Vanadate Activates Platelets by Enhancing Arachidonic Acid Release

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Human platelet activation is associated with, and regulated by, the phosphorylation of a number of proteins. Recently, attention has been focused on tyrosine phosphorylation of proteins and their function in platelet activation. Here vanadate, an inhibitor of tyrosine phosphohydrolase, was used to examine the role that tyrosine phosphorylation plays in platelet activation. Vanadate (7.5 to 100 μmol/L) stimulated the dose-dependent aggregation of saponin-permeabilized, platelet proteins and their function in platelet activation. Vanadate-induced aggregation was inhibited by pretreatment with prostacyclin (1 to 10 nmol/L), genistein (1 to 10 μg/mL), aspirin (100 μmol/L), or BW755C (80 μmol/L). Aggregation was associated with the aspirin-sensitive formation of [32P]-phosphatidic acid and the phosphorylation of platelet proteins, notably pleckstrin and myosin light chain. Immunoblotting studies indicated that vanadate caused the tyrosine phosphorylation of proteins of approximate molecular weights 26, 29, 32, 40, 42, 80, and 90 Kd. Preincubation with BW755C abolished the phosphorylation of the 26-, 29-, 32-, 40-, and 42-Kd proteins but not the 80- and 90-Kd proteins. Vanadate stimulated the release of [3H]-arachidonic acid that was not affected by pretreatment with BW755C. The subsequent conversion of [3H]-arachidonic acid to [3H]-thromboxane A2 was significantly inhibited. These findings show that vanadate stimulates platelets by promoting arachidonic acid release from phospholipids. Tyrosine phosphorylation, potentially of the 80- or 90-Kd proteins, may regulate a platelet phospholipase A2. The released arachidonic acid was converted to thromboxane A2 that produced secondary effects such as phospholipase C activation, protein phosphorylation, and aggregation, and was associated with the tyrosine phosphorylation of the 26-, 29-, 32-, 40-, and 42-Kd proteins.

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MATERIALS AND METHODS

Materials. Sodium orthovanadate, saponin, prostacyclin, and aspirin were obtained from Sigma (St Louis, MO). Antiphosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology Inc (Lake Placid, NY), and genistein from GIBCO (Gaithersburg, MD). BW755C was a gift from Dr V.G. Mahadevappa (University of Waterloo, Ontario, Canada). Carrier-free [32P]-orthophosphate was from ICN Radiochemicals (Mississauga, Ontario, Canada) and [5,6,9,1,12,14,15-3H(N)]-arachidonic acid was from New England Nuclear (Mississauga, Ontario, Canada). Phospholipid standards were purchased from Serdary Research (London, Ontario, Canada) and thromboxane (Tx) B2 from Cayman Chemicals (Ann Arbor, MI). Electron-microscopic supplies were from J.B. E.M. Supplies (Montreal, Quebec, Canada); electrophoresis and immunoblotting supplies were from Bio-Rad (Mississauga, Ontario, Canada); enhanced chemiluminescence (ECL) Western blotting detection system was from Amersham (Oakville, Ontario, Canada).

Preparation of platelets. Blood, obtained by venipuncture from healthy human volunteers who had not taken medication known to
 interfere with platelet function within the previous 2 weeks, was collected into acid-citrate dextrose anticoagulant (3.8 mmol/L citric acid, 7.5 mmol/L trisodium citrate, 125 mmol/L dextrose; 1.9 mL anticoagulant per 8.1 mL whole blood). Platelet-rich plasma (PRP) was obtained by centrifugation at 800g for 5 minutes. Plasma-free platelet suspensions were prepared by centrifugation (800g for 10 minutes) and the platelet pellet was resuspended in a high-citrate washing buffer. The platelets were pelleted by centrifugation (800g for 10 minutes) and the resultant pellet resuspended in a high-potassium buffer that resembles the cytoplasm.

Platelet aggregation. Platelets were placed in an aggregometer cuvette and stirred with 10 to 14 μg/mL saponin for 2 minutes before the addition of vanadate. Aggregation was monitored photometrically with continuous stirring at 37°C in a Payton dual-channel aggregometer (Payton Associates, Scarborough, Ontario, Canada). Saponin alone did not cause aggregation and its concentration was carefully titrated in each experiment to avoid cell lysis.

Phosphoinositide metabolism. PRP was centrifuged (800g for 10 minutes). The resultant pellet was then resuspended in 1 mL of platelet poor plasma (PPP) and labeled with [32P]-orthophosphate and [32P]-orthophosphate for 60 minutes, washed, and aggregation was performed as outlined above for phosphoinositide metabolism. The samples were denatured by boiling for 3 minutes in a solubilizing solution (2% sodium dodecyl sulfate [SDS], 2% β-mercaptoethanol, 10% glycerol, 1 mmol/L EDTA, 4 mmol/L EGTA, 0.01% bromophenol blue, 150 mmol/L Tris HCl; pH 6.8). Proteins were separated on a 10% polyacrylamide/SDS vertical slab gel that was dried and autoradiographed as previously described.

Immunoblotting. Permeabilized platelets were prepared and aggregation studies performed as described above. The samples were then denatured by boiling in the solubilization buffer. The proteins were separated on a 10% polyacrylamide/SDS vertical slab gel and then transferred to nitrocellulose at 100 V for 1 hour at 15°C. The nitrocellulose was blocked by incubation for 1 hour with 10% nonfat powdered Carnation milk (Carnation Inc, Toronto, Ontario, Canada) in TBS (20 mmol/L Trizma base, 130 mmol/L NaCl; pH 7.4) and incubated with antiphosphotyrosine antibody 4G10 (1 μg/mL) at 4°C for 12 hours. The nitrocellulose was washed thoroughly with TBS containing 0.1% Tween 20 (Sigma, St Louis, MO), incubated with horseradish peroxidase-conjugated goat anti-mouse IgG for 1
Fig 2. Effects of inhibitors on vanadate-induced aggregation. Photometric recording of the aggregation of human platelets. (A) Platelets stirred in the presence of saponin before the addition of prostacyclin (1 to 10 nmol/L) followed 30 seconds later by the addition of 100 pmol/L vanadate. (B) Platelets stirred in the presence of saponin before the addition of genistein (1 to 30 μg/ml) followed 30 seconds later by 100 μmol/L vanadate.

RESULTS

Effects of vanadate on platelet aggregation. The addition of vanadate (7.5 to 100 μmol/L) to saponin-permeabilized, but not to intact (Fig 1A), human platelets caused aggregation in a concentration-dependent manner (Fig 1B), indicating the intracellular nature of the vanadate substrate. Vanadate has been reported to inhibit ATPases, notably the Na⁺/K⁺ ATPase. However, ouabain (100 μmol/L), another Na⁺/K⁺ ATPase inhibitor, failed to stimulate aggregation in intact or permeabilized platelets (data not shown).

The concentration of vanadate (100 μmol/L) shown to cause maximal platelet aggregation (Fig 1B) was used for all subsequent studies. Aggregation in response to vanadate was...
Inhibited by pretreatment with prostacyclin at concentrations (1 to 10 nmol/L) consistent with adenylate cyclase activation (Fig 2A), or with genistein (1 to 30 μg/mL; Fig 2B), a tyrosine kinase inhibitor that has recently been shown also to antagonize the Tx receptor. In the latter case the platelets remained responsive to 1 U/mL thrombin (data not shown), indicating that the effects of genistein were not caused by toxicity on the permeabilized platelets. Furthermore, the data suggests that thrombin-induced platelet aggregation is associated with multiple intracellular pathways and occurs when both the Tx receptor is blocked and tyrosine phosphorylation is inhibited. Vanadate-induced aggregation of permeabilized platelets was also inhibited by pretreatment with either the cyclo-oxgenase inhibitor aspirin (100 μmol/L; Fig 3A) or the dual cyclo-oxgenase and lipoxygenase inhibitor BW755C (80 μmol/L; Fig 3B).

Effects of vanadate on platelet ultrastructure. Electron-microscopic examination showed that saponin-permeabilized platelets retain a homogeneous distribution of their organelles (Fig 4A). The addition of vanadate caused the platelets to extend pseudopods, centralize and evacuate their granules, and form small aggregates (Fig 4B).

Effects of vanadate on phospholipase C. Vanadate (100 μmol/L) stimulated the formation of [32P]-phosphatidic acid in permeabilized platelets prelabeled with [32P]-orthophosphate (Fig 5, A and B). This formation of [32P]-phosphatidic acid was inhibited by preincubation with aspirin (100 μmol/L) (Fig 5B).

Effects of vanadate on protein phosphorylation. Vanadate (100 μmol/L) caused the phosphorylation of platelet proteins, notably of pleckstrin (an index of protein kinase C activity) and, to a variable extent, of myosin light chain (an index of...
myosin light chain (MLC) kinase activity) (Fig 6A). This phosphorylation, believed to be on threonine and serine residues, was inhibited by aspirin (Fig 6B).

Western blotting with the antiphosphotyrosine antibody 4G10 indicates the enhanced presence of proteins of approximate molecular weights 29, 32, 40, 42, and 80 Kd (Fig 7) and the appearance of two faint proteins of molecular weights 26 and 90 Kd (Fig 7) after the stimulation of the platelets with vanadate. Pretreatment of the platelets with BW755C (80 μmol/L), which abolished aggregation, diminished the presence of the 26-, 29-, 32-, 40-, and 42-Kd proteins to a level comparable with unstimulated platelets. In contrast, the 80- and 90-Kd proteins, although diminished, remained at an elevated level (Fig 7).

Effects of vanadate on arachidonate metabolism. When compared with vehicle control, vanadate (100 μmol/L) caused an approximate 40% release of [3H]-arachidonic acid from [3H]-arachidonic acid prelabeled-permeabilized platelets and this was associated with a twofold increase in [3H]-TxB2 levels (Fig 8). Pretreatment with the BW755C (80 μmol/L) abolished the increase in [3H]-TxB2 levels but did not affect [3H]-arachidonic acid release (Fig 8).

DISCUSSION

The present study has extended previous reports of the stimulatory effects of vanadate in human platelets. Vanadate stimulates platelet aggregation in a concentration-dependent manner, and this effect was mediated by the liberation of arachidonic acid and its subsequent conversion to Tx.

Vanadate is known to affect Na+/K+ ATPases, GTP-binding proteins, and PTPases. Several lines of evidence in the present study indicate that inhibition of PTPases is associated with the stimulation of platelet aggregation. Under the conditions used (ie, cytosolic potassium buffer), the sodium and potassium gradients are lost and vanadate was still able to elicit a response. In addition, the cardiac glycoside ouabain, a known inhibitor of Na+/K+ ATPases, was unable to mimic the stimulatory effects of vanadate. The relevance of inhibition of platelet Na+/K+ ATPases is questionable, as platelet activation occurs in the absence of the depolarization that would accompany such an inhibition. The concentration of vanadate required to cause aggregation of permeabilized platelets is consistent with that required to inhibit PTPases in platelet lysates but 10-fold lower than affects GTP-binding proteins. Furthermore, vanadate caused the phosphorylation of several phosphotyrosines identified by Western blotting.

One major question addressed by the present study is the site of action of vanadate and, by implication, the role of phosphotyrosine phosphorylation in platelet activation. The vanadate-induced platelet aggregation was associated with the stimulation of phospholipase C (as monitored by the for-

Fig 4. Effects of vanadate on platelet ultrastructure. Platelets were stirred in the presence of saponin before the addition of 100 μmol/L vanadate. Samples were fixed, stained, dehydrated, embedded, and examined under a transmission electron microscope. (A) Control platelet. (B) Vanadate stimulated the formation of platelet clumps with evidence of pseudopod extension (p), degranulation (d), and granule redistribution (r) (the bar represents 1 μm).
Effects of vanadate ± aspirin on [32P]-phosphatidic acid formation. [32P]-orthophosphate prelabeled platelets (in the presence or absence of 100 μmol/L aspirin) were stirred in the presence of saponin before the addition of 100 μmol/L vanadate. Phospholipids were extracted, separated by thin-layer chromatography, autoradiographed, and quantified by radiochromatographic scanning. (A) Autoradiograph of time course (seconds) of [32P]-phosphatidic acid (PtdOH) formation. (B) Radiochromatogram of control (upper), vanadate-stimulated (2 minutes) (middle), and aspirin pretreated, vanadate-stimulated (2 minutes) (lower) platelets. All data is representative of two similar experiments each with triplicate determinations.

Immunoblot analysis indicated that vanadate caused tyrosine phosphorylation of several proteins. These proteins fall into two classes: those of approximate molecular weight 26, 29, 32, 40, and 42 Kd, the phosphorylation of which was attenuated by BW755C; and those of approximate molecular weights of 80 and 90 Kd, which were less affected by inhibition of cyclo-oxygenase. The results show that the latter group of phosphotyrosines is associated with the liberation of arachidonic acid (phosphotyrosine[s] a in Fig 9), whereas the former group is involved at some later stage in the aggregation process (phosphotyrosine[s] b in Fig 9). This is consistent with the observation that aspirin does not affect tyrosine phosphory-

Fig 5. Effects of vanadate ± aspirin on [32P]-phosphatidic acid formation. [32P]-orthophosphate prelabeled platelets (in the presence or absence of 100 μmol/L aspirin) were stirred in the presence of saponin before the addition of 100 μmol/L vanadate. Phospholipids were extracted, separated by thin-layer chromatography, autoradiographed, and quantified by radiochromatographic scanning. (A) Autoradiograph of time course (seconds) of [32P]-phosphatidic acid (PtdOH) formation. (B) Radiochromatogram of control (upper), vanadate-stimulated (2 minutes) (middle), and aspirin pretreated, vanadate-stimulated (2 minutes) (lower) platelets. All data is representative of two similar experiments each with triplicate determinations.
VANADATE-STIMULATED HUMAN PLATELETS

Fig 6. Effects of vanadate ± aspirin on protein phosphorylation. [32P]-orthophosphate prelabeled platelets (in the presence or absence of 100 μmol/L aspirin) were stirred in the presence of saponin before the addition of 100 μmol/L vanadate. Proteins were denatured and separated by SDS-PAGE (10% gel). The gel was stained, destained, dried, and autoradiographed. (A) Autoradiograph of a time course (seconds). (B) Autoradiograph of the effects of aspirin: lane A, control; lane B, vanadate (2 minutes); lane C, aspirin control; lane D, aspirin plus vanadate (2 minutes). Molecular weights (Kd), plekstrin (P), and MLC are indicated. All data is representative of two similar experiments each with triplicate determinations.

The possible regulation of neutrophil phospholipase A2, the principle enzyme involved in arachidonic acid release, by a phosphotyrosine was recently hypothesized.6 The present data supports such a role for a phosphotyrosine, which is regulated by a vanadate-sensitive PTPase, in stimulated human platelets.

The regulation of phospholipase A2 activity in platelets is not well defined. Lipocortins, or calpactins, have been re-
Fig 7. Effects of vanadate ± BW755C on antiphosphotyrosine immunoblotting. Platelets were stirred in the presence of saponin in the presence or absence of 80 μmol/L BW755C for 60 seconds before the addition of 100 μmol/L vanadate. Proteins were denatured, separated by SDS-PAGE (10% gel), transferred to nitrocellulose, immunoblotted with antiphosphotyrosine antibody 4G10, and visualized by ECL. Lane A, control; lane B, vanadate; lane C, vanadate plus BW755C. Molecular weights (Kd) and phosphotyrosines of 26, 29, 32, 40, 42, 80, and 90 Kd are indicated. Data is representative of four similar experiments.

Fig 8. Effects of vanadate ± BW755C on arachidonic acid metabolites. [3H]-arachidonic acid prelabeled platelets were stirred in the presence of saponin in the presence or absence of 80 μmol/L BW755C for 60 seconds before the addition of 100 μmol/L vanadate. The arachidonic metabolites were extracted, separated by thin-layer chromatography, and quantified by liquid-scintillation counting. Values are expressed as a percentage of the water control. Upper panel, [3H]-arachidonic acid levels; lower panel, [3H]-TxB2 levels. Solid bars, control; shaded bars, vanadate; hatched bars, BW755C plus vanadate. Data are the mean of four similar experiments.

Fig 9. Potential involvement of phosphotyrosines in vanadate-induced platelet activation. Proposed relationship of tyrosine phosphorylation to other biochemical processes in platelet activation. See text for details. Abbreviations: P-Tyr, phosphotyrosines; DAG, diacylglycerol; IP3, inositol 1,4,5 triphosphate.
The combination of orthovanadate and H$_2$O$_2$ has been reported to stimulate platelet activation, albeit after a considerable lag phase, independently of Tx production. This may reflect differences in the method of administration of the vanadate; saponin-permeabilization versus the combination of vanadate and H$_2$O$_2$. Both processes may have inherent difficulties in interpretation as both saponin and H$_2$O$_2$ may have detrimental effects on the cell membrane, although under the permeabilization conditions used in the present study little cell damage has been observed. Alternatively, the discrepancy may indicate that phospholipase A$_2$ activity and Tx generation is critical in the saponin-permeabilized system, reflecting the substantially lower elevation of cytosolic calcium levels achieved after stimulation. Regardless of the method of administration, vanadate clearly stimulates platelet activation and presents evidence for a role for phosphorylation in this process.

In conclusion, the present study shows that vanadate-induced platelet aggregation is mediated by Tx$_2$A$_3$, the production of which is associated with an inhibition of PTase activity. This is the first evidence for the regulation of arachidonic acid release by a phosphorytrosine in human platelets. The phosphoprotein is potentially of 80 or 90 Kd that may be a form of phospholipase A$_2$ or some regulatory protein. This further emphasizes both the complex nature of arachidonic acid release from human platelets and the crucial role that phosphotirosines play in signal transduction in nonproliferative cells.

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Vanadate activates platelets by enhancing arachidonic acid release

A McNicol, C Robertson and JM Gerrard