Dipyridamole Enhances NO/cGMP-Mediated Vasodilator-Stimulated Phosphoprotein Phosphorylation and Signaling in Human Platelets

In Vitro and In Vivo/Ex Vivo Studies

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Background and Purpose—Dipyridamole and in particular dipyridamole in combination with low-dose aspirin are very effective in preventing recurrent stroke. However, the mechanism(s) underlying this dipyridamole effect have not been elucidated. Since dipyridamole inhibits the cGMP-specific phosphodiesterase type V in vitro, we hypothesized and tested whether therapeutically relevant dipyridamole concentrations enhance NO/cGMP-mediated effects in intact human platelets studied ex vivo.

Methods—Phosphorylation of vasodilator-stimulated phosphoprotein (VASP), an established marker of NO/cGMP effects in human platelets, was quantified by phosphorylation-specific antibodies and Western blots. Serotonin secretion and thromboxane synthase activity were determined by fluorometric quantification of derivatized serotonin and synthase products, respectively.

Results—Endothelium-derived factors such as NO and prostaglandin I\textsubscript{2} are known to elevate both cGMP and cAMP levels with concomitant platelet inhibition and VASP phosphorylation. In our in vitro experiments, therapeutically relevant concentrations (3.5 \textmu mol/L) of dipyridamole amplified only cGMP-mediated VASP phosphorylation due to the NO donor sodium nitroprusside, but not cAMP-mediated effects. Furthermore, thromboxane synthase activity and serotonin secretion, events important for initial platelet activation, were inhibited by sodium nitroprusside, an effect also enhanced by dipyridamole, demonstrating the functional relevance of these observations. Finally, the ex vivo enhancement of NO/cGMP effects was also observed with platelets obtained from healthy volunteers treated with extended-release dipyridamole.

Conclusions—Under therapeutically relevant conditions, dipyridamole enhances platelet inhibition by amplifying the signaling of the NO donor sodium nitroprusside. These data support the concept that enhancement of endothelium-dependent NO/cGMP-mediated signaling may be an important in vivo component of dipyridamole action. (Stroke. 2003;34:764-769.)

Key Words: dipyridamole ▪ phosphodiesterase inhibitors ▪ platelet aggregation inhibitors ▪ stroke

The role of activated platelets in acute and chronic vascular diseases is well established. Accordingly, antiplatelet drugs significantly reduce the risk of severe events, such as ischemic stroke, myocardial infarction, and vascular death in atherosclerotic vascular disease. In numerous clinical studies, the efficacy of antiplatelet drugs in the treatment and secondary prevention of vascular diseases, particularly stroke, has been proven.\textsuperscript{1-3}

Platelet activation is inhibited by factors released from endothelial cells that constitute the inner cell layer of the vessel wall. The most important inhibitory endothelium-derived factors are NO and prostaglandin I\textsubscript{2} (PGL\textsubscript{2}), which inhibit platelets by increasing the level of intracellular cyclic nucleotides.\textsuperscript{4,5} Subsequent stimulation of cGMP- and cAMP-dependent protein kinases leads to the phosphorylation of a variety of proteins.\textsuperscript{6} In platelets, this inhibits agonist-stimulated calcium signaling,\textsuperscript{7,8} fibrinogen binding,\textsuperscript{9} adhesion,\textsuperscript{10} and aggregation\textsuperscript{3} and stimulates phosphorylation of the cytoskeletal and focal adhesion protein vasodilator-stimulated phosphoprotein (VASP).\textsuperscript{4,5,11} This phosphorylation correlates strongly with the inhibition of platelets.\textsuperscript{9,11} Interestingly, antiplatelet treatment with clopidogrel or ticlopidine also increases VASP phosphorylation by attenuating inhibitory ADP effects.\textsuperscript{12,13} There is increasing evidence that phosphorylation of VASP can serve as a marker for cyclic nucleotide and especially cGMP signaling not only in platelets but also in other cell types. Physiological and defective NO/cGMP signaling\textsuperscript{4,11} as well as endothelial function/
dysfunction\textsuperscript{16} could be monitored by measuring vessel wall VASP serine 239 phosphorylation. In addition, impaired signaling of endothelial cells (ie, endothelial dysfunction) in hyperlipidemic rabbits,\textsuperscript{16} in hypertensive rats,\textsuperscript{17} and in nitrate tolerance of both rabbits\textsuperscript{18} and humans\textsuperscript{19} could be detected by VASP phosphorylation.

Endothelial dysfunction during acute or chronic vascular diseases increases platelet activity. Accordingly, antiplatelet drugs significantly reduce the risk of severe vascular events, such as ischemic stroke, myocardial infarction, and vascular death.\textsuperscript{1–3} Dipyridamole, which has been used clinically since the early 1960s as a coronary vasodilator, had a renaissance as an antithrombotic drug because of the positive results of the European Stroke Prevention Study 2 (ESP\textsuperscript{2}S-2).\textsuperscript{2} Treatment with extended-release dipyridamole alone was as effective as low-dose aspirin in the reduction of recurrent stroke, and the combination of extended-release dipyridamole and low-dose aspirin (Aggrenox) was more than twice as effective as aspirin alone.\textsuperscript{1–3} Several possible mechanisms underlying these recognized clinical benefits have been described in earlier studies.\textsuperscript{20} Dipyridamole increases the plasma concentration of the endogenous platelet inhibitor adenosine by inhibition of adenosine uptake into red blood cells\textsuperscript{21,22} and attenuation of adenosine catabolism.\textsuperscript{23} In addition, it directly stimulates the release of endothelial PGI\textsubscript{2},\textsuperscript{24–26} Dipyridamole has also been reported to inhibit cyclic nucleotide degradation by phosphodiesterases (PDE), including the cGMP-specific PDE type V.\textsuperscript{27–29} However, it is not clear whether these mechanisms contribute to the clinical benefits of dipyridamole reported in ESPS-2 because treatment with extended-release dipyridamole achieves only relatively low plasma concentrations of 0.8 to 3.6 \(\mu\text{mol/L}\).\textsuperscript{20,30}

The aim of this study was therefore to demonstrate an antiplatelet effect of dipyridamole at therapeutically relevant concentrations. We hypothesized and tested whether such dipyridamole concentrations, delivered to human platelets ex vivo or found in volunteers treated with extended-release dipyridamole, enhance NO/cGMP-mediated effects in intact human platelets studied ex vivo. In these experiments, the labile and short-lived endothelial-derived NO was replaced by the well-characterized platelet inhibitor and NO donor sodium nitroprusside (SNP). As biochemical marker for NO/cGMP effects, we analyzed VASP serine 239 phosphorylation. In addition, serotonin secretion from platelet granules and thromboxane synthase activity were measured as functional correlates. Finally, platelets from healthy volunteers treated with extended-release dipyridamole were analyzed ex vivo to confirm our in vitro data.

Materials and Methods

Materials and Platelet Preparation

Platelets were prepared from whole human blood as previously described.\textsuperscript{8} Briefly, the blood was collected in a citrate buffer (100 \(\mu\text{mol/L}\) sodium citrate, 7 \(\mu\text{mol/L}\) citric acid, 140 \(\mu\text{mol/L}\) glucose, pH 6.5). Platelet-rich plasma (PRP) was separated by centrifugation for 20 minutes at 300g. The PRP was removed with a plastic pipette. For the preparation of washed platelets, blood was collected in a citrate buffer as described above that additionally contained 15 \(\mu\text{mol/L}\) EGTA. After preparation of PRP, platelet suspension was centrifuged for 10 minutes at 500g at room temperature. The platelet pellet was resuspended in HEPES buffer (145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl\textsubscript{2}, 10 mmol/L HEPES, 10 mmol/L glucose, pH 7.4).

VASP Phosphorylation

The extent of VASP phosphorylation was determined in platelets obtained from PRP that had been incubated with dipyridamole or vehicle. Then the platelet guanylyl cyclase activator SNP or the platelet adenyl cyclase activator prostaglandin E\textsubscript{1} (PGE\textsubscript{1}) was added to the platelet suspension, which was incubated for an additional 4 minutes (in the case of SNP) or 2 minutes (in the case of PGE\textsubscript{1}) or in the vehicle alone. Platelets were then sedimented by centrifugation, the supernatant plasma was rapidly removed, and the platelet pellet was solubilized in a hot sodium dodecyl sulfate-containing stop solution. Platelet proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted onto nitrocellulose. VASP phosphorylation and VASP were detected with the phosphorylation specific monoclonal VASP antibody 16C2 directed against the serine 239 phosphorylation site of VASP (46 kDa) and the polyclonal antibody M4 recognizing VASP (46 kDa) and VASP phosphorylated at serine 157 (50 kDa).\textsuperscript{24,15,31}

Thromboxane Synthase Activity

Thromboxane synthase activity was determined by fluorometric quantification of derivatized malondialdehyde according to the method of Ledergerber and Hartmann.\textsuperscript{32} For the experiments, 0.7-mL aliquots of washed human platelets resuspended in HEPES buffer were incubated with the reagents at 37°C in a thermostixer and stopped by addition of 0.4 mL ice-cold trichloroacetic acid (20% wt/vol). The samples were kept on ice for 10 minutes and then centrifuged for 10 minutes at 14 000 rpm in an Eppendorf 5415C table centrifuge. Equal volumes (0.6 mL) of the derivatization reagent thiobarbituric acid (0.53% wt/vol) and the supernatant were combined and incubated for 30 minutes at 70°C. The mixture was incubated for an additional 30 minutes at room temperature. The fluorometric quantification was performed with 0.7 mL of undiluted sample or with a 1:5 dilution of the samples. Fluorescence was measured with a Perkin-Elmer LS-50 luminometer at an excitation wavelength of 533 nm with a slit width of 2.5 nm and at an emission wavelength of 550 nm with a slit width of 15.0 nm. The standards were prepared from the stable malondialdehyde derivative tetraethoxypropane with thiobarbituric acid in presence of trichloroacetic acid (20% wt/vol).

Serotonin Secretion

Serotonin secretion was determined according to Holmsen and Dangelmaier\textsuperscript{33} with a Wallac Victor\textsuperscript{2} 1420 Multilabel Counter. Before platelet stimulation, 2 \(\mu\text{mol/L}\) fluoxetine, a selective serotonin reuptake inhibitor, was added to the platelet suspension. An aliquot (350 \(\mu\text{L}\)) of washed platelets was stimulated with various agonists as described in the figure legends. This was stopped with an ice-cold mixture of 35 \(\mu\text{L}\) EDTA (50 mmol/L, pH 7.4) and 100 \(\mu\text{L}\) silicon oil (Merck). Samples (amount of secreted serotonin after stimulation of platelet suspension with agonist) and totals (total amount of serotonin present in 350 \(\mu\text{L}\) platelet suspension) were mixed for 15 seconds, and only the samples were additionally centrifuged for 30 seconds at 5000g (Eppendorf 5415C centrifuge). Supernatants (270 \(\mu\text{L}\)) were transferred to an Eppendorf tube containing 60 \(\mu\text{L}\) of 100% trichloroacetic acid and then centrifuged at 5000g for 2 minutes. An aliquot (250 \(\mu\text{L}\)) of these supernatants was added to 1 mL of ortho-phthalaldehyde reagent (0.5% wt/wt) in ethanol, then mixed with 10 volumes of 8N HCl and heated at 95°C for 10 minutes. Samples and totals (each 200 \(\mu\text{L}\)) were washed twice with 5 mL of chloroform, and fluorescence emission of the upper water phase was measured at an excitation wavelength of 355 nm and an emission wavelength of 475 nm.

Volunteers

Four healthy volunteers (3 women and 1 man, with an age range of 22 to 45 years, who did not take any platelet-affecting drugs 2 weeks
before testing) entered this study with their written, informed consent. This volunteer study was approved by the ethics committee of our university. The volunteers took 1 capsule of 200 mg extended-release dipyridamole (Persantin Retard; provided via a local pharmacy). Blood was drawn 21 hours before and 3 hours after the intake. Dipyridamole plasma levels were measured at AAI Applied Industries, Neu-Ulm, Germany.

Statistical Analysis
The biochemical data obtained represent mean±SD and were statistically evaluated by 2-way ANOVA. The ANOVA analysis was used to test the hypothesis of whether dipyridamole would affect the SNP action on platelets. Therefore, we applied the test to the factors SNP and dipyridamole with 2 levels each (+/−). As a basis for the statistical analysis, we used a linear model with the hypothesis of no interaction. Data with P<0.05 were considered significant. Since the ANOVA analysis clearly indicated a significant effect of dipyridamole concentrations on SNP-induced VASP phosphorylation was even more striking at a threshold concentration of SNP (Figure 2); while 0.3 μmol/L SNP did not cause significant phosphorylation of VASP at serine 157 and/or serine 239, additional pretreatment of dipyridamole caused significant VASP phosphorylation at serine 157 (Figure 2; data for serine 239 phosphorylation not shown). In contrast, dipyridamole did not affect PGE1-induced VASP serine 157 phosphorylation (Figure 2). However, the synergistic effect of cGMP-mediated (in response to 0.3 μmol/L SNP) and cAMP-mediated (in response to 3 nmol/L PGE) VASP serine 239 phosphorylation was further enhanced by dipyridamole (Figure 2). Effects of dipyridamole at concentrations lower than 3.5 μmol/L were not observed. For example, preincubation (up to 60 minutes with 1.5 μmol/L dipyridamole) did not potentiate VASP phosphorylation induced by 1 μmol/L SNP (data not shown).

Results

In Vitro Experiments With 3.5 μmol/L Dipyridamole
When incubated alone, dipyridamole at a therapeutically relevant concentration (3.5 μmol/L) did not affect basal cyclic nucleotide content (not shown) or basal VASP phosphorylation (Figure 1). However, VASP serine 239 phosphorylation caused by 1 μmol/L SNP was significantly increased after pretreatment of PRP with 3.5 μmol/L dipyridamole (Figure 1). This amplifying effect of low dipyridamole concentrations on SNP-induced VASP phosphorylation was even more striking at a threshold concentration of SNP (Figure 2); while 0.3 μmol/L SNP did not cause significant phosphorylation of VASP at serine 157 and/or serine 239, additional pretreatment of dipyridamole caused significant VASP phosphorylation at serine 157 (Figure 2; data for serine 239 phosphorylation not shown). In contrast, dipyridamole did not affect PGE1-induced VASP serine 157 phosphorylation (Figure 2). However, the synergistic effect of cGMP-mediated (in response to 0.3 μmol/L SNP) and cAMP-mediated (in response to 3 nmol/L PGE) VASP serine 239 phosphorylation was further enhanced by dipyridamole (Figure 2). Effects of dipyridamole at concentrations lower than 3.5 μmol/L were not observed. For example, preincubation (up to 60 minutes with 1.5 μmol/L dipyridamole) did not potentiate VASP phosphorylation induced by 1 μmol/L SNP (data not shown).

![Figure 1. Dipyridamole potentiates SNP-induced VASP serine 239 phosphorylation in human platelets. PRP was incubated in the absence (control [Co]) or presence of dipyridamole (DP) (3.5 μmol/L) for 20 minutes and then with SNP (1 μmol/L) as indicated. Platelet suspensions were stopped at 2, 4, and 9 minutes after stimulation with SNP. Platelet VASP phosphorylation was then determined by Western blot analysis as described in Materials and Methods with the use of the monoclonal 16C2 antibody and quantitative densitometry with the NIH Image 1.62 system. In the representative Western blot (bottom), the time course of SNP-induced VASP phosphorylation in the presence and absence of dipyridamole is shown. Both 46- and 50-kDa bands are VASP phosphorylated at serine 239 because additional phosphorylation at serine 157 causes a shift of the protein from the 46-kDa to the 50-kDa band. The quantitative data (top) represent 4-minute incubations with SNP and are expressed as x-fold increase of the amount of phosphorylated VASP (P-VASP) at serine 239 compared with basal P-VASP. Data (mean of duplicate) are mean±SD of 4 separate experiments (P<0.05).](http://stroke.ahajournals.org/)

![Figure 2. Dipyridamole potentiates SNP-, but not PGE1-,induced VASP serine 157 (Ser157) phosphorylation in human platelets. PRP was incubated with dipyridamole (3.5 μmol/L) for 20 minutes and then with SNP (0.3 μmol/L) or PGE, (3 nmol/L) for 4 minutes or 2 minutes, respectively. VASP serine 157 phosphorylation was detected with the polyclonal M4 antibody and quantitatively determined by densitometry with the NIH Image 1.62 system. Other abbreviations are as defined in Figure 1. Data (mean of duplicate) are mean±SD of 4 separate experiments (P<0.05).](http://stroke.ahajournals.org/)
The effects of dipyridamole on platelet function were investigated. We examined serotonin secretion from platelet granules and thromboxane synthase activity. Activation of both events is important to amplify initial platelet response. Dipyridamole (3.5 μmol/L) did not alter the activation of thromboxane synthase by thrombin but enhanced the SNP-caused inhibition of synthase activity (Figure 3). Analogously, stimulation of serotonin secretion by the stable thromboxane analogue U46619 was not affected by dipyridamole. However, the SNP-caused inhibition of U46619-stimulated serotonin release was significantly enhanced in the presence of dipyridamole at a therapeutically relevant concentration (3.5 μmol/L) (Figure 4).

Effects of Treatment With Extended-Release Dipyridamole in Healthy Volunteers on Platelet VASP Phosphorylation

SNP-induced VASP serine 239 phosphorylation was studied 21 hours before and 3 hours after treatment with 1 capsule of extended-release dipyridamole (Persantin Retard). Dipyridamole plasma levels were analyzed and detected in all 4 subjects. On stimulation of human PRP with 1 μmol/L SNP, VASP serine 239 phosphorylation was stimulated approximately 7-fold or 15-fold without or with dipyridamole treatment, respectively, compared with basal VASP phosphorylation in each case (Figure 5). Therefore, dipyridamole treatment of volunteers enhanced the SNP-stimulated VASP phosphorylation 2-fold.

Discussion

In platelets, elevation of cyclic nucleotide levels on stimulation with endothelium-derived factors causes inhibition of platelet function and phosphorylation of target proteins such as VASP.4–6 Antiplatelet compounds interfere with cyclic nucleotide–mediated pathways and therefore represent useful tools in the therapy and prevention of vascular diseases. In particular, treatment with extended-release dipyridamole alone and in combination with low-dose aspirin significantly reduced the risk of recurrent stroke.2 A variety of biochemical mechanisms have been reported for dipyridamole,20 but it is not clear whether these mechanisms contribute to the observed clinical benefits because under treatment with extended-release dipyridamole only very low plasma concentrations of 0.8 to 3.6 μmol/L20,30 are achieved.

In this study we established a method that allows the quantitative determination of cyclic nucleotide–mediated kinase activation in platelets by measuring the phosphorylation of VASP, which is a substrate of both cAMP-dependent
(cAK) and cGMP-dependent protein kinase (cGK). In vitro incubation of platelets with the NO donor SNP and the PGI2 analogue PGE1 caused a rapid phosphorylation of VASP at serine 239 and serine 157 (Figures 1 and 2). However, dipyridamole, at a therapeutically relevant concentration of 3.5 μmol/L, amplified only NO/cGMP-mediated (Figures 1 and 2), but not PGE1/cAMP-mediated, PGI2 phosphorylation (Figure 2). Dipyridamole did not affect basal VASP phosphorylation (Figure 1) or basal cGMP levels (not shown). These findings indicate that a cGMP-selective PDE is inhibited by dipyridamole. Therefore, we conclude that dipyridamole at therapeutically relevant concentrations selectively inhibits PDE V (IC50=1.6 μmol/L) and not other PDE isoforms present in platelets.

In earlier studies, the synergistic effects of NO and PGI2 on platelet inhibition and on cyclic nucleotide–dependent protein kinase–mediated phosphorylation have been demonstrated. This synergism is mainly caused by the inhibition of PDE III by cGMP, which enhances PGI2/cAMP signaling. Accordingly, an increased concentration of cGMP due to PDE V inhibition by dipyridamole caused an enhanced synergism of cGK and cAK (Figure 2). Interestingly, elevation of cGMP appears insufficient to increase basal cAMP levels and cAK effects (Figure 2). Therefore, our data do not support the hypothesis that all cGMP effects are mediated by cAK.

To demonstrate a functional relevance of the aforementioned observed phosphorylation data, we investigated the effects of dipyridamole on the secretion of serotonin from platelet granules and on thromboxane synthase activity. Activation of both events is important to amplify initial platelet response. For example, impairment of degranulation by congenital defects causes severe bleeding problems, and inhibition of thromboxane synthase represents the main mechanism contributing to the antiplatelet effects of acetylsalicylic acid, which is still the gold standard in the treatment of vascular diseases. SNP inhibited in a dose-dependent manner the activation of thromboxane synthase by thrombin (not shown); this was clearly enhanced by additional incubation of dipyridamole (Figure 3). Similar results have been obtained by measuring serotonin secretion: the SNP-induced inhibition of U46619-stimulated serotonin release is amplified by dipyridamole at a therapeutically relevant concentration (3.5 μmol/L) (Figure 4). In contrast to the activation of thromboxane synthase, serotonin secretion is triggered by various agonists including thrombin, collagen, and U46619 (not shown), which indicates different underlying signaling pathways. However, both thromboxane synthase and serotonin secretion appear to be sensitive to NO/cGMP-mediated inhibition. These findings are in accord with previous studies reporting that dipyridamole enhances NO-induced inhibition of platelet aggregation.

Finally, our in vitro data could be confirmed by in vivo/ex vivo studies with healthy volunteers treated with extended-release dipyridamole (Persiant Retard). This treatment caused a clear, statistically significant 2-fold enhancement of SNP-induced VASP phosphorylation measured in platelets ex vivo compared with platelets obtained before drug intake (Figure 5).

In summary, we could demonstrate a clear antplatelet effect of dipyridamole under therapeutically relevant conditions, as summarized in Figure 6. In human platelets under in vitro conditions, dipyridamole selectively enhanced NO/cGMP signaling and its consequence such as VASP serine 239 phosphorylation and inhibition of thromboxane synthase and serotonin secretion. We have shown these dipyridamole effects on NO/cGMP signaling with human platelets ex vivo only but expect that these effects also occur in human blood vessels and vessel walls in vivo. This is supported by our observation that enhanced NO/cGMP signaling is clearly observed in platelets from volunteers treated with extended-release dipyridamole. When one considers the well-accepted fact that major diseases such as arteriosclerosis, diabetes, heart failure, and hypertension are characterized by impaired NO/cGMP signaling, it appears likely that improved/enhanced NO/cGMP signaling is a major component of the beneficial clinical effects observed with dipyridamole.

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