in contrast to P2 and Y2/51, does not bind to resting platelets. Background binding values were obtained by analyzing platelets stained with appropriate nonspecific isotype controls.

Statistical Analysis—Data are expressed as mean ± S.E. Data were one- or two-way analysis of variance as appropriate. The results of an analysis were considered significant when p values were <0.05.

RESULTS

Inhibition of TRAP-stimulated p85/P13-Kinase Activity in Platelets by NO—The p85/P13-kinase is stimulated primarily when its SH2 domains bind phosphotyrosine moieties on other proteins (6–8). To determine if NO has an effect on P13-kinase activation, we therefore examined the effect of the NO donor S-nitrosglutathione (S-NO-glutathione, 10 µM) on antiphosphotyrosine-immunoprecipitable P13-kinase activity. Control platelets and platelets pretreated with S-NO-glutathione were read as...
incubated for 1 min with TRAP, and lipid kinase assays were carried out on antiphosphotyrosine immunoprecipitates from Triton X-100-soluble extracts. Although PI3-kinase has been reported to translocate into the cytoskeletal fraction after TRAP stimulation, this process required longer than 1 min (19), an observation that was confirmed in control studies (data not shown). As shown in Fig. 1A (left panel), TRAP significantly stimulated phosphotyrosine-associated PI3-kinase activity in the soluble fraction compared with that in unactivated platelets (CON), and this effect was blocked by preincubation of platelets with S-NO-glutathione (TRAP + S-NO).

To identify specific tyrosine kinases involved in the thrombin-induced activation of platelet p85/PI3-kinase, we carried out lipid kinase assays on immunoprecipitates of the SRC family kinase LYN. Following platelet activation, LYN is activated and associates with several signaling molecules that are known to be present in complexes with activated PI3-kinase, including focal adhesion kinase (26), the SRC family kinase SYK (11, 26), and several membrane glycoproteins involved in outside-in signaling, including GPIIb-IIIa (27–29). Moreover, the binding of the SH3 domain of LYN to purified PI3-kinase leads to a severalfold increase in its specific activity (30). Fig. 1A (right panel) shows a 4-fold increase in LYN-associated PI3-kinase activity after TRAP stimulation compared with unactivated platelets (CON), which was significantly attenuated by preincubation with S-NO-glutathione (TRAP + S-NO). Similar inhibitory effects were noted using a different NO donor, diethyleniminonoate (data not shown). Thus, at least one PI3-kinase target subpopulation for the inhibitory effect of NO is the LYN-associated PI3-kinase pool.

To determine if the effect of NO on phosphotyrosine and LYN-associated PI3-kinase was due to nonspecific inhibition of total PI3-kinase, total PI3-kinase activity was assayed in p85 regulatory subunit immunoprecipitates from TRAP-stimulated platelets that had been previously incubated with or without S-NO-glutathione. In contrast to the results in phosphotyrosine and LYN immunoprecipitates, total PI3-kinase activity decreased by 38% upon exposure to TRAP and, more importantly, S-NO-glutathione had no effect on p85 activity, either by itself (S-NO) or in combination with TRAP (TRAP + S-NO) (Fig. 1B). Since the activity of the PI3-kinase that commumunoprecipitated with LYN was between 0.7 and 3.1% of the p85-immunoprecipitable activity, and since both the p85 and LYN immunoprecipitations were essentially quantitative (see Fig. 3B), the results in Fig. 1 are consistent with the hypothesis that TRAP selectively activates and NO inhibits only a small subpopulation of the p85/PI3-kinase enzyme, including that in LYN signaling complexes. Although it is not known what other signaling complexes that contain PI3-kinase are affected by S-NO-glutathione, it should be noted that SRC itself, as a prototypical SRC kinase family member, does not associate with measurable PI3-kinase activity in immunoprecipitates under any condition (data not shown).

Association of p85 and LYN Is Not Altered by NO—Fig. 2 shows an anti-p85 Western blot that demonstrates the presence of p85 in LYN immunoprecipitates from resting platelets (CON) and platelets preincubated with buffer (TRAP) or S-NO-glutathione (TRAP + S-NO) for 10 min and then stimulated with TRAP. Densitometry showed that the amount of PI3-kinase associated with LYN remains constant regardless of agonist exposure or preincubation with S-NO-glutathione, indicating that neither thrombin nor NO affects the physical association of the two enzymes. Similar results were obtained in Western analyses of LYN-associated PI3-kinase in neutrophils.2

The data in Fig. 1 suggest that LYN-associated PI3-kinase accounts for only a fraction of the total PI3-kinase pool. To confirm this hypothesis, LYN and p85 Western blots were carried out on platelet extracts before and after LYN was removed by immunoprecipitation. The result (Fig. 3A) indi-

---

2 G. M. Bokoch, personal communication.
Gel-filtered platelets were preincubated with or without 10 pM pool.

Platelets stimulated with 10 μM TRAP for 1 min were subjected to immunoprecipitations with anti-LYN monoclonal antibody, and TRAP-stimulated and unstimulated platelets were conducted to assess the relative size of the pool of LYN immunoprecipitates. Thus, in at least some of the signal-competing complexes containing PI3-kinase and LYN, the two are likely to be bridged by c-CBL.

**Effect of Nitric Oxide on LYN-associated PI3-Kinase Involves cGMP-dependent Protein Kinase**—In order to test the hypothesis that the effect of NO on LYN-associated PI3-kinase is mediated by cGMP-dependent protein kinase, platelets were preincubated with the cGMP analog 8-bromo-cyclic GMP (8-Br-cGMP) for 30 min, and PI3-kinase activity was measured in LYN immunoprecipitates. Fig. 5 shows that 8-Br-cGMP inhibited the stimulation by TRAP of LYN-associated PI3-kinase activity to a similar extent as S-NO-glutathione, indicating that the NO effect was mediated, at least in part, by cGMP-dependent protein kinase.

**Modulation of LYN Kinase Activity by TRAP and NO**—The most direct mechanism by which TRAP may stimulate LYN-associated PI3-kinase activity is that LYN becomes activated by upstream signaling elements and then itself activates PI3-kinase. To confirm that LYN, indeed, becomes activated, we assessed LYN kinase activity using an in vitro histone H1 phosphorylation assay. As shown in Fig. 6A, the phosphorylation of histone H1 increased 2-fold in TRAP-stimulated platelets (TRAP) as compared with unstimulated platelets (CON), an effect that was blocked by S-NO-glutathione (TRAP + S-NO). Thus, LYN is activated by TRAP, and this activation is blocked by S-NO-glutathione.

In the process of obtaining LYN kinase activity measurements, we observed that a number of proteins in the LYN immunoprecipitates were phosphorylated in vitro. Fig. 6B is an autoradiogram showing the results of in vitro protein kinase assays in LYN immunoprecipitates from resting platelets (CON) and platelets stimulated with TRAP after a 10-min preincubation with buffer (TRAP) or S-NO-glutathione (TRAP + S-NO). Interestingly, p53/p56 LYN was less phosphorylated in TRAP-stimulated than in control platelets, whereas S-NO-glutathione partially blocked the effect of TRAP. These effects were not due to changes in the quantity of LYN protein in the immunoprecipitate, as shown in Fig. 6C. In contrast, TRAP stimulation increased the phosphorylation of a 70-kDa protein running just above LYN that was completely blocked by S-NO-glutathione preincubation. The identity of this protein remains likely to be bridged by c-CBL.

**Effect of Nitric Oxide on LYN-associated PI3-Kinase Involves cGMP-dependent Protein Kinase**—In order to test the hypothesis that the effect of NO on LYN-associated PI3-kinase is mediated by cGMP-dependent protein kinase, platelets were preincubated with the cGMP analog 8-bromo-cyclic GMP (8-Br-cGMP) for 30 min, and PI3-kinase activity was measured in LYN immunoprecipitates. Fig. 5 shows that 8-Br-cGMP inhibited the stimulation by TRAP of LYN-associated PI3-kinase activity to a similar extent as S-NO-glutathione, indicating that the NO effect was mediated, at least in part, by cGMP-dependent protein kinase.

**Modulation of LYN Kinase Activity by TRAP and NO**—The most direct mechanism by which TRAP may stimulate LYN-associated PI3-kinase activity is that LYN becomes activated by upstream signaling elements and then itself activates PI3-kinase. To confirm that LYN, indeed, becomes activated, we assessed LYN kinase activity using an in vitro histone H1 phosphorylation assay. As shown in Fig. 6A, the phosphorylation of histone H1 increased 2-fold in TRAP-stimulated platelets (TRAP) as compared with unstimulated platelets (CON), an effect that was blocked by S-NO-glutathione (TRAP + S-NO). Thus, LYN is activated by TRAP, and this activation is blocked by S-NO-glutathione.

In the process of obtaining LYN kinase activity measurements, we observed that a number of proteins in the LYN immunoprecipitates were phosphorylated in vitro. Fig. 6B is an autoradiogram showing the results of in vitro protein kinase assays in LYN immunoprecipitates from resting platelets (CON) and platelets stimulated with TRAP after a 10-min preincubation with buffer (TRAP) or S-NO-glutathione (TRAP + S-NO). Interestingly, p53/p56 LYN was less phosphorylated in TRAP-stimulated than in control platelets, whereas S-NO-glutathione partially blocked the effect of TRAP. These effects were not due to changes in the quantity of LYN protein in the immunoprecipitate, as shown in Fig. 6C. In contrast, TRAP stimulation increased the phosphorylation of a 70-kDa protein running just above LYN that was completely blocked by S-NO-glutathione preincubation.
ANOVA.

compared with TRAP-stimulated platelets; each by repeated measures

A, proteins in LYN immunoprecipitates.

m platelets preincubated with 10

GMP for 30 min prior to stimulation with TRAP as in Fig. 1. Resting

mulations (as in Fig. 1. The figure shows the average results from three experi-

iments (as in Fig. 1. The figure shows the average results from three experi-

periments were performed

TRAP stimulated LYN-associated PI3-kinase 2–3-fold. Genis-

is required for the activation of PI3-kinase in LYN signaling

plexes, platelets were preincubated for 30 min with the
tyrosine kinase inhibitor genistein (5 μM) or the SRC family kinase-selective inhibitor PP2, and PI3-kinase activity was measured in LYN immunoprecipitates. As shown in Fig. 7, TRAP stimulated LYN-associated PI3-kinase 2–3-fold. Genis-

tein had a small stimulatory effect (60%) on LYN-associated PI3-kinase when added alone but completely blocked its ac-
tivation by TRAP (Fig. 7A). Similarly, Fig. 7B demonstrates that PP2 blunted the stimulation of TRAP of PI3-kinase, while having no significant effect of its own. The data indicate that

ivation of an SRC family kinase, most likely LYN, is neces-

ary for the stimulation of LYN-associated PI3-kinase.

Effects of NO and Wortmannin on Platelet Aggregation and Surface Glycoprotein Expression—Since cGMP-dependent pro-

tein kinase activation is known to inactivate the protein kinase 
C (PKC)/inositol trisphosphate pathway, PI3-kinase is not likely to be the only site at which NO donors act to inhibit platelet activation (20). For this reason, we evaluated the antiplatelet effects of wortmannin, a relatively selective PI3-kinase inhibitor (at concentrations <100 nM), and S-NO-glutathione, used alone and in combination. We reasoned that if the S-NO-glutathione inhibited platelet aggregation principally through its effect on PI3-kinase, then the two treatments would not be additive. The inhibitory dose responses for S-NO-gluta-

thione and wortmannin were first determined (wortmannin, 0.1 nM to 1 μM; S-NO-glutathione, 0.1 nM to 10 μM). Fig. 8 shows that wortmannin inhibited TRAP-induced aggregation by over 80% at 100 nM and that its IC50 was 10 nM. For S-NO-glutathione, there was nearly complete inhibition of aggregation by concentrations as low as 1 μM, and its IC50 was 120 nM. We found an additive response when platelets were preincubated in the presence of 10 nM wortmannin and 10 nM S-NO-glutathi-
mechanisms underlying the antiplatelet activity of NO. While

one. In addition, there was also a trend toward synergy between 1 nM wortmannin and 10 nM S-NO-glutathione. Taken together, these results suggest that while both NO and wortmannin inhibit PI3-kinase associated with LYN and other tyrosine-phosphorylated proteins, they also act at unique sites, as well.

These functional inhibitory effects were confirmed by examining the molecular inhibitory effects of S-NO-glutathione and wortmannin on the surface expression of active GPIIb-IIIa and P-selectin, both important markers of platelet activation (25). Flow cytometric analysis shown in Fig. 9 demonstrates that both S-NO-glutathione and wortmannin significantly prevented the TRAP-induced surface expression of P-selectin and of the active conformation of GPIIb-IIIa, and that the effect of both added together was greater than either alone.

DISCUSSION

The major finding of this study is that NO inhibits the thrombin-induced activation of the p85/PI3-kinase pathway in platelets. Owing to the important role played by PI3-kinase in the aggregation response, this effect is likely to be one of the mechanisms underlying the antiplatelet activity of NO. While suppression of PI3-kinase activity has been shown to inhibit the production of NO in certain tissues (32), this is the first report of an effect of NO on PI3-kinase.

Although the activation of PI3-kinase in LYN signaling complexes has been demonstrated in neutrophils and other cell types, its role in platelet activation has not previously been established. In neutrophils, LYN has been thought to act as a “link” between the G protein-coupled chemoattractant receptors and the Ras/mitogen-activated protein kinase and PI3-kinase pathways (9). Since the effectors and adaptors involved in thrombin-induced p85/PI3-kinase activation in the platelet are largely unknown, it is logical to propose a similar role for LYN in signal propagation from the G protein-coupled thrombin receptor. In vitro analyses have demonstrated that PI3-kinase is stimulated when it associates with the SH3 domain of LYN (30) or (potentially) when its SH2 domains interact with phosphotyrosine moieties on c-CBL (31). Our data indicate that at least in some signaling complexes, the latter mechanism may be active. c-CBL was present in LYN signaling complexes, and since the quantity of the p85 subunit in LYN signaling complexes does not change with TRAP and/or NO exposure, it is highly likely that the increase in PI3-kinase activity results from direct activation of the enzyme.

One consistent and surprising finding was that TRAP induced a slight decrease in p85-immunoprecipitable (i.e. total) PI3-kinase activity despite stimulating the much smaller LYN-immunoprecipitable pool. Studies in other cell types have concluded that only a relatively small fraction of the p85/PI3-kinase is activated by some agonists; thus, the activation of PI3-kinase in LYN and other positive signaling complexes may not be detectable in the total p85 immunoprecipitate. Although the mechanism by which TRAP decreases total activity is not known, it is possible that one or more downstream pathways in the platelet activation cascade may induce the serine phos-
phorylation of some p85 subpopulations, leading to their inactivation (4).

Protein kinase assays (Fig. 6) suggest that TRAP differentially regulates the phosphorylation of LYN and other proteins in LYN immunoprecipitates and that NO antagonizes these effects. The changes in LYN phosphorylation were not due to measurable changes in LYN protein in the cytosol because its levels remain constant, with or without S-NO-glutathione, in both the cytoplasm and cytoskeleton (data not shown). TRAP exposure led to a decrease in the in vitro phosphorylation of p53/p6 LYN compared with resting platelets, while dramatically increasing the phosphorylation of an as yet unidentified 70-kDa protein. Thus, these data suggest that there must be either additional TRAP-regulatable kinases or at least one TRAP-regulatable phosphatase in LYN immunoprecipitates. These findings are consistent with the model first proposed by Sotirellis and colleagues (33) in which LYN phosphorylated at the inhibitory C-terminal tyrosine (Tyr-508) is inactive, whereas dephosphorylation at this site by an as yet unidentified phosphatase in combination with the dissociation of the C-terminal kinase CHK from the LYN signaling complex (34) leads to its activation. Further work will be required to prove that this model applies to the platelet. By whatever mechanism LYN signaling complexes are activated, the observation that NO pretreatment blocks both the positive and negative effects of TRAP on protein phosphorylation indicates that NO coordinately inhibits all of the TRAP-induced signals in the platelet that converge on LYN.

Once activated, LYN may stimulate PI3-kinase by way of the interaction between its SH3 domain and the proline-rich region of p85 or via interactions between the SH2 domains of p85 and phosphotyrosyl moieties on a third protein. Recent work has demonstrated a strong interaction between LYN and c-CBL using the yeast two-hybrid system (31) leading to a model in which LYN tyrosine phosphorylates c-CBL, which in turn activates PI3-kinase via the SH2 domains of p85. As alluded to above, this model is supported by our data. Fig. 7 clearly demonstrates that tyrosine kinase activation is necessary for the stimulation of Lyn-associated PI3-kinase in platelets, whereas Fig. 4 shows that c-BCL is present in LYN signaling complexes. However, these data do not exclude the possibility that PI3-kinase is activated by way of an interaction with the p70 protein, which is markedly phosphorylated with TRAP stimulation and may itself be a LYN substrate. She is another candidate substrate, since it has been shown to become tyrosine-phosphorylated in LYN-PI3-kinase signaling complexes in N-formyl peptide-stimulated neutrophils (9). Whatever the precise molecular mechanism, NO’s inhibition of TRAP-induced PI3-kinase may be explained by the ability of NO to maintain p53/p6 LYN in a phosphorylation state similar to that observed in unstimulated platelets.

The importance of PI3-kinase in platelet function has only recently been recognized. PI3-kinase is believed to maintain the thrombin-induced activation of GPIb-IIIa and, in turn, to mediate in signaling events initiated by GPIb-IIIa ligand binding, such as filopodial extension (1, 35, 36). It, thus, acts both in the “inside-out” and “outside-in” signaling pathways across this integrin receptor. The fact that the inhibitory effects of NO occur during the 1st min of TRAP stimulation indicates that NO affects the early signals of the thrombin receptor, i.e., in inside-out signaling occurring prior to completion of the GPIb-IIIa-dependent translocation of PI3-kinase to the cytoskeleton (19).

The data demonstrating the additive inhibition of platelet aggregation, P-selectin expression, and active glycoprotein IIb-IIIa expression by NO and wortmannin are consistent with earlier reports showing the individual inhibitory effects of the two compounds (1, 21). The best studied effect of nitric oxide is the stimulation of soluble guanylyl cyclase, responsible for the production of cGMP and, thus, activation of cGMP-dependent protein kinase. One effect of this serine/threonine kinase is an inhibition of the PKC/inositol phosphate pathway, as evidenced by a blunted rise in intracellular calcium and an inactivation of classical and novel protein kinase C (20, 37–40), both of which lead to an inhibition of platelet aggregation. It is becoming increasingly apparent, however, that the PI3-kinase pathway and some PKC signaling pathways can overlap. For example, the 3-phosphorylated phosphoinositide products of PI3-kinase are able to activate a number of novel and atypical PKCs (41, 42). Moreover, the phorbol ester-induced phosphorylation of pleckstrin, generally considered to be a PKC-dependent event, is wortmannin-sensitive (43). Thus, there is evidence that PI3-kinase can evoke effects and responses both upstream and downstream of the PKC family taken as a whole. However, in the present study, the additive of wortmannin and S-NO-glutathione in inhibiting platelet aggregation indicates that NO and cyclic GMP-dependent protein kinase must exert their inhibitory actions at multiple sites in the platelet, not exclusively via PI3-kinase. Teleologically, such redundancy would serve to ensure that NO or NO donors effectively inhibit platelet activation regardless of the pathway of activation. The identification of other NO-modulated pathways will, therefore, be of great importance in deciphering the overall antiplatelet and antithrombotic effects of NO.

Acknowledgments—We thank C. R. Kahn, Joslin Diabetes Center, Boston, for help and discussion on preliminary experiments. We also thank Stephanie Tribuna and Michael Hollywood for expert technical assistance.

REFERENCES

1. Kovacsiovics, T., Bachelot, C., Toker, A., Vlahos, C., Duckworth, B., Cantley, L. C., and Hartwig, J. (1995) J. Biol. Chem. 270, 11358–11366

2. Toker, A., and Cantley, L. C. (1997) Nature 387, 673–676

3. Quon, M., Chen, H., Ing, B., Liu, M., Zarnowski, M., Yonezawa, K., Kasuga, M., Cushman, S., and Taylor, S. (1995) Mol. Cell. Biol. 15, 5409–5411

4. Carpenter, C. L., Auger, R. K., Duckworth, B., Hou, W. M., Schaffhausen, B., and Cantley, L. C. (1993) Mol. Cell. Biol. 13, 1657–1665

5. Lam, K., Carpenter, C., Ruderman, N., Friel, J., and Kelly, K. (1994) J. Biol. Chem. 269, 20648–20656

6. Carpenter, C. L., Auger, R. K., Chanudthuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S., and Cantley, L. C. (1993) J. Biol. Chem. 268, 9478–9483

7. Meng, M. G., Jr., Becher, J. M., Xie, Y. J., Shoemaker, S., Ho, P., Schlessinger, J., Yoakim, M., Schaffhausen, B., and White, M. F. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10350–10354

8. Hu, P., Margolis, B., Skolnik, E. Y., Lammers, R., Ulrich, A., and Schlessinger, J. (1992) Mol. Cell. Biol. 12, 10350–10354

9. Ptasznik, A., Traynor-Kaplan, A., and Bokoch, G. (1995) J. Biol. Chem. 270, 19693–19697

10. Kocera, G., and Rittenhouse, S. (1990) J. Biol. Chem. 265, 5354–5358

11. Yanagi, S., Sada, Y., Tohyma, Y., Tsubokawa, M., Nagai, K., Yonezawa, K., and Yamamura, H. (1994) Eur. J. Biochem. 224, 329–333

12. Kocsis, H., Maeshima, T., Okada, T., Yamamoto, T., Hoshio, S., Fukui, Y., Uji, M., Hazeki, O., and Katada, T. (1997) J. Biol. Chem. 272, 24252–24256

13. Hazeki, O., Okada, T., Kuros, H., Takasuga, S., Suzuki, T., and Katada, T. (1998) Life Sci. 62, 1555–1559

14. Stephano, L. R., Eguinoa, A., Erdjument-Bromage, H., Lui, M., Cooke, F., Coadwell, J., Smrcka, A. S., Thelen, M., Caldwellwar, K., Tempst, P., and Hawkins, P. T. (1997) Cell 89, 105–114

15. Tang, X., and Downes, C. (1995) J. Biol. Chem. 270, 14193–14199

16. Thomason, T., James, S., Casey, P., and Downes, C. (1994) J. Biol. Chem. 269, 16525–16529

17. Zhang, J., Zhang, J., Shattil, S., Cunningham, M., and Rittenhouse, S. (1996) J. Biol. Chem. 271, 6265–6272

18. Zhang, J., Zhang, J., Fry, J., Waterfield, M., Jakens, S., Liao, L., Fox, J., and Rittenhouse, S. (1992) J. Biol. Chem. 267, 4686–4692

19. Guinebaut, C., Payraste, B., Racad-Sultan, C., Mazarguil, H., Breton, M., Maucou, G., Plantavid, M., and Chap, H. (1995) J. Cell Biol. 129, 831–842

20. Mendesohn, M. E., O’Neill, S., George, D., and Loscalzo, J. (1990) J. Biol. Chem. 265, 19028–19034

21. Michelon, A., Bencot, S., Farnan, M., Brockwoldt, W., Rohrer, M., Barnard, M., and Loscalzo, J. (1996) Am. J. Physiol. 270, 1640–1648

22. Freedman, J. E., Loscalzo, J., Barnard, B., Alpert, C., Keaney, J. F., Jr., and Michelson, A. (1997) J. Clin. Invest. 100, 350–356

23. Folli, F., Saad, M. J., Becher, J. M., and Kahn, C. R. (1992) J. Biol. Chem. 267, 22171–22177

24. Born, G. V., and Cross, M. J. (1963) J. Physiol. (Lond.) 168, 178–195
NO and PI3-Kinase in Platelets

25. Frenette, P., Mayadas, T., Rayburn, H., Hynes, R., and Wagner, D. (1996) Cell 84, 563–574
26. Chang, J., Gao, A. G., and Frazier, W. A. (1997) J. Biol. Chem. 272, 14740–14746
27. Dorahy, D. J., Lincz, L. F., Meldrum, C. J., and Burns, G. F. (1997) Biochem. J. 319, 67–72
28. Dorahy, D. J., Berndt, M. C., Shafren, D. R., and Burns, G. F. (1996) Biochem. Biophys. Res. Commun. 218, 575–581
29. Dorahy, D. J., Berndt, M. C., and Burns, G. F. (1995) Biochem. J. 309, 481–490
30. Pleiman, C., Hertz, W., and Cambier, J. (1994) Science 263, 1609–1612
31. Dombrosky-Ferlan, P. M., and Corey, S. J. (1997) Oncogene 14, 2019–2024
32. Zeng, G., and Quon, M. (1996) J. Clin. Invest. 98, 894–898
33. Sotirellis, N., Johnson, T., Hibbs, M., Stanley, I., Stanley, E., Dunn, A., and Cheng, H. (1995) J. Biol. Chem. 270, 29773–29780
34. Hirao, A., Hamaguchi, I., Suda, T., and Yamaguchi, N. (1997) EMBO J. 16, 2342–2351
35. Rittenhouse, S. (1996) Blood 88, 4401–4414
36. Hartwig, J., Kung, S., Kovacsovics, T., Janney, P., Cantley, L. C., Stossel, T., and Toker, A. (1996) J. Biol. Chem. 271, 32886–32893
37. Vaandrager, A., and de Jonge, H. (1996) Mol. Cell. Biochem. 157, 23–30
38. Arnold, W., Mittal, C., Katsuki, S., and Murad, F. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 3203–3207
39. Geiger, J., Nolte, C., Butt, E., Sage, S., and Walter, U. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1031–1035
40. Gopalakrishna, R., Chen, Z., and Gundimeda, H. (1993) J. Biol. Chem. 268, 27180–27185
41. Nakanishi, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16
42. Toker, A., Meyer, M., Reddy, K. K., Falck, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) J. Biol. Chem. 269, 32358–32367
43. Yatomi, Y., Hazeki, O., Kume, S., and Ui, M. (1992) Biochem. J. 285, 745–751