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PROTEIN-TYROSINE PHOSPHORYLATION AND AGGREGATION OF INTACT HUMAN PLATELETS BY VANADATE WITH H_2O_2

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SUMMARY: The ability of vanadate with H_2O_2 to stimulate protein-tyrosine phosphorylation and aggregation in intact human platelets was observed. Upon stimulation by these agents, there was a change in the amount of protein-tyrosine phosphorylation of four bands with molecular masses of 80-, 76-, 53- and 38-kDa proteins. The tyrosine phosphorylation in these four bands increased gradually and reached a maximum level at 2 min and then decreased. Aggregation of platelets was also induced by these agents in a dose dependent manner. The observation that the aggregation was preceded by the increase in tyrosine phosphorylation of these proteins suggests that tyrosine phosphorylation may be involved in an early stage of platelet aggregation.

Protein-tyrosine kinase activity was found to be associated with transforming gene product of certain viruses as well as receptors for several mitogenic polypeptides (1). These enzymes, therefore, are believed to be involved in transformation and proliferation of cells. The observation that high levels of protein-tyrosine kinase activity are present in platelets (2-5) strongly suggests that the role of these enzymes is not limited to transformation and proliferation. Moreover several investigators have recently shown that activators of platelets, such as thrombin and collagen, stimulate protein-tyrosine phosphorylation in intact human platelets (6-8).

Vanadate is well known as a protein-tyrosine phosphatase inhibitor (9), however the induction of certain biological effects by vanadate alone requires prolonged incubations (10,11), relatively high concentrations (12) or artificial electropermeabilization (13) in 3T3 L1 adipocytes, IM-9 lymphocytes, rat hepatocytes and human platelets, respectively. Nevertheless, in the presence of H_2O_2 which is well known as an insulinomimetic agent (14,15), orthovanadate is oxidized to pervanadate (16) which could facilitate it's entry into cells. To investigate the role of protein-tyrosine phosphorylation in platelets, we have studied employing protein-tyrosine phosphatase inhibitor as a powerful tool. In this communication we have reported that a combination of vanadate with H_2O_2 stimulates protein-tyrosine phosphorylation and aggregation in intact human platelets.

The abbreviation used is : Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

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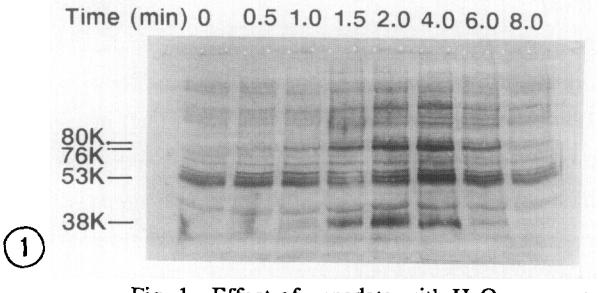
EXPERIMENTAL PROCEDURES

Materials and chemicals-----Sodium orthovanadate and H_2O_2 (30 %) were obtained from Wako pure chemicals. Prostaglandin E1 was purchased from Sigma. Other chemicals were reagent grade. Antibodies to phosphotyrosine conjugated to human immunoglobulin were prepared by the method of Ek and Heldin (17).
Isolation of human platelets-----Human blood was drawn from healthy volunteers who had not taken medication for at least the previous 2 weeks. Prostaglandin E1 (500 nM) was added to platelet-rich-plasma, and platelets were centrifuged for 15 min at 800 x g. Platelets were resuspended in a buffer (135 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 20 mM Hepes, 5 mM glucose, pH 7.4). After centrifugation, the platelets were finally suspended at a concentration of 5 x 10^8 cells / ml in the same buffer containing 1 mM $CaCl_2$.
Immunoblot procedure-----Isolated platelets were stimulated by H_2O_2, vanadate or both for appropriate dose and intervals in aggregometer cuvette at 37°C with stirring. The reactions were terminated by boiling for 3 min with a Laemmli sample buffer, and proteins were separated using 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18). Immunoblot analysis was carried out as described previously (8).
Platelet aggregation-----Isolated platelets (1.0 ml) were transferred into aggregometer cuvette and incubated for 2 min at 37°C with stirring before addition of aggregating agents. Aggregation was measured with the platelet aggregometer (Bryston Manufacturing LTD).

RESULTS

Effect of vanadate with H_2O_2 on protein-tyrosine phosphorylation in intact human platelets-----Upon stimulation by vanadate with H_2O_2, the tyrosine phosphorylation



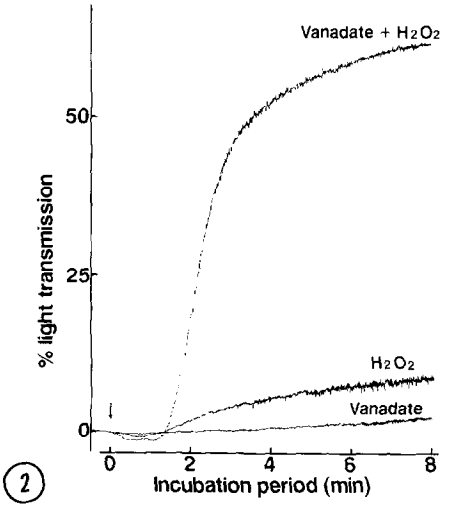


Fig. 1. Effect of vanadate with H_2O_2 on protein tyrosine phosphorylation in intact human platelets. Isolated platelets were incubated at 37°C with stirring for the indicated time in minutes in the presence of vanadate (100 μM) with H_2O_2 (1 mM). The samples were then processed for immunoblots with antibodies to phosphotyrosine as described under "EXPERIMENTAL PROCEDURES". The lines indicate the positions of proteins of molecular masses of 80-, 76-, 53- and 38-kDa that undergo tyrosine phosphorylation in response to vanadate with H_2O_2.

Fig. 2. Effect of vanadate with H_2O_2 on platelet aggregation. Isolated platelets (1 ml) were placed in aggregometer cuvette and either vanadate (100 μM), H_2O_2 (1 mM) or both was added. The aggregation was recorded as an increase in light transmission through the platelet suspension. The arrow indicates the addition of the agents.

increased in 80-, 76-, 53- and 38-kDa proteins in a time dependent manner until 2 min and after 4 min gradually decreased (Fig. 1). In particular, the phosphorylation of 76-kDa protein appeared faster than that of other proteins. In contrast, vanadate alone or H_2O_2 alone induced practically no tyrosine phosphorylation (data not shown).

Effect of vanadate with H_2O_2 on platelet aggregation-----Fig. 2 showed that vanadate with H_2O_2 induced platelet aggregation with a lag time of 1-2 min. However, vanadate alone or H_2O_2 alone had little effect on platelet aggregation. Although H_2O_2 is well known as an insulinomimetic agent, addition of vanadate (100 μM) and insulin (500 nM) to platelets had no effect on aggregation (data not shown).

Dose response for the effect of vanadate and H_2O_2 on platelet aggregation-----The effect of vanadate on platelet aggregation in the presence of 1 mM H_2O_2 was dependent on the concentration (Fig. 3A). Maximum stimulation was observed at the concentration of 100 μM. And similar results were obtained when H_2O_2 concentration was varied from 0.25 mM to 1 mM in the presence of 50 μM vanadate (Fig. 3B). Maximum stimulation was observed at the concentration of 1 mM H_2O_2.

Relationship between tyrosine phosphorylation and aggregation induced by vanadate with H_2O_2-----Fig. 4 compared the level of phosphotyrosine with a molecular mass of 76-kDa and platelet aggregation induced by these agents. The increase in tyrosine phosphorylation of 76-kDa protein preceded aggregation. Similar tendency was observed with the level of phosphotyrosine of other three proteins (data not shown). These results suggest that protein-tyrosine phosphorylation may trigger platelet aggregation.

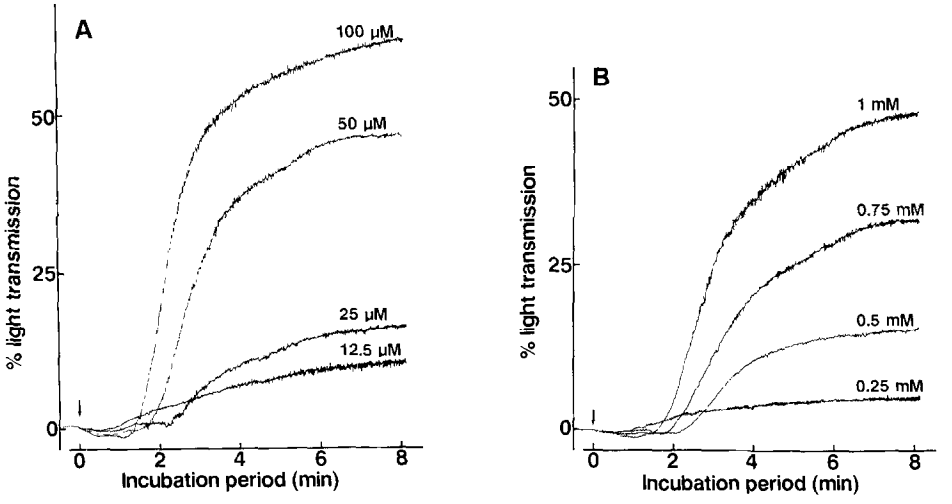


Fig. 3. Dose response for the effect of vanadate and H_2O_2 on platelet aggregation. (A) Vanadate at the indicated final concentration was added to human platelets together with 1 mM H_2O_2 as in Fig. 2. (B) H_2O_2 at the indicated final concentration was added to human platelets together with 50 μM vanadate as in Fig. 2.

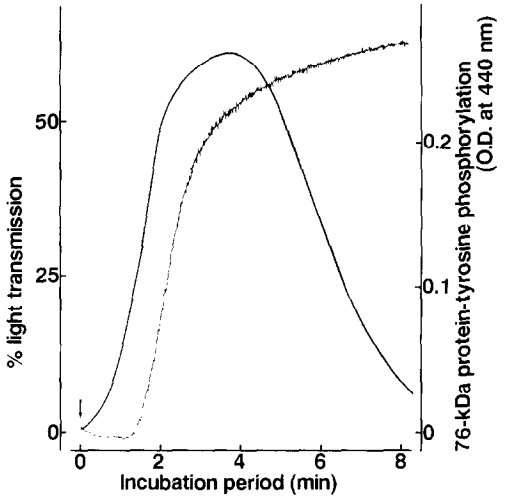


Fig. 4. Relationship between tyrosine phosphorylation and aggregation induced by vanadate with H_2O_2. The change in phosphotyrosine in the 76 kDa protein induced by vanadate with H_2O_2 as in the legend to Fig.1 was quantitated by densitometric tracing at 440 nm using a chromatogram scanner (Toyo Scientific Industry Co., model DMU-33C). Aggregation was performed as in the legend to Fig. 2.

DISCUSSION

In the present study we demonstrated that vanadate with H_2O_2 increased tyrosine phosphorylation and induced platelet aggregation. According to recent reports (19,20), vanadate with H_2O_2 did not activate protein-tyrosine kinase(s) but inhibited protein-tyrosine phosphatase(s). The ability of these agents to increase in protein-tyrosine phosphorylation and to induce platelet aggregation is probably due to inhibition of protein-tyrosine phosphatase(s). We previously reported the increase in tyrosine phosphorylation of 135-, 124- and 76-kDa proteins was observed in activations of human platelets by thrombin and collagen (8). On the other hand, in the case of vanadate with H_2O_2, we demonstrated that lower molecular weight proteins were also phosphorylated. This discrepancy may result from the difference between the use of physiological activators and pharmacological agents. Lerea *et al.* (13) showed that 50- and 38-kDa proteins were phosphorylated on tyrosine residues by the addition of vanadate and molybdate in electropermeabilized human platelets. This difference from our results is probably reflected from the following reasons; 1) difference of specificity of protein-tyrosine phosphatase inhibitors. In fact Fantus *et al.* (19) found that the action of vanadate for an inhibition of phosphotyrosine phosphatases activity was distinct from that of pervanadate. 2) Difference between electropermeabilized platelets and intact platelets employed.

In the case of platelet activation, it is believed to be the hydrolysis of phosphatidylinositol 4,5-bisphosphate to form inositol 1,4,5-triphosphate and diacylglycerol (21-24). Inositol 1,4,5-triphosphate causes Ca^{2+} release from the platelet-

dense tubular system, contributing thereby to the increase in the cytosolic free Ca^{2+} concentration (25) and diacylglycerol directly activates protein kinase C (26). On the other hand, protein-tyrosine phosphorylation occurs when platelets are stimulated by physiological activators (6-8). However, the crosstalk among these systems is not fully understood at present. In this experiment, the mechanism of platelet aggregation by vanadate with H_2O_2 is precisely unknown. It has been reported that phospholipase C is phosphorylated on tyrosine residues in cultured cells in response to platelet derived growth factor and epidermal growth factor (27,28). Thus, tyrosine phosphorylation may influence the degradation of phosphoinositides. Further studies are necessary to elucidate the crosstalk among protein-tyrosine phosphorylation, protein serine/threonine phosphorylation, phosphoinositides breakdown and so on.

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REFERENCES

1. Hunter, T. and Cooper, J.A. (1985) Annu. Rev. Biochem. 54, 897-930.
2. Tuy, F.P.D., Henry, J., Rosenfeld, C. and Kahn, A. (1983) Nature 305, 435-438.
3. Nakamura, S., Takeuchi, F., Tomizawa, T., Takasaki, N., Kondo, H. and Yamamura, H. (1985) FEBS Lett. 184, 56-59.
4. Nakamura, S., Yanagi, S. and Yamamura, H. (1988) Eur. J. Biochem. 174, 471-477.
5. Golden, A., Nemeth, S.P. and Brugge, J.S. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 852-856.
6. Ferrel, J.E., Jr. and Martin, G.S. (1988) Mol. Cell. Biol. 8, 3603-3610.
7. Golden, A. and Brugge, J.S. (1989) Proc. Natl. Acad. Sci. USA 86, 901-905.
8. Nakamura, S. and Yamamura, H. (1989) J. Biol. Chem. 264, 7089-7091.
9. Swarup, G., Cohen, S. and Garbers, D.L. (1982) Biochem. Biophys. Res. Commun. 107, 1104-1109.
10. Frost, S.C., Kohanski, R.A. and Lane, D.M. (1987) J. Biol. Chem. 262, 9872-9876.
11. Torossian, K., Freedman, D. and Fantus, I.G. (1988) J. Biol. Chem. 263, 9353-9359.
12. Seglen, P.O. and Gordon, P.B. (1987) J. Biol. Chem. 256, 7699-7701.
13. Lerea, K.M., Tonks, N.K., Krebs, E.G., Fischer, E.H. and Glomset, J.A. (1989) Biochemistry 28, 9286-9292.
14. Czeck, M.P., Lawrence, J.C., Jr. and Lynn, W.S. (1974) Proc. Natl. Acad. Sci. USA 71, 4173-4177.
15. Little, S.A. and de Haën, C. (1980) J. Biol. Chem. 255, 10888-10895.
16. Howarth, O.W. and Hunt, J.R. (1979) J. Chem. Soc. Dalton. Trans. 1388-1391.
17. Ek, B. and Heldin, C.-H. (1984) J. Biol. Chem. 259, 11145-11152.
18. Laemmli, U.K. (1970) Nature 227, 680-685.
19. Fantus, I.G., Kadota, S., Deragon, G., Foster, B. and Posner, B.I. (1989) Biochemistry 28, 8864-8871.
20. Heffetz, D., Bushkin, I., Dror, R. and Zick, Y. (1990) J. Biol. Chem. 265, 2896-2902.
21. Rittenhouse-Simmons, S. (1979) J. Clin. Invest. 63, 580-587.
22. Broekmann, J., Ward, J. and Marcus, A. (1980) J. Clin. Invest. 66, 275-283.
23. Billah, M. and Lapetina, E. (1982) J. Biol. Chem. 257, 12705-12708.
24. Agranoff, B., Murthy, P. and Seguin, E. (1983) J. Biol. Chem. 258, 2076-2078.
25. Berridge, M.J., Dawson, R.M.C., Downes, C.P., Heslop, J.P. and Irvine, R.F. (1983) Biochem. J. 212, 473-482.
26. Nishizuka, Y. (1984) Nature 308, 693-698.
27. Wahl, M.I., Daniel, T.O. and Carpenter, G. (1988) Science 241, 968-970.
28. Meisenhelder, J., Suh, P.-G., Rhee, S.G. and Hunter, T. (1989) Cell 57, 1109-1122.