Biochimica et Biophysica Acta, 363 (1974) 287–292

BBA Report

BBA 71207

PREFERENTIAL INCORPORATION OF FATTY ACIDS AT THE INSIDE OF HUMAN ERYTHROCYTE MEMBRANES

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(Received June 18th, 1974)

Summary

1. Phospholipase A_2 isolated from *Naja naja* venom was used as a tool to discriminate between the outer and inner lipid monolayer of the membranes of human erythrocytes.

2. Incubation of human erythorcytes with radioactive fatty acids resulted in the formation of labelled lecithin in the membrane. The label was found predominantly in that pool of lecithin which is localized in the inner monolayer of the membrane.

3. Lecithins isolated from the total erythrocyte membrane and from the inner monolayer of the membrane possess identical fatty acid patterns and identical positional distributions of their fatty acyl constituents.

Circulating red blood cells should be capable of incorporating fatty acids into their phospholipids since variations in the diet can induce alterations in the fatty acid pattern of the phospholipids within a much shorter period than the average lifespan of the erythrocyte [1,2]. Two mechanisms have been described to play a major role in the renewal of the phospholipids of the erythrocyte membrane: (1) an exchange of phospholipids between serum lipoproteins and the red cell membrane [3–5] and (2) the incorporation of fatty acids into lysophospholipids which are either formed in the membrane or supplied by the surrounding serum [6,7]. Shohet [8,9] proposed the existence of two compartments of lecithin in the erythrocyte membrane: one of these is thought to be in equilibrium with lecithin from

serum lipoproteins by passive exchange (Mechanism 1), while the other compartment is involved in the active incorporation of fatty acids (Mechanism 2). This second compartment of lecithin was suggested to be located "deeper" in the membrane. Zwaal et al. [10] and Verkleij et al. [11] showed that treatment of intact human erythrocytes with phospholipase A_2 from *Naja naja* venom (EC 3.1.1.4) resulted in the degradation of two-third of the lecithin without affecting phosphatidylserine and phosphatidylethanolamine. On the other hand, essentially all the phospholipids were accessible to the enzyme if erythrocyte ghosts were used. From these results they concluded that at least two thirds of the lecithin is localized in the outer monolayer, whereas phosphatidylserine and phosphatidylethanolamine are largely confined to the membrane interior of the human erythrocyte. A similar conclusion regarding the non-random distribution of phospholipids was reached by Bretscher [12] and also by Gordeski and Marinetti [13]. In these latter studies the observation was mainly based on indirect evidence obtained by labelling the cell with relatively non-permeant reagents. In the present study phospholipase A_2 from *N. naja* has been used as a tool to establish that fatty acids incorporate preferentially at the inside of human erythrocyte membranes.

Fresh samples of human blood were obtained by vena punction, using acid–citrate–dextrose as anticoagulant. The cells were spun down in a clinical centrifuge at maximal speed for 15 min. After removal of the plasma and the fluffy layer, the cells were washed three times with 0.9% NaCl. Incubations were carried out with 2 ml of packed cells for 2 h at 37 °C in 3 ml of human plasma, to which ATP (3 mM), CoASH (50 μM) and 10 μCi (172 nmoles) of [1-^{14}C]palmitic acid or [1-^{14}C]linoleic acid were added. After the incubation the cells were spun down and washed 3 times with 0.9% NaCl. Aliquots of 0.25 ml of the radioactive cells were subsequently incubated for 1 h at 37 °C in 5 ml 0.87% NaCl, 10 mM $CaCl_2$, 0.25 mM $MgCl_2$ (adjusted to pH 7.4 with Tris) containing 10 I.U. of phospholipase A_2. This enzyme had been purified from *N. naja* venom as described by Cremona and Kearny [14]. Parallel experiments were conducted in exactly the same medium except that phospholipase A_2 had been omitted. After the incubation the erythrocytes were spun down and EDTA (final concentration 5 mM) was added to terminate the reaction. The phospholipids were extracted according to the procedure of Reed et al. [15] and separated via two-dimensional silica thin-layer chromatography as described by Broekhuyse [16]. The individual phospholipid constituents were assayed for radioactivity using a Packard liquid Scintillation Counter Model 2425 B [17] and for the amount of inorganic phosphorus according to the procedure described by Bartlett [18].

In agreement with previous publications [10,11], 65% of the lecithin of human red cells can be degraded by phospholipase A_2 from *N. naja* (Table I). It is important to emphasize that no haemolysis occurred during this treatment. Lecithin is converted quantitatively into lysolecithin which remains in the membrane without further breakdown. The amount of lecithin

TABLE I

NON-LYTIC DEGRADATION OF THE PHOSPHOLIPIDS OF INTACT HUMAN ERYTHROCYTES WITH PHOSPHOLIPASE A_2 FROM *N. NAJA* VENOM AFTER LABELLING WITH [1-^{14}C] PALMITATE OR [1-^{14}C]LINOLEATE

	Radioactivity incorporated before degradation (%)		Phospholipid composition (% of phosphorus)		% phospholipid not degraded	% radioactivity not degraded	
	16:0	18:2	Before degradation	After degradation		16:0	18:2
Lecithin	68	68	32	11	35	76	68
Lysolecithin	1	1	2	23	—	—	—
Sphingomyelin	8	14	24	24	100	100	100
Phosphatidylserine *	4	6	14	15	100	100	100
Phosphatidylethanolamine	19	11	28	27	97	98	97

* including phosphatidylinositol

which is broken down may correspond with the lecithin pool in the outer monolayer of the erythrocyte membrane. No degradation of phosphatidylserine or phosphatidylethanolamine was observed. In contrast to lecithin, these latter phospholipids were suggested to be located almost exclusively at the inside of the erythrocyte membrane. Whereas the lecithin of the membrane exterior (representing at least 65% of the total lecithin of the human red cell) is converted into lysolecithin, the majority of the labelled lecithin is not degraded. This point is even clearer when the specific activities of lecithin of the inner and outer monolayer are calculated from the data in Table I. In the case of palmitic acid the specific activity of lecithin at the inside is 5.8 times higher than that at the outside. Also phosphatidylserine and phosphatidylethanolamine which are located almost exclusively at the membrane interior are labelled by incubating the red cells with radioactive palmitic or linoleic acid. From these results it may be concluded that the incorporation of palmitic and linoleic acid takes place preferentially into the phospholipids of the inner monolayer of the human erythrocyte. A possible explanation is that the acyltransferases are located at the inside of the membrane. It has to be realised however that the fatty acids must be converted into their corresponding acyl-CoA esters prior to incorporation into the phospholipids. It is possible that this activation process is located exclusively at the inside of the membrane. The much lower labelling of lecithin in the outer layer of the membrane is either due to a slow translocation of the acyl-CoA ester from the inside to the outside of the membrane, or might be the result of a rate limiting flip-flop mechanism of labelled lecithins formed in the inner layer of the membrane.

The positional distribution of the fatty acids incorporated into lecithin was also measured. Therefore the labelled lecithins of red cells before and after treatment of the intact cells with phospholipase A_2 from *N. naja* were isolated via 2-dimensional thin-layer chromatography. The lecithins were then degraded with phospholipase A_2 and assayed for radioactivity [17]. The details of this procedure have been described previously [19]. The positional distribution of the fatty acids incorporated into the lecithin located at the inside of the membrane is not different from that observed for the total lecithin of the erythrocyte. Palmitic acid is incorporated predominantly at the 1-position (86%) and linoleic acid mainly at the 2-position (61%) of lecithin. This observation is in agreement with previous reports by Mulder and Van Deenen [6] and Van den Bergh [20] and in disagreement with Oliveira and Vaughan [21]. These authors reported that both palmitic and linoleic acid incorporate at the 2-position mainly.

It is known [19] that lecithin of the erythrocyte represents a heterogeneous collection of molecules differing with respect to their fatty acyl-constituents. In this respect it could be speculated that the various individual molecular species of lecithin could be distributed in a non-random way among the outer and inner layer of the erythrocyte membrane. The results compiled in Table II show that the fatty acid patterns of lecithin of the inner

TABLE II

FATTY ACID COMPOSITION OF LECITHIN FROM THE TOTAL MEMBRANE AND FROM THE INSIDE OF THE MEMBRANE OF HUMAN ERYTHROCYTES

	Total membrane			Inner Layer		
		1-position	2-position		1-position	2-position
14:0	0.7	1.5	—	+	+	—
16:0	33.3	60.4	12.0	40.9	62.6	15.1
16:1	0.6	+	2.3	+	—	1.8
17:0	3.1	2.2	1.2	2.9	1.4	1.0
18:0	15.6	29.0	5.2	12.5	27.8	4.1
18:1	15.9	6.4	26.7	16.4	6.1	26.3
18:2	22.6	0.6	40.2	18.4	1.7	36.6
18:3	1.4	+	1.0	+	+	+
20:3	1.1	+	1.5	2.2	+	3.0
20:4	5.7	+	10.0	7.0	+	12.0

layer of the membrane and of the total lecithin pool of the erythrocyte are very similar. Also the positional distribution of the fatty acyl chains is identical, this being in agreement with the results obtained with radioactive fatty acids. These results strongly suggest that the various molecular species of lecithin are distributed randomly among the outer and inner layer of the membrane.

This report shows that in vitro incorporation of fatty acids into lecithin of human erythrocytes occurs predominantly at the inner part of the membrane. This may well account for the "deeper pool" for fatty acid incorporation into phospholipids suggested by Shohet [8,9]. On the other hand there is a good possibility that renewal of phospholipids by exchange of the whole molecule between plasma lipoproteins and red cell membrane [3—5,22] takes place in the outer monolayer only. There is a good correlation between the 65% of the lecithin which is located in the outer part of the membrane and the exchangeable pool of lecithin, which size has been calculated by Reed [22] to be 63% of the total lecithin in human erythrocytes. Phospholipase A_2 from *N. naja* venom may be a useful tool to study the relative contribution of exchange processes and fatty acid incorporation to the renewal of phospholipids in erythrocyte membranes.

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