BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **219,** 1–5 (1996)
ARTICLE NO. 0171

Nitric Oxide Stimulates the Phosphorylation of rap1b in Human Platelets and Acts Synergistically with Iloprost

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Received December 5, 1995

Phosphorylation of rap1b in human platelets correlates with both an upward shift of the protein on sodium dodecyl sulfate polyacrylamide gels and the translocation of the phosphorylated protein to the cytosolic fraction of platelets. We reported that this phenomenon occurs in platelets in response to agents that stimulate adenylate cyclase and thereby activate the cyclic AMP-dependent protein kinase. We now have evidence that phosphorylation of rap1b in platelets is also induced by nitric oxide generating compounds through stimulation of guanylate cyclase and activation of the cyclic GMP-dependent protein kinase. We observed time-dependent phosphorylation of rap1b and dose-dependent inhibition of collagen-stimulated aggregation in washed platelets incubated with S-nitroso serum albumin. In the presence of a combination of iloprost and 3-morpholino-sydnonimine, when both PKA and PKG are activated, phosphorylation of rap1b increased synergistically to a level three times higher than the sum of their individual actions.

Proper platelet function requires a delicate balance between activation and inhibition. Platelets aggregate within seconds after encountering an injury to the endothelial lining of blood vessels, but aggregation in normal circulation must be prevented. Endothelial cells release prostacyclin and nitric oxide, which act synergistically to inhibit aggregation of circulating platelets (1). When stimulated, platelets also produce nitric oxide internally, which serves to modulate their aggregation (2).

Nitric oxide, initially described as the endothelium-derived relaxing factor (3), is a free radical gas produced from arginine by nitric oxide synthase. With effects on immune function, blood vessel dilation, and neurotransmission, nitric oxide is now recognized as an important second messenger molecule in systems throughout the body. Nitric oxide is a very reactive molecule that binds readily to heme iron and reduced sulfhydryl groups (4). By binding to iron in the heme of guanylyl cyclase, nitric oxide stimulates the enzyme, causing an increase in cyclic GMP levels (5). Nitric oxide has been shown to increase cyclic GMP levels in platelets (6, 7).

Rap1b, a ras-related, low molecular weight GTP-binding protein, is targeted to the cell membrane by a post-translational modification (i.e., geranylgeranylation) of its C-terminal CAAX motif (8, 9, 10). The activated, GTP-bound form of rap1b can be deactivated by a specific GTPase-activating protein (11, 12). Purified rap1b is phosphorylated *in vitro* by PKA (13) and PKG (14) at Ser^{179}. We previously demonstrated that when intact platelets are treated with iloprost, a stable prostacyclin analog that increases cyclic AMP levels, rap1b is phosphorylated. The ^{32}P-labeled rap1b migrates with an upward shift on SDS-PAGE[1] and appears in the cytosolic as well as the particulate fractions of platelets (15, 16, 17).

We now report that rap1b is phosphorylated in a time-dependent manner when platelets are treated with nitric oxide generating compounds. We demonstrate a physiological effect of S-nitroso

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The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NO-BSA, S-nitroso serum albumin; SIN-1, 3-morpholino-sydnonimine; SNAP, N-acetyl-3-(nitrosothio)-D,L-valine(+2-(acetylamino)-2-carboxy-1,1-dimethylethyl-thionitrite; PKA, cyclic AMP-dependent protein kinase; PKG, cyclic GMP-dependent protein kinase; CAAX, Cysteine-aliphatic amino acid-aliphatic amino acid-last amino acid; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; ECL, enhanced chemiluminescent; BSA, bovine serum albumin.

0006-291X/96 $18.00

albumin, a naturally occurring nitric oxide donor (4), by showing the dose-dependent inhibition of collagen-induced platelet aggregation. We also show that the combination of nitric oxide and iloprost synergistically increases the phosphorylation of rap1b in platelets.

MATERIALS AND METHODS

Materials. 3-Morpholinosydnonimine was from Casella AG, Frankfurt. N-Acetyl-3-(nitrosothio)-D,L-valine(+2-(acetylamino)-2-carboxy-1,1-dimethylethyl-thionitrite was synthesized by Wellcome Laboratories, Beckenham. Carrier free [^{32}P]orthophosphate was purchased from ICN. Collagen was from Hormon-Chemie, Munich. Iloprost was from Schering AG, Berlin. Bio-Gel A-50m was purchased from Bio-Rad. The ECL detection kit was purchased from Amersham.

Isolation of human platelets. Blood from human donors was collected, using 1/10 volume of trisodium citrate (3.8%) as an anticoagulant, and centrifuged 20 min at 180 × g to pack the red cells. The platelet-rich plasma was treated with EDTA (5 mM final conc.) and centrifuged 15 min at 1000 × g to pellet the platelets. At this stage, the platelets were either washed (for aggregation studies) or labeled with [^{32}P]orthophosphate and washed (for phosphorylation studies). To wash the cells, the pellet was resuspended in Tyrode's buffer (pH 7.4) containing 5 mM EDTA and either centrifuged 10 min at 800 × g or gel filtered on a Bio-Gel A-50m column. Tyrode's buffer consisted of 138 mM NaCl, 12 mM $NaHCO_3$, 10 mM glucose, 5 mM HEPES, 2.9 mM KCl, 1 mM $MgCl_2$, and 0.36 mM $NaH_2PO_4 \cdot H_2O$.

Labeling of platelets with [^{32}P]orthophosphate. Platelets from 100 ml blood were resuspended in 1.8 ml Tyrode's buffer containing 5 mM EDTA. Additions were: 0.2 ml platelet-poor plasma, 6 units apyrase, and 2 mCi [^{32}P]orthophosphate. Platelets were incubated 1 h at 37°C, washed, and resuspended in 5 ml Tyrode's buffer with 5 mM EDTA.

Preparation of NO-BSA. Equimolar amounts of bovine serum albumin and $NaNO_2$ were incubated in 0.5 M HCl for 30 min at room temperature (18). The solution was then dialyzed for 3 h in three changes of 0.01 M phosphate buffer, pH 7.5. The stoichiometry of the S-nitrosylation, determined by the method of Saville (19), was approximately 0.5 mol NO/mol BSA. The NO-BSA was used on the day of preparation.

Measurement of platelet aggregation. Washed platelets from 100 ml blood were resuspended in 10 ml Tyrode's buffer without EDTA. Platelet aggregation in response to collagen stimulation (1–5 μg/ml) was followed using a Chrono-log aggregometer, which measures increased light transmission through the platelet suspension.

Analysis of rap1b mobility, localization, and phosphorylation. ^{32}P-labeled platelet samples (typically 0.5 ml) were incubated at 37°C with or without the addition of a nitric oxide generator (SIN-1 or SNAP) for the desired period of time. The platelets were pelleted in a microfuge, resuspended in 1 volume of hypotonic buffer (5 mM Tris, 5 mM EDTA), and lysed with five cycles of freezing in liquid nitrogen and thawing in a 37°C bath. After centrifugation for 10 min at 800 × g to remove any unbroken cells, the platelet homogenates were centrifuged 15 min at 170,000 × g in a Beckman airfuge to separate cytosolic and particulate fractions. The particulate pellet was washed by resuspension in hypotonic buffer and centrifugation for 10 min at 170,000 × g. The proteins were dissolved in SDS-sample buffer and separated by SDS-PAGE (11%, 16 cm). From an initial homogenate volume of 175 μl, all of the particulate pellet and 25 μl (1/7 vol) of the cytosol were used in an effort to equalize the amount of protein loaded in each lane. The proteins were transferred to nitrocellulose using a semi-dry blotter, and the blots were probed with the anti-ras mouse monoclonal antibody M90, which recognizes an epitope (amino acids 107–130) in the GTP-binding region of ras p21 protein (20) and also recognizes rap1b (15, 16, 17). The rap1b bands were visualized using either an alkaline phosphatase staining reaction or an enhanced chemiluminescent reaction. The blots were then exposed to film to identify the ^{32}P-labeled bands, and rap1b phosphorylation was quantitated by scanning the autoradiographs in a Shimadzu scanning densitometer.

RESULTS AND DISCUSSION

The monoclonal antibody M90 was made to recognize ras p21 protein (20), but it also identifies rap1b well on Western blots (15, 16, 17). Unphosphorylated rap1b is detected as a 22-kDa protein in the particulate fraction of human platelets by M90. After treatment of platelets with iloprost,

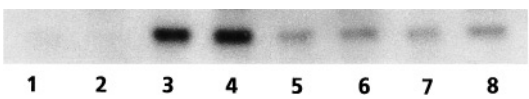

FIG. 1. Phosphorylation of rap1b induced by iloprost, SIN-1, and SNAP. ^{32}P-labeled platelets were incubated 2 h at 37°C alone (lanes 1 and 2), with 10 μM iloprost (lanes 3 and 4), with 100 μM SIN-1 (lanes 5 and 6), or with 200 μM SNAP (lanes 7 and 8). The cytosolic fractions (lanes 1, 3, 5, and 7) and particulate fractions (lanes 2, 4, 6, and 8) were blotted onto nitrocellulose after SDS–PAGE and exposed to film for autoradiography. The phosphorylation of rap1b in platelets by PKG was much weaker than the phosphorylation we observed with PKA. We determined, from previous experiments, that unphosphorylated rap1b is only observed in the particulate fraction of platelets, while phosphorylated rap1b is observed in both the particulate and cytosolic fractions.

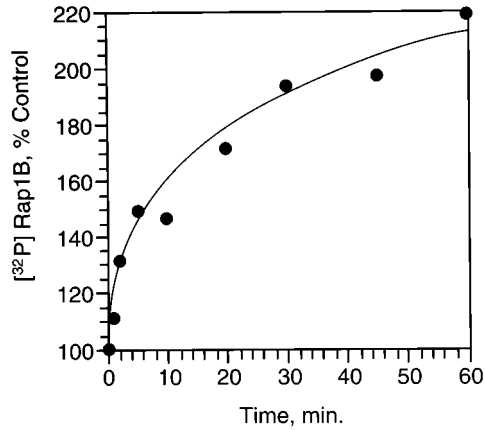

FIG. 2. Time course of rap1b phosphorylation induced by NO-BSA. ^{32}P-labeled platelets were incubated with 30 μM NO-BSA for the indicated times. Protein samples were resolved by SDS–PAGE, blotted onto nitrocellulose, and exposed to film. The autoradiograph was quantitated by scanning densitometry.

phosphorylated rap1b is detected in the cytosolic and particulate fractions as a 24-kDa band (15). Prostacyclin, and the more stable analog iloprost, induce phosphorylation of rap1b by increasing cAMP levels (21) and thereby activating PKA. Nitric oxide generating compounds such as SNAP, SIN-1, and NO-BSA increase levels of cGMP and activate PKG (5, 7). Both kinases phosphorylate rap1b at Ser^{179} (14). Figure 1 shows that the phosphorylation induced by iloprost was much stronger than the phosphorylation induced by SIN-1 and SNAP. The phosphorylation of rap1b in platelets by PKG resulted in a shift and translocation of the rap1b band on Western blots (data not shown), but it was much weaker than the effect we observed with PKA phosphorylation. This lesser effect may be reflected in the fact that iloprost is 1000 times more potent in inhibiting platelet aggregation than are nitric oxide generating compounds (22).

Nitric oxide is most likely presented to the cell as an adduct instead of a free radical. S-nitroso-serum albumin is a naturally occurring nitric oxide carrier (4). We showed that NO-BSA can transfer nitric oxide to intact washed platelets, induce rap1b phosphorylation, and inhibit platelet aggregation. Figure 2 shows the time course of the phosphorylation of rap1b caused by NO-BSA. Rap1b phosphorylation increased to more than two times the basal level during a 1 h incubation of

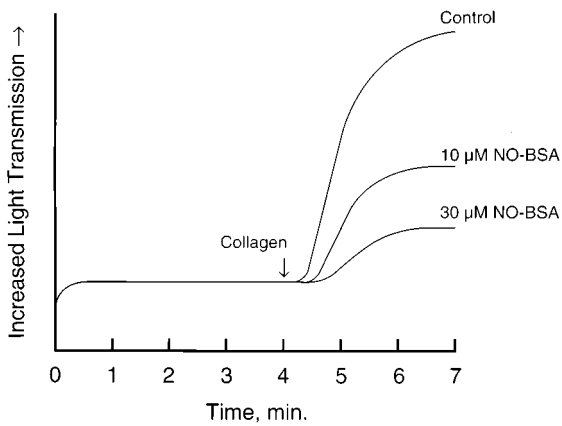

FIG. 3. Inhibition of platelet aggregation by NO-BSA. Washed platelets were preincubated 4 min with NO-BSA (0, 10 μM, or 30 μM) and stimulated with collagen (2 μg/ml) in a Chrono-log aggregometer. BSA alone had no effect on platelet aggregation.

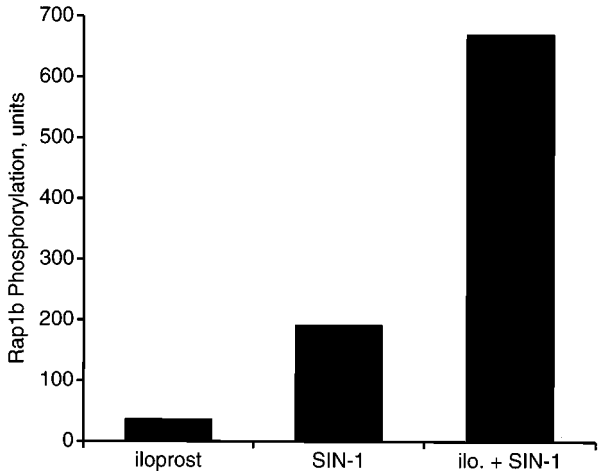

FIG. 4. Synergism of rap1b phosphorylation induced by iloprost and SIN-1. ^{32}P-labeled platelets were incubated 90 min at 37°C alone, with 50 pM iloprost, with 100 μM SIN-1, or with 50 pM iloprost and 100 μM SIN-1. The samples were analyzed by SDS–PAGE, blotted onto nitrocellulose, and exposed to film for autoradiography. The autoradiograph was quantitated by scanning densitometry.

platelets with NO-BSA. Although maximum phosphorylation occurred too slowly to correlate it with inhibition of platelet aggregation, phosphorylation of only a small percentage of the total rap1b may be sufficient to indicate an inhibitory effect. The effect of NO-BSA on platelet aggregation induced by collagen is shown in Figure 3. NO-BSA inhibited collagen-induced platelet aggregation in a dose-dependent manner. Equivalent amounts of control BSA, other than slightly dampening the oscillations on the aggregometer tracing, had no effect on the level of platelet aggregation (data not shown).

The combination of iloprost and nitric oxide, while having only an additive effect on relaxation of vascular smooth muscle, acts synergistically to inhibit platelet aggregation at a level two- to threefold higher than the sum of their individual actions (22). Our experiments showed that nitric oxide generating compounds and iloprost act synergistically to increase the phosphorylation of rap1b. When submaximal concentrations of iloprost and SIN-1 were added to platelets in combination, the resulting ^{32}P-rap1b level was approximately threefold higher than that predicted from the sum of their effects alone (Figure 4). It seems likely that phosphorylation of rap1b is associated with the inhibition of platelet aggregation. Our observations indicate that phosphorylation of platelet rap1b is a sensitive marker for the effect of agents that increase cyclic AMP or cyclic GMP levels, and that it is even more sensitive to the combined effects of agents that elevate levels of cyclic AMP and cyclic GMP at the same time.

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