Trakia Journal of Sciences, Vol. 12, Suppl. 1, pp 20-24, 2014

Available online at:
http://www.uni-sz.bg

ISSN 1313-7050 (print)
ISSN 1313-3551 (online)

INFLUENCE OF MODULATORS OF PROTEIN PHOSPHORYLATION AND ION TRANSPORT ON PLATELET GLYCOLYSIS

K. Boyanov*, A. Maneva

Department of Biochemistry, Pharmaceutical Faculty, Medical University, Plovdiv, Bulgaria

ABSTRACT

PURPOSE: We investigate influence of wortmannin (inhibitor of phosphoinositide 3-kinase – PI3K), caffeine (phosphodiesterase inhibitor and modulator of protein phosphorylation), quercetin (redox system and modulator of phosphorylation), 5-(N,N-Dimethyl)amiloride hydrochloride (inhibitor of Na^+/H^+-antiport) and ouabain (inhibitor of Na^+/K^+-ATPase and modulator of phosphorylation) on platelet glycolysis. **METHODS:** *1. Isolation of platelets* – Platelets, obtained from human blood of healthy donors, were washed and suspended in PBS to a final concentration 25×10^6 cells/ml. *2. Incubation experiments* – 25×10^6 platelets were incubated with 10 mM glucose, agents and 50 mM PBS for 30 min at 37°C. Control does not contain agents. *3. Determination of lactate content* – The method was based on pyruvate reduction in the presence of NAD.H and lactate dehydrogenase. To estimate lactate, the reaction was carried out in the excess of NAD^+, and the absorbance of NAD.H was measured at 340 nm wavelength. **RESULTS:** All modulators have reliable stimulating effect on glycolysis, which is most strongly manifested on exposure to wortmannin (185%, $p < 0.01$) and amiloride (146%, $p < 0.001$). **CONCLUSIONS:** These results indicate that, in the regulation of platelet glycolysis take part signaling pathways involving PI3K and cyclic AMP (cAMP), systems for ion transport and maintenance of cell redox status.

Key words: Platelets, Glycolysis, Lactate, Modulators

INTRODUCTION

The main intracellular pathways are regulated by phosphorylation/dephosphorylation and changes in ionic balance. We use some modulators of these processes to study their interference on platelet glycolisys – the metabolic pathway for energy supply.

Wortmannin is a potent and specific inhibitor of PI3K (1). PI3K can phosphorylate the D3 position of phosphatidylinositol (Ptdlns), Ptdlns (4) phosphate (Ptdlns (4) P), or Ptdlns (4, 5) bisphosphate (Ptdlns (4, 5) P_2) to produce Ptdlns (3) P, Ptdlns (3, 4) P_2, or Ptdlns (3, 4, 5)trisphosphate (Ptdlns (3, 4, 5) P_3), respectively (1). Human platelets synthesize PtdIns (3, 4, 5) P_3 and PtdIns (3, 4) P_2 in response to thrombin and these PI3K products are involved in modifications of the cytoskeleton (2).

Caffeine is an example of non-specific phosphodiesterase inhibitor (3). Phosphodiesterase is a ubiquitous enzyme that catalyses the hydrolysis of phosphodiester bonds. It is responsible for the hydrolysis of cAMP (3). Protein kinase A (PKA) translates cAMP level into protein phosphorylation pattern. Human platelets contain micromolar concentrations of PKA (4). cAMP is also known as powerful inhibitor of platelet aggregation (4). Thus caffeine is responsible for the accumulation of cAMP and subsequent inhibition of platelet aggregation.

Quercetin is a bioflavonoid that has an antioxidant activity; suppress collagen, thrombin, or ADP-induced platelet aggregation; inhibits whole-cell tyrosine protein phosphorylation in platelets (5) and attenuated

***Correspondence to**: *Krasimir Boyanov, Department of Biochemistry, Pharmaceutical Faculty, Medical University-Plovdiv, Bulgaria, krasi.boyanov@abv.bg, +a_maneva@gbg.bg*

PI3K, Akt, ERK2, JNK1, and p38 MAPK activations (6). This result suggests quercetin may have a potential to treat cardiovascular diseases involving aberrant platelet activation and inflammation (6).

Amiloride is a potent inhibitor of human platelet Na^+/H^+ exchanger (NHE) involved in intracellular pH regulation (7). Activation of NHE results from activation of human platelets (8). Inhibition of this transport, inhibits aggregation and secretion, and prevents cytoskeletal assembly in thrombin- or ADP-activated platelets (8).

Ouabain, as an inhibitior of plasma membrane Na^+/K^+-ATPase, enhance intracellular sodium ion concentration and induce a time-dependent increase in basal and thrombin-stimulated cytosolic calcium concentration in human platelets. In that manner the glycoside influences Na^+-Ca^{2+} exchange which play an important role in Ca^{2+} homeostasis of the human platelet (9).

It was previously shown that these modulators interfere with erythrocyte glycolisys – quercetin itself enhanced the ATPase activity but did not affect the lactate formation (10). Caffeine decreased significantly the lactate content in the presence of lactoferrin (Lf) (11). Amiloride is able to decline the stimulatory effect of Lf on the lactate generation (11). Ouabain take part in cell signals that antagonize or compete with Lf stimulatory effect on erythrocyte glycolysis; wortmannin reduced with 45% this effect of Lf (11).

That's the way the aim of this study came out – to determine the influence of the chosen concentrations of the modulators of protein phosphorylation and ion transport on glycolysis in resting platelets.

MATERIALS AND METHODS

Isolation of platelets – Human platelets were obtained from healthy donor blood using Na-citrate as an anticoagulant. Platelet rich plasma was separated by centrifugation at 400 g for 10 min (12). The supernatant then was centrifuged (15 min, 1100 g) to obtain platelets (12). After washing three times in 1mM EDTA/phosphate buffered saline – PBS (pH=7.4) and once in PBS only, cells were suspended in 50 mM PBS to a final concentration 25×10^6 cells (12). All procedures were performed at room temperature.

Incubation experiments – Incubation mixture (1 ml) contained: platelets – 25×10^6 cells; glucose – 10 mM; agents; 50 mM PBS pH 7,4. The incubation time was 30 min at 37°C (13). Control does not contain modulators. The mentioned above agents were used in the following concentrations: wortmannin - 50 nM; caffeine - 20 mM; quercetin - 1,5 μM; amiloride - 1mM and Ouabain - 0,5 mM.

Determination of lactate content – After centrifugation of the incubation mixture for 10 min at 2000 g platelets were resuspended in 0.4 ml 10% trichloroacetic acid (14). For entire precipitation samples were cooled on ice 10 min and than centrifuged again at the same conditions (14). 0.1 ml from the supernatant was used further according to the prescription of the test kit obtained from Sigma-Aldrich. The method was based on pyruvate reduction in the presence of NAD.H and lactate dehydrogenase. To estimate lactate, the reaction was carried out in the excess of NAD^+, and the absorbance of NAD.H was measured at 340 nm wavelength. The results were calculated according to the formula applied in the test and presented in μM.

RESULTS

The effect of the agents on platelet glycolysis was estimated by measuring the lactate formed. The results are presented in **Table 1 and Figure 1.**

Table 1. *Production of lactate and corresponding stimulation of glycolysis in the presence of various modulators. n - number of repetitions; SD - standard deviation; p - level of significance*

Agents, nM (n=6)	Lactate, μM (x ± SD)	p	Stimulation, %
Without an agent	1.823 ± 0.911	0	–
Wortmannin, 50 nM	5.191 ± 2.890	< 0.01	+ 185
Caffeine, 20 mM	2.708 ± 0.246	< 0.05	+ 48
Quercetin, 1,5 μM	3.360 ± 1.508	< 0.05	+ 84
Amiloride, 1 mM	4.485 ± 0.671	< 0.001	+ 146
Ouabain, 0.5 mM	3.159 ± 0.722	< 0.05	+ 73

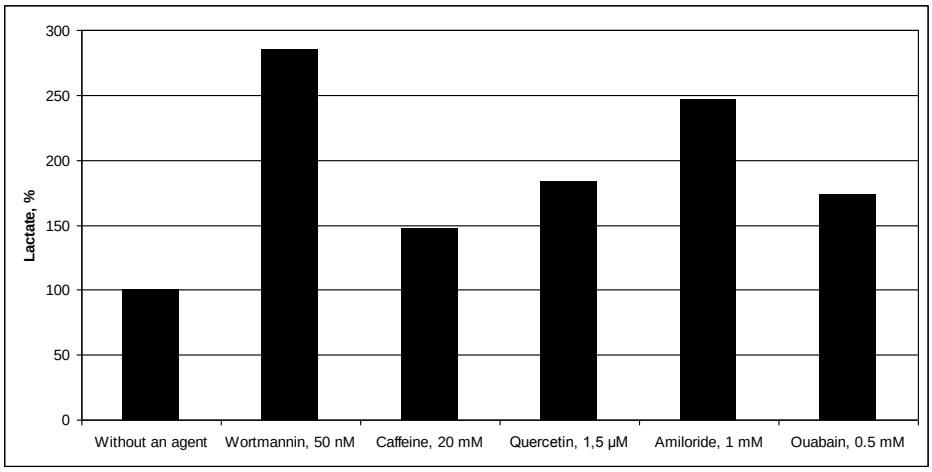

Figure 1. Stimulation lactate production in the presence of various modulators. The concentration of lactate in the absence of an agent is 100%.

All modulators used have reliable stimulating effect on glycolysis, in the appended concentrations, which is most strongly manifested on exposure to wortmannin (185%, $p < 0.01$) and amiloride (146%, $p < 0.001$).

DISCUSSION

Platelets have an active bioenergetics. They can support both anaerobic and aerobic glycolysis (15). There is no consensus regarding which of the two processes dominates. End product of anaerobic glycolysis is lactate. Pyruvate is exported to oxidative phosphorylation in mitochondria under aerobic conditions. In our experiments equilibrium of lactate dehydrogenase reaction shifts to the formation of lactate. Under such conditions pyruvate does not penetrate into the mitochondria and the entire amount was reduced to lactate (13).

Glycolytic pathway is the major pathway involved in energy exchange and therefore highly regulated. It is known that there are differences in the regulation mechanisms, depending on the cell type (16).

Based on *in vitro* studies using wortmannin and LY294002, there is evidence for an important role for PI3K in regulating a broad range of functional platelet responses, including primary platelet adhesion, cytoskeletal remodelling and platelet aggregation. One or more PI3K isoforms appears to be important for G_i-dependent activation of Rap1b and adhesion receptor activation of PLCγ isoforms (17). Our results show stimulation of the formation of lactate in the presence wortmannin, indicating that the cellular PI3K signals inhibit the formation of lactate **(Figure 1 and Table 1).**

Caffeine stimulates glycolysis. Caffeine is a phosphodiesterase inhibitor and modulator of the phosphorylation. It is known that the methylxanthines are able to inhibit the cyclic nucleotide-independent protein kinases present in the cytosol and erythrocyte membrane (18). The regulatory enzymes in glycolysis are active dephosphorylated. It is possible caffeine to inhibit some protein kinases and so to activate platelet glycolysis **(Figure 1 and Table 1)**.

Inhibitory potencies of structurally distinct flavonoids (quercetin, apigenin and catechin) and plasma metabolites (tamarixetin, quercetin-3'-sulphate and quercetin-3-glucuronide) for collagen-stimulated platelet aggregation and 5-hydroxytryptamine secretion included blocking Fyn kinase activity and the tyrosine phosphorylation of Syk and PLCγ2 following internalization (9). Dual roles of quercetin in platelets was found: PI3K and MAPK inhibition, and cAMP-dependent vasodilator-stimulated phosphoprotein stimulation (11). Quercetin can stimulate glycolysis **(Figure 1 and Table 1)** as an inhibitor of some of these kinases, or as a final acceptor for electrons, formed in the glyceraldehyde-3P-dehydrogenase reaction (19). Amiloride is an inhibitor of Na^+/H^+-antiport in platelets (7). Our results show stimulation of lactate formation in the presence of amiloride **(Figure 1 and Table 1).** Amiloride interrupts the

removal of the protons out of the cell, thereby increasing their intracellular concentration and hence the ability to reduce pyruvate.

Ouabain is a highly specific inhibitor of Na^+/K^+-ATPase and signal transducer. Studies with [3H]ouabain revealed an increase in the number of accessible ouabain binding sites from approximately 55 per resting platelet to approximately 625 per ADP-activated platelet (20). Binding of ouabain to Na^+/K^+-ATPase changes the interaction of the enzyme with neighboring membrane proteins and induces the formation of multiple signaling modules, resulting in activation of Src, transactivation of the EGF receptor, and increased production of reactive oxygen species (21). There is no data about the cellular signals that ouabain uses in platelets. Probably, the observed stimulating effect on platelet glycolysis is independent of its effect on the sodium pump (figure 1 and table 1).

CONCLUSIONS

These results indicate that, in the regulation of platelet glycolysis signaling pathways involving PI3K and cyclic AMP, systems for ion transport and maintenance of cell redox status take part.

REFERENCES

1. Wymann M.P., Bulgarelli-Leva G., Zvelebil M.J., Pirola L., Vanhaesebroeck B., Waterfield M.D., Panayotou G., Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol Cell Biol.* 16:1722-33. 1996.
2. Zhang J., King W.G., Dillon S., Hall A., Feig L., Rittenhouse S.E., Activation of platelet phosphatidylinositide 3-kinase requires the small GTP-binding protein Rho. *J Biol Chem.* 268:22251-4. 1993.
3. Feneck R., MB BS FESC FRCA, Phosphodiesterase inhibitors and the cardiovascular system. *Contin Educ Anaesth Crit Care Pain*. 7:203-207. 2007.
4. Smolenski A., Novel roles of cAMP/cGMP-dependent signaling in platelets. *J Thromb Haemost*. 10:67-76. 2012.
5. Hubbard G.P., Wolffram S., Lovegrove J.A., Gibbins J.M., The role of polyphenolic compounds in the diet as inhibitors of platelet function. *Proc Nutr Soc*. 62:469-78. 2003.
6. Oh W.J., Endale M., Park S.C., Cho J.Y., Rhee M.H., Dual Roles of Quercetin in Platelets: Phosphoinositide-3-Kinase and MAP Kinases Inhibition, and cAMP-Dependent Vasodilator-Stimulated Phosphoprotein Stimulation. *Evid Based Complement Alternat Med*. 2012;2012:485262. doi: 10.1155/2012/485262. Epub 2012 Dec 17.
7. Laeckmann D., Rogister F., Dejardin J.V., Prosperi-Meys C., Géczy J., Delarge J., Masereel B., Synthesis and biological evaluation of aroylguanidines related to amiloride as inhibitors of the human platelet Na(+)/H(+) exchanger. *Bioorg Med Chem*. 10:1793-804. 2002.
8. Siffert W., Gengenbach S., Scheid P., Inhibition of platelet aggregation by amiloride. *Thromb Res*. 44:235–240. 1986.
9. Roevens P., de Chaffoy de Courcelles D., Ouabain increases the calcium concentration in intracellular stores involved in stimulus-response coupling in human platelets. *Circ Res*. 67:1494-502. 1990.
10. Maneva A., Taleva B., Effect of some flavonic compounds and ascorbic acid on lactoferrin stimulation of erythrocyte glycolysis and Na+/K+-atpase activity. *Z Naturforsch C*. 63:773-9. 2008.
11. *Taleva Z.B., Maneva A., Interference of some modulators of protein phosphorylation and ion transport with lactoferrin stimulatory effect on erythrocyte glycolysis.* Biotechnol. & Biotechnol. Eq. *23:498-501. 2009.*
12. Maneva A., Taleva B., Manev V., Sirakov L., Lactoferrin binding to human platelets. *Int J Biochem*. 25:707-712. 1993.
13. Cesar J.M., Pallares E., Rubi J., Navarro J.L., Lactate production by thrombin-activated platelets of patients with primary thrombocythemia. *Thromb Res*. 118:335-339. 2006.
14. Maneva A., Taleva B., Maneva L., Lactoferrin-Protector against Oxidative Stress and Regulator of Glycolysis in Human Erythrocytes. *Z Naturforsch C*. 58:256-262. 2003.
15. Kramer P.A., Ravi S., Chacko B., Johnson M.S., Darley-Usmar V.M., A review of the mitochondrial and glycolytic metabolism in human platelets and leukocytes: Implications

for their use as bioenergetic biomarkers. *Redox Biol*. 10:206-210. 2014.
16. Sorbara L.R., Davies-Hill T.M., Koehler-Stec E.M., Vannucci S.J., Horne M.K., Simpson I.A., Thrombin-induced translocation of GLUT3 glucose transporters in human platelets. *Biochem J*. 328:511-6. 1997.
17. Schoenwaelder S.M., Ono A., Sturgeon S., Chan S.M., Mangin P., Maxwell M.J., Turnbull S., Mulchandani M., Anderson K., Kauffenstein G., Rewcastle G.W., Kendall J., Gachet C., Salem H.H., Jackson S.P., Identification of a unique co-operative phosphoinositide 3-kinase signaling mechanism regulating integrin alpha IIb beta 3 adhesive function in platelets. *J Biol Chem*. 282:28648-58. 2007.
18. Biovin P., Lecomte M.C., Galand C., Methylxanthines and phosphorylation of the constituents of the membrane of the human red blood cell. *Pathol Biol*. 36:1042-6. 1988.
19. Chen Y., Deuster P., Comparison of quercetin and dihydroquercetin: antioxidant-independent actions on erythrocyte and platelet membrane. *Chem Biol Interact*. 182:7-12. 2009.
20. Bork T., Mrsny R.J., Appearance of additional ouabain binding sites on platelet surfaces following activation. *Exp Cell Res*. 208:189-96. 1993.
21. Xie Z., Molecular mechanisms of Na/K-ATPase-mediated signal transduction. *Ann N Y Acad Sci*. 986:497-503. 2003.