Vol. 187, No. 1, 1992 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1992 Pages 353-358

ACYL-TRAFFICKING IN MEMBRANE PHOSPHOLIPID FATTY ACID TURNOVER: THE TRANSFER OF FATTY ACID FROM THE ACYL-L-CARNITINE POOL TO MEMBRANE PHOSPHOLIPIDS IN INTACT HUMAN ERYTHROCYTES

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Received July 13, 1992

In this work we have investigated the transfer of radioactive palmitic acid between membrane phospholipids and acyl-L-carnitines in intact human erythrocytes. During the incubation period of labeled erythrocyte in non-defatted bovine serum albumin, radioactivity in phosphatidylcholine and phosphatidylethanolamine increased. On the contrary, a decrease of radioactivity in erythrocyte palmitoyl-L-carnitine was observed. 2-Tetradecylglycidic acid, an irreversible erythrocyte carnitine palmitoyltransferase inhibitor, abolished any radioactivity changes in both phospholipids and palmitoyl-L-carnitine. Similar findings were obtained by using erythrocytes labeled with radioactive oleic acid. Our data suggest that in human erythrocytes a carnitine palmitoyltransferase-catalyzed acyl transfer from acyl-L-carnitine to phospholipids, rather than a previously described fatty acid transfer from phosphatidylcholine to phosphatidylethanolamine, is operative.

Fatty acid incorporation into erythrocyte membrane phospholipids occurs mainly through the combined action of two enzymes: a phospholipase A_2, which removes the fatty acid esterified into membrane phospholipids, and an acyl-CoA lysophospholipid acyltransferase, which reacylates the corresponding lyso-derivative [1-3]. In addition, since the latter enzyme requires acyl-CoA, an ATP-dependent reaction, catalyzed by a long-chain acyl-CoA synthetase, provides acyl-CoA. However, previous studies from our laboratory have indicated that erythrocyte carnitine palmitoyltransferase (CPT) may also be involved in the membrane phospholipid fatty acid turnover of human erythrocytes [4-6]. We have hypothesized that the reversible transfer of long chain fatty acids from CoA to L-carnitine, catalyzed by erythrocyte CPT, may finely tune the acyl-CoA/free CoA ratio which is critical for the physiological expression of the membrane phospholipid fatty acid turnover. An important aspect of our hypothesis is that acyl-L-carnitines may serve as a reservoir of acyl-units for the

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The abbreviations used are: PLC, palmitoyl-L-carnitine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TDGA, 2-tetradecylglycidic acid; CPT, carnitine palmitoyltransferase.

0006-291X/92 $4.00

reacylation of membrane phospholipids at no ATP cost. In other words, the fatty acid would be transfered from the acyl-L-carnitine pool to free CoA by CPT, and the acyl-CoA produced would then be the substrate of the well known lysophospholipid acyl-CoA transferase-mediated transfer of the acyl unit from acyl-CoA to lysophospholipids. However, Shohet showed that, in addition to the major routes for membrane phopholipid renewal in human erythrocyte (the afore mentioned ATP-dependent acylation of erythrocyte membrane phospholipids, and the passive exchange of intact phosphatidylcholine (PC) between plasma lipoprotein and erythrocyte membrane), the transfer of the fatty acid from PC to lyso-phosphatidylethanolamine in the erythrocyte membrane would also take place, most probably by a not well defined transacylase reaction [7,8].

In this work we demonstrate that, in pulse-chase experiments, a transfer of fatty acid from the acyl-L-carnitine pool to membrane phospholipids occurrs in intact human erythrocytes. This process can be completely abolished by pretreating erythrocyte, before the chase period, with a CPT inhibitor. Finally, our data show that the apparent transfer of fatty acid from PC to phosphatidylethanolamine (PE) is not operative, at least in the early phase of the chasing.

Materials and Methods

Chemicals. Essentially fatty acid-free BSA and BSA fraction V (non-defatted BSA) were obtained from SIGMA Chemical Company, St. Louis, Mo. [1-^{14}C]Palmitic acid (58 Ci/mol), [9,10-^{3}H]oleic acid (7 Ci/mmol) and [1-^{14}C]palmitoyl-L-carnitine (58 Ci/mol) were obtained from New England Nuclear Corporation, Boston, Mass. Thin-layer plates, Whatman LK6 (silica gel) (20x20 cm) with a pre-absorbant layer were obtained from Carlo Erba, Milan. 2-Tetradecylglycidic acid (TDGA) was given by McNeil Pharmaceuticals, Spring House, PA, U.S.A. All other compounds used were reagent grade.

Experimental Procedures. Heparinized blood was collected daily from normal human volunteers. White blood cells and platelets were removed with a column containing a mixture of microcrystalline cellulose and α-cellulose (1:1, w/w), and filtered blood cells were washed three times with 4 vol. of cold 0.9% NaCl [9]. Isolated erythrocytes were once again washed with incubation buffer (NaCl 120 mM, KCl 5 mM, $MgSO_4$ 1 mM, NaH_2PO_4 1 mM, saccharose 40 mM, 5 mM glucose, Tris-HCl 10 mM, at pH 7.4) and resuspended in the same buffer at a final hematocrit of 5%. The presence of saccharose in the incubation buffer prevented colloid-osmotic lysis of the cell. A Rotabath shaking bath at 37 °C was used for the incubations. Erythrocytes were labeled (pulse phase) with radioactive fatty acids by incubating them for four hours with [1-^{14}C]palmitic acid or [9,10-^{3}H]oleic acid (10 μM), complexed to fatty acid free bovine serum albumin (1.65 mg/ml). Incubations were ended by washing cells once with cold incubation buffer, three times with fatty acid free BSA 1% in incubation buffer, and finally once again with incubation buffer. Labeled erythrocytes were then reincubated (chase phase) either with non-defatted BSA (1.65 mg/ml) or autologous fresh platelet free plasma. In TDGA studies, labeled erythrocytes were preincubated with 15 μM TDGA for 1 hour at 37 °C [6], and then reincubated as above. The final hematocrit was 20 %. Incubations were ended by washing cells three times with cold incubation buffer.

Erythrocyte lipids, immediately extracted from intact cells [10], were separeted by two dimensional thin layer chromatography [11,12]. Aliquots of the lipid extract were used for determination of lipid phosphorus content [13]. The chromatograms were developed using chloroform/methanol/28% ammonia (65:25:5, v/v) in the first dimension, and then developed using chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, v/v) in the second dimension. PC and PE were visualized by brief exposure of the plates to iodine and identified using standards as a reference. PLC and oleyl-L-carnitine were observed by adding 0.1 mg of genuine PLC to the lipid extracts immediatly before the application to the TLC plates [6].

Individual phospholipid and acyl-L-carnitines spots were scraped off into vials containing scintillation fluid and radioactivity was determined by liquid scintillation counting. Counting efficiency was evaluated by an external standard. Calculations are based on the specific activity of [1-^{14}C]palmitic acid, [9,10-^{3}H]oleic acid.

Results and Discussion

Recent studies from our laboratory support the concept that erythrocyte CPT, an enzyme essentially known for its shuttling function of fatty acids through mitochondrial membranes, may be considered an additional component of the metabolic pathway of the membrane phospholipid fatty acid turnover [4-6]. Erythrocyte CPT, like the other subcellular acyl-L-carnitine transferases, catalyzes the reversible transfer of the acyl moiety from carnitine to CoA, and because of its sensitivity to the mass action ratio of the reactants, it would modulate the size of the acyl-CoA and free CoA pool, which are critical for the reacylation process of membrane phospholipids [6,14]. Our view would also imply an active acyl trafficking between acyl-L-carnitines and membrane phospholipids. To explore this metabolic pathway, we have conducted pulse-chase studies. This experimental approach allows one to "chase" the metabolic fate of a radioactive fatty acid, previously incorporated into membrane phospholipids and carnitine of intact erythrocyte in the so called "pulse" phase. Thus, the pulsed red cells are washed with defatted BSA and reincubated with either non-defatted BSA or autologous plasma.

Fig. 1 shows a typical pulse-chase experiment in which we chased radioactive palmitate present in PE, PC and PLC, by reincubating the pulsed cellular suspension with non-defatted BSA. Radioactivity increases linearly with time in both PE and PC, though the magnitude of the process is higher in PE than PC. In contrast, the radioactivity found in PLC decreased (Fig. 1B), and the extent of this loss matched the corresponding increase of radioactivity in PE plus PC (Fig. 2). The pretreatment of the red cells with TDGA, a specific CPT inhibitor, before the chasing period, almost

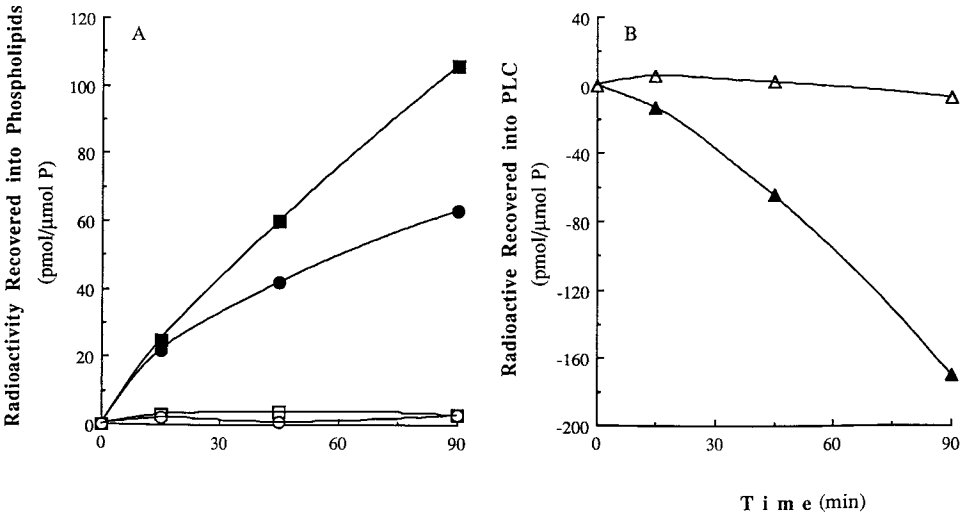

Fig. 1. Radioactivity recovered into PE, PC, and PLC during the chasing period in control and TDGA-treated red cells. Erythrocyte suspensions labeled with radioactive palmitic acid, either treated or not with TDGA, were incubated in non-defatted BSA, and at fixed times aliquots were removed and processed as described under "Materials and Methods". Results are expressed as the difference of pmol [1-^{14}C]palmitic acid/μmol lipid phosphorus incorporated or lost between time zero and the next time value of the chase. (A) Radioactivity recovered into PE (■) and PC (●). (B) Radioactivity recovered into PLC (▲). Control, closed symbols; TDGA treated erythrocytes, open symbols. Values are the average of three experiments done in duplicate. The variation between experiments was not more than 5 %.

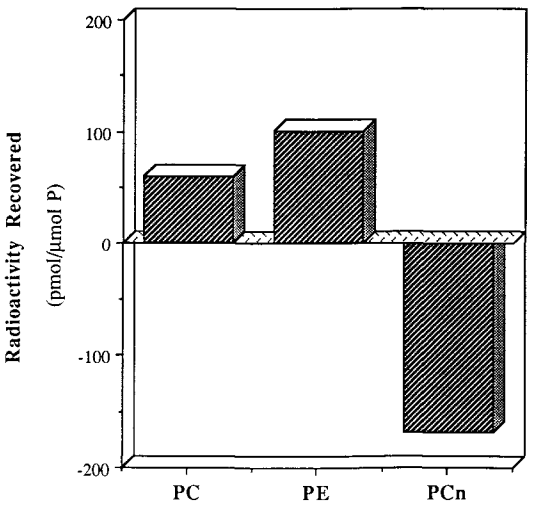

Fig. 2. Radioactivity recovered into PE, PC, and PLC at the end of the chasing period (90 min) in control erythrocytes. Values obtained from the experiments reported in Fig. 1.

abolished radioactivity changes in PE, PC and PLC, previously detected in untreated red cells (Fig. 1). It should be said that TDGA does not inhibit either the reacylation step of the membrane phospholipids, or the activation step of the fatty acid [6]. These results strongly suggest that the rise in radioactivity observed in both PE and PC derives from a metabolic route in which the first step would be the CPT-catalyzed mobilization of the radioactive acyl units from the radioactive acyl-L-carnitine pool to free CoA. The second step, instead, would be mediated by lysophospholipid acyl-CoA transferase which transfers the radioactive acyl units from acyl-CoA to lyso-PE and lyso-PC. In addition, similar results were obtained either using radioactive oleate as "pulser" or by replacing non-defatted BSA with autologous platelet free plasma (data not shown).

An early account of Shohet showed that upon reincubation in fresh autologous serum of either red cells containing PC double labeled with choline-1,2-^{14}C and tritiated palmitate, or simply red cells pulsed with tritiated palmitate, a rise of radioactive palmitate in PE accompained with a fall of radioactivity in PC was detected [8]. Since the radioactivity present in the choline group of PC was not recovered in PE, the author ruled out the possibility that the increase in PE radioactivity was related to either a base exchange reaction between PC and PE, or a demethylation reaction of PC. Therefore, to explain the transfer of the fatty acid from PC to PE, a transacylase reaction occurring between PC and lyso-PE was postulated. The apparent discrepancy between these data and our findings has to be ascribed to the chromatographic procedure adopted by this author to separate erythrocyte lipid extracts [15]. In fact, Fig. 3 clearly demonstrates that the one dimension TLC system utilized, does not separate genuine PLC from erythrocyte PC. In other words, the radioactivity found in PC from erythrocyte labeled with tritiated palmitate was heavily contaminated by radiolabeled PLC. We have shown that after incubating intact human erythrocyte with [1-^{14}C]palmitic acid complexed to fatty acid free BSA, three major radioactive spots, identified as PC, PE and PLC, could be observed in the autoradiographic pattern of a two-dimensional TLC chromatogram [6]. PLC contained the highest amount of radioactive palmitate, when compared to PC and PE, and the incorporation of radioactive palmitate into PC is usually only slightly higher than PE.

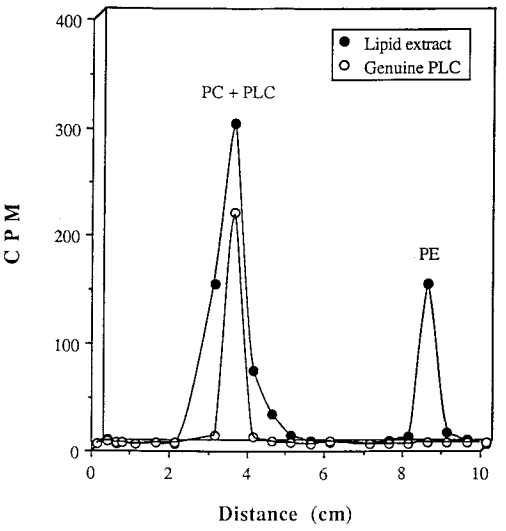

Fig. 3. Radioactive profiles of one dimension TLC analysis [15] of [1-^{14}C]palmitic acid incorporated into erythrocyte membrane phospholipids and genuine [1-^{14}C]palmitoyl-L-carnitine. The scans were performed with a System 200 Imaging Scanner, Bioscan Inc. (U.S.A.).

On the contrary, after pulsing erythrocyte with tritiated palmitate (beginning of the chase), it has been shown that PC incorporated about 13 times more radioactivity than PE [8]. This finding suggests that radiolabeled PLC is contributing for a large proportion of the radioactivity recovered in the PC, since PLC is not separated from PC in the one dimension TLC system.

A further confirmation of such an artifactual observation, derives from the chase studies, in which the extent of radioactive increase into PE accounts for only a relatively small percentage of the fall in PC radioactivity (26 % and 40 % after 4 and 16 hours chase, respectively) [8]. The rest of the radioactive fall in PC cannot be explained by an exchange of the radiolabeled PC pool present in the erythrocyte membrane with serum. According to the data reported in a previous study [7], the release of radioactivity into serum from actively labeled erythrocyte PC would account for about 12 % and 16 % after 4 and 16 hours chase, respectively. Taking into account our findings, the apparent decrease of radioactivity observed in erythrocyte PC during the chase period is most likely caused by a decrease of radiolabeled PLC, at least in the early phase of the chase. One should also consider that during the later stages of the chase studies, a passive exchange of radiolabeled PLC from erythrocyte to serum may occur. In fact, it has been demonstrated that PLC cross erythrocyte membrane passively, and the half time translocation rate of PLC from one emileaflet to the other of the membrane is 2.6 hr [16]. This would further complicate the interpretation of the data, since the decrease of radioactivity in PC may be partly connected with the exchange of radioactive PLC from the erythrocyte to the serum. In our chase studies, we did not recover any radioactive PLC in non-defatted BSA or autologous plasma (data not shown).

Although, it is not possible to rule out *a priori* that erythrocytes are able to express transacylation reactions between phospholipids, the data presented in this work suggest that during the early stages of chase, acyl-L-carnitines are potential acyl donors for the ATP-independent reacylation of membrane phospholipids, and that this process is mediated by CPT.

Acknowledgments

This research was supported in part by Consiglio Nazionale delle Ricerche (Progetto Strategico per il Mezzogiorno) and Sigma Tau S.p.A., Pomezia, Italy.

References

1. Shohet, S.B., Nathan, D.G., and Karnovsky, M.L. (1968) *J. Clin. Invest.* **47**, 1096-1108.
2. Lands, W.E.M., and Crawford, C.G. (1976) in *The Enzymes of the Biological Membranes* (Martonosi, A., ed) Vol. 2, pp. 3-85, Plenum Press Publishing, New York.
3. Dise, C.A., Goodman, D.B.P., and Rasmussen, H. (1980) *J. Lipid Res.* **21**, 292-300.
4. Arduini, A., Mancinelli, G., and Ramsay, R. R. (1990) *Biochem. Biophys. Res. Commun.* **173**, 212-217.
5. Arduini, A. (1992) *Am. Heart J.* **123**, 1726-1727.
6. Arduini, A., Mancinelli, G., Radatti, G., Dottori, S., and Ramsay R. (1992) *J. Biol. Chem.* in press.
7. Shohet, S.B. (1970) J. Clin. Invest. **49**, 1668-1678.
8. Shohet, S.B. (1971) J. Lipid. Res. **12**, 139-142.
9. Beutler, E., West, C. and Blume, K. G. (1976) *J. Lab. Clin. Med.* **88**, 328-333.
10. Rose, H.G., Oklander, M. (1965) *J. Lipid Res.* **6**, 428-431.
11. Rouser, G., Kritohevsky, G., Yamamoto, A., Simon, G.,Calli, C., Bauman, A. J. (1969) *Methods Enzymol.* **14**, 272-317.
12. Rouser, G., Fleisher, S., and Yamamoto, A. (1970) *Lipids* **5**, 494-496.
13. Bottcher, C.J.F., Van Gent, C.M., and Pries, C. (1961) *Anal. Chim. Acta* **24**, 203-208.
14. Ramsay, R.R., Mancinelli, G., and Arduini, A. (1991) *Biochem. J.* **275**, 685-688.
15. Skipski, V.P., Peterson, R.F., and Barclay, M. (1964) Biochem. J. **90**, 374-378.
16. Classen, J., Deuticke, B., and Haest., C.W.M. (1989) *J. Membrane Biol.* **111**, 169-178.