J. Physiol. Biochem., 52 (1), 45-48, 1996
Revista española de Fisiología

Platelets isolated by albumin gradient retain normal activation pathways*

A. M. Vecino, J. L. Navarro, and J. M. Cesar

Departamento de Hematología, Hospital Ramón y Cajal
28034 Madrid (Spain)

(Received on November 8, 1995)

A. M. VECINO, J. L. NAVARRO and J. M. CESAR. *Platelets isolated by albumin gradient retain normal activation pathways.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 52 (1), 45-48, 1996.

Isolated platelets from samples with low counts produce technical problems. Albumin gradient (AG) has been shown to be useful for this purpose, preserving the aggregating response of these cells. The influence of this method in the enzymatic pathways that regulate the platelet activation is studied. Platelets were isolated by either AG or conventional centrifugation methods and labelled with C-14-arachidonic acid (C-14-AA). Isolated platelets were activated with thrombin (5 U/ml) and lipids were extracted according to Bligh and Dyer. Platelet phospholipids and prostanoids were resolved by TLC. The incorporation of C-14-AA by platelets was similar by both methods (31.7 ± 18 % versus 47.2 ± 6.9 %), as well as the distribution of C-14-AA in the five major platelet phospholipids. Formation of radioactive thromboxane B2, hydroxyheptadecatrienoic acid and hydroxyeicosatetraenoic acid by activated platelets was also similar by both methods. These findings suggest that platelet isolation by albumin gradient preserves the enzymatic pathways responsible for the activation of these cells.

Key words: Platelet isolation, Albumin gradient.

Studies of platelet metabolism are usually performed in plasma free medium. Isolation of these cells produces technical problems in samples with low platelet counts. Albumin gradient has been shown to be useful for concentrating these cells, maintaining the platelet availability to aggregate in response to physiological agonists (15). We have studied the generation of active metabolites in platelets isolated by this procedure to evaluate whether this method preserves the function of these metabolic pathways.

Correspondence to J. M. Cesar (Tel.: 34 1 336 82 24; Fax: 34 1 336 90 16).
*This work was supported by FIS 92/0405, INSALUD (Spain).

Materials and Methods

Thin-layer chromatography (TLC) plates (Silica gel 60) and solvents were supplied by Merck (Darmstadt, Germany). C-14-Arachidonic acid (C-14-AA) was obtained from New England Nuclear (Southampton, U.K.). Phospholipid standards and bovine albumin were purchased from Sigma. ADP and collagen were supplied by Stago (Asnieres, France), calcium ionophore A23187 and arachidonic acid (AA) were obtained from Menarini (Firenze, Italy).

Blood was collected from six healthy volunteers in silicone-coated tubes containing trisodium citrate 3.8 %. Samples were centrifuged at 150 x *g* for 15 min to obtain platelet rich plasma (PRP). Residual blood was centrifuged at 1500 x *g* for 15 min to obtain platelet poor plasma (PPP). Albumin gradient was performed according to WALSH (15). For this purpose 9 ml of PRP were layered over 1 ml of 50 % bovine albumin in a 10 ml plastic tube. Tubes were centrifuged at 2700 x *g* for 15 min. A platelet band of 0.5 cm just above the albumin layer was carefully removed.

For aggregation studies, PRP or albumin-isolated platelets were adjusted to 300 x 10^9/L with autologous plasma. The aggregation tests were carried out in an aggrecorder II PA-3220 (Kyoto, Japan) according to BORN (4). The final concentration of aggregating agents were AA 1.4 mM, ADP 5 μM, collagen 10 μg/ml and ionophore A-23187 5 μg/ml.

For metabolic studies, PRP was added to EDTA 5 mM (final concentration). After 10 min samples were divided into two aliquots, and platelets were processed by albumin gradient or isolated by centrifugation at 1200 x *g* for seven min (samples of 3 ml). Both, the platelet pellet and the albumin-isolated platelets were washed three times in Tris-saline-glucose-EDTA (Tris 15 mM, sodium chloride 0.134 M, glucose 5 mM, EDTA 1 mM, pH 7.4). Washed platelets were resuspended in Tris-saline-glucose albumin 1.5 % and incubated with 0.5 μCi of C-14-arachidonic acid (C-14-AA 880 mCi/mmol) for 1 h at 37 °C. Labelled platelets were washed three times and resuspended in tris-saline-glucose at 800 x 10^9/L. Finally, samples of 1 ml were incubated at 37 °C with saline solution or thrombin (5 U/ml) for 10 min. The incubation was terminated adding iced chloroform:methanol 1:2 (v/v) and lipids were extracted according to BLIGH and DYER (5). Platelet phospholipids (PLs) were resolved by a two dimensional thin-layer-chromatography (TLC) as described (6). This method resolves phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS). Prostanoids were chromatographed on single TLC as previously reported (6). Spots were visualized by iodine or autoradiography. Statistic analyses were made by the Student's paired-*t*-test ($n = 6$).

Results

Platelets isolated in albumin gradient retained the aggregating response for all agonists even though there was a diminution in the increase of transmittance with respect to the conventional procedure (table I). Minor changes were observed in C-14-AA incorporation by platelets isolated by albumin gradient with regard to the conventional isolated cells (31.7 ± 18 % versus 47.2 ± 6.9 % of total radioactivity respectively, $p < 0.05$). There were no changes in the distribution of C-14-AA in platelet phospholipids between both

Table I. *Platelet aggregation by agonists.* Platelet rich plasma or platelets obtained by albumin gradient were adjusted to 300 10^9/L with autologous plasma for aggregation studies. Data indicate increased of light transmittance (mean ± S.D., n = 6).

	Albumin Gradient	Platelet Rich Plasma
Arach. acid, 1.4 mM	59.5 ± 12	77.3 ± 14
ADP, 5 µM	44.6 ± 7	73.0 ± 11*
Collagen, 10 µg/ml	45.6 ± 6	83.0 ± 10**
Ionophore, 5 µg/ml	58.3 ± 16	77.0 ± 11

*p < 0.01; **p < 0.001.

Table II. *C-14-AA incorporation in platelet phospholipids of rest platelet.* Washed platelets were resuspended in Tris-saline-glucose albumin 1.5 % and incubated with 0.5 µCi of C-14-arachidonic acid for 1 h at 37 °C. Platelet phospholipids were resolved by two dimensional thin-layer chromatography. Results indicate % of total radioactivity (mean ± S.D., n = 6).

	Albumin Gradient	Conventional isolated cells
Phosphatidylserine	3.9 ± 1.0	3.6 ± 0.2
Sphingomyelin	traces	traces
Phosphatidylcholine	67.5 ± 1.8	67.6 ± 2.4
Phosphatidylinositol	12.6 ± 3.1	11.9 ± 1.9
Phosphatidylethanolamine	16.0 ± 3.1	16.9 ± 1.9

Table III. *Prostanoid synthesis by thrombin-stimulated platelets.* Samples of 1 ml contained 800 x 10^9 platelets were incubated with thrombin (5 UI/ml) for 10 min at 37 °C. Results are expressed as percentage of radioactivity (mean ± S.D., n = 6).

	Albumin Gradient	Conventional Isolated cells
TxB2	1.0 ± 0.9	1.6 ± 0.5
HHT	1.1 ± 0.9	1.5 ± 0.4
HETE	2.0 ± 0.5	3.5 ± 2.2

methods (table II). The generation of radioactive metabolites by thrombin stimulated platelets was similar by both procedures (table III).

Discussion

Results of the aggregation test of platelets obtained by albumin gradient are in agreement with the normal range of aggregation although they were lower than those obtained by conventional methods and those described by WALSH for this procedure (15). We did not wash the platelets after the albumin gradient for aggregation tests. This circumstance probably caused the presence of residual albumin in the samples which could decrease the platelet response to agonists (9). C-14-AA is mainly incorporated in platelet phospholipids by both acylation and transacylation pathways (3, 10, 14). Minor differences were observed in the platelet incorporation of C-14-AA between both methods which probably results from the binding of arachidonic acid to residual albumin. However the distribution of radioactivity in platelet phospholipids was similar by both methods and it was in agreement with others (10). Activated platelets release C-14-AA from membrane phospholipids by two routes, 1) the consecutive action of phospholipase C (which generates diacylglycerol) and glycerol lipases (1, 7, 11, 13); and 2) the activation of phospholipase A-2 which cleaves the position 2 of platelet phospholipids (2). C-14—AA is further converted to thromboxane or 12-L-hydroxy-5,8,10-heptadecatrienoic acid (HHT) by prostaglandinsynthethase and thromboxane synthetase (8), or to a 12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) by lipoxygenase (12). Formation of these

metabolites by platelets isolated by albumin gradient was normal, indicating a normal function of these pathways. Summarizing, this study suggests that gradient albumin is a useful method to perform metabolic studies of platelets in samples with low counts.

A. M. VECINO, J. L. NAVARRO, y J. M. CESAR. *Plaquetas aisladas por gradiente de albúmina mantienen las vías de activación normales.* J. Physiol. Biochem. (Rev. esp. Fisiol.), 52 (1), 45-48, 1996

La presencia de trombopenia plantea problemas técnicos en la obtención de plaquetas para estudios funcionales. El gradiente de albúmina (AG) permite la concentración de estas células y es útil para realizar pruebas de agregación. Se estudia aquí la eficacia de este método en las vías enzimáticas que regulan la activación plaquetaria. Las plaquetas se aíslan por AG o por métodos convencionales y se marcan con C-14-ácido araquidónico (C-14-AA). Las plaquetas se activan con trombina (5 U/ml) y los lípidos se extraen por el método de Bligh y Dyer. Los fosfolípidos y los prostanoides se separaran por cromatografía de capa fina. La incorporación de C-14-AA es similar por ambos métodos (31,7 ± 18 % respecto de 47,2 ± 6,9 %), así como la distribución de la radioactividad en los principales fosfolípidos plaquetarios. No se observan cambios en la generación de metabolitos del C-14-AA. Estos resultados indican que la separación en gradiente de albúmina es útil para estudios bioquímicos plaquetarios.

Palabras clave: Gradiente de albúmina, Separación de plaquetas.

References

1. Bell, R..L., Kennerly, R..A. and Standford, N. (1979): *Proc. Natl. Acad. Sci. USA*, 76, 3238-3241.
2. Bills, T. K., Smith, J. B. and Silver, M. J. (1977): *J. Clin. Invest.*, 60, 1-6.
3. Bills, T., Smith, J. B. and Silver, J. M. (1976): *Biochim. Biophys. Acta*, 424, 302-314.
4. Born, G. V. R. (1962): *Nature*, 194, 927-928.
5. Bligh, E. G. and Dyer, W. J. (1959): *Can. J. Biochem. Physiol.*, 37, 911-917.
6. Cesar, J. M. and Navarro, J. L. (1990): *Br. J. Haematol.*, 74, 295-299.
7. Chau, L. Y. and Tai, H. H. (1981): *Biochem. Biophys. Res. Commun.* 100, 1688-1695.
8. Hamberg, M., Svensson, J. and Samuelsson, B. (1975): *Proc. Natl. Acad. Sci USA*, 72, 2994-2998.
9. Jorgensen, K. J.and Stoffersen, E. (1980): *Thromb. Res.*, 17, 13-18.
10. Kramer, R. M., Pritzker, C. R. and Deykin, D. (1984): J. Biol. Chem., 259, 2403-2406.
11. Lapetina, E. G. and Cuatrecasas, P. (1979): *Biochim. Biophys. Acta*, 573, 394-402.
12. Nutgeren, D. H. (1975): *Biochim. Biophys. Acta*, 380, 299-307.
13. Rittenhouse-Simmons, S. (1979): *J. Clin. Invest.*, 63, 580-587.
14. Silver, M. J., Smith, J. B., McKean, M. L. and Bills, T. K. (1980): In "Hemostasis, prostaglandins, and renal diseases". (Remuzzi, G., Mecca, G. and Gaetano, G., eds.). Raven Press, New York, pp. 159-173.
15. Walsh, P. N. (1972): *Brit. J. Haematol.*, 22, 205-217.