Asymmetric Metabolism of Phosphatidylethanolamine in the Human Red Cell Membrane*

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The incorporation of labeled fatty acids into phosphatidylethanolamine (PE) on the two sides of the human red cell membrane was studied by use of the vectorial probe trinitrobenzene sulfonate (TNBS). A small population of PE molecules on the outer surface of the membrane has a 4-fold higher turnover rate than the remaining PE molecules. This effect is greatest with palmitic acid, less with linoleic and linolenic acids, and not seen with stearic acid. By use of the hydrophobic penetrating probe fluorodinitrobenzene (FDNB), we find a second larger population of PE and phosphatidylserine (PS) molecules which reacts with FDNB and has a higher specific activity than the PE and PS molecules which do not react. With human polymorphonuclear cells, the labeled PE molecules inside the cell have a higher specific activity than the PE molecules located on the outer cell surface. These results suggest that there are heterogeneous populations of PE and PS on both halves of the red cell membrane which show different metabolic turnover rates of their fatty acids.

The red cell membrane has an asymmetric distribution of its phospholipids (1-5). The question arises how this asymmetry is made and maintained and whether these phospholipids are differently metabolized and renewed. Renooij and van Golde (6) found that the specific activities of the more unsaturated classes of PC of rat red cells labeled from PC were higher on the outer layer of the red cell membrane as compared to the inner layer, but that the disaturated PC had the highest activity on the inner layer. They proposed that the acylation activity of the erythrocyte was directed toward the formation of disaturated PC on the inner layer of the membrane. Renooij et al. (7, 8) reported that the incorporation of different fatty acids into PC of isolated rat red cells occurred predominantly on the inner layer of the membrane. They state that two mechanisms may account for the renewal of phospholipids in the red cell membrane, these being the acylation of lysophospholipids which occurs in the membrane and the exchange of phospholipids between serum lipoproteins and the red cell membrane (9). Shohet (10, 11) also reported that these two processes occur in two different compartments in the red cell membrane.

In order to further explore the origin of the asymmetry of the red cell membrane, we have studied the incorporation of different fatty acids into the various phospholipids of the isolated human red cell. We have found an asymmetric labeling of PE on the two halves of the membrane by use of vectorial chemical probes. Our results show that a small population of PE molecules on the outer surface of the red cell membrane has a more rapid turnover than the other PE molecules either on the outer or inner surface of the membrane. We also provide evidence for heterogeneous populations of PE and PS in the red cell membrane. With human PMN cells, the labeled PE molecules inside the cell have a higher specific activity than the PE molecules on the outer cell surface.

MATERIALS AND METHODS

Human blood was obtained from the Red Cross Blood Bank and used within 2 weeks. The cells were washed three times with Krebs-Ringer phosphate (KRP) buffer, pH 7.4, containing 5 mM glucose and 1% BSA prior to use. The white cells and platelets were removed from the red cells by three washings in KRP buffer and centrifugation. Human PMN cells were prepared by the method of Boyum (12). The cells after appropriate incubation with labeled fatty acids or with chemical probes were lysed in 10 mM Tris buffer containing 1 mM EDTA by the method of Dodge et al. (13). The ghosts were extracted with chloroform/methanol by the Folch et al. method (14) and the lipids separated by two-dimensional or one-dimensional TLC (15, 16). The lipid spots were eluted with hot methanol/HCl and lipid P was reported that these two processes occur in two different compartments in the red cell membrane.

RESULTS

The nonpermeable probe TNBS was used to measure the specific activity of PE and PS on the outer surface of the red cell membrane. The PE and PS which do not react with
TNBS at 0 °C are localized on the inner surface of the membrane and/or are tightly bound to membrane protein (18). The time course profile in Fig. 1 shows that PE on the outer membrane surface has a much higher specific activity than the remaining PE in the membrane when intact cells are incubated with $[^{3}H]$palmitate. These PE molecules represent only 1–2% of the total PE molecules. These results demonstrate an asymmetric rapid turnover of a small population of PE molecules. The amount of PS which reacts with TNBS at 0 °C is too small to measure. The labeling of red cells with TNBS was, therefore, done at 21 °C in order to react sufficient FS molecules for analysis. At 21 °C, there is slow penetration of TNBS into the cell so that saturation of PE labeling is not attained as it is at 0 °C (Fig. 2). However, we did carry out some experiments with TNBS labeling at 21 °C in order to compare the results with those at 0 °C.

In order to see whether the PE and PS molecules localized on the inner membrane surface also have heterogeneous turnover which show a different turnover, cells were labeled with $[^{3}H]$palmitate and then reacted with FDNB and TNBS. FDNB is a penetrating probe which reacts on both sides of the membrane and which may react with PE and PS molecules buried more deeply in the hydrophobic part of the membrane. The results in Table I show that the PE and PS molecules which react with FDNB or TNBS have a higher specific activity than the remaining unreactive PE and PS molecules. Since FDNB reacts on both sides of the membrane, the increase in specific activity of the DNP-PE and possibly DNP-PS molecules is due in part to a small population of molecules localized on the outer membrane surface as seen in both Fig. 1 and Table I. However, since the extent of reaction of PE and PS with FDNB is much greater than that of TNBS, the results show that another population of PE and PS molecules on the inner surface of the membrane or more deeply buried in the membrane is accessible to FDNB and TNBS and has a higher turnover of their fatty acids than the remaining PE and PS molecules which do not react with these probes.

In order to see whether the labeling of PE and PS was due to acylation of lyso-PE and lyso-PS in the membrane, the red cells were pretreated with purified phospholipase A$_{2}$ and then incubated with $[^{3}H]$palmitate. The results in Table II show that phospholipase action led to a small decrease in the amount of PC, PE, and PS and that phospholipase enhanced the labeling of PC, PE, and PS. The results in Table III show that phospholipase treatment also enhanced the specific activity of the PE which reacted with TNBS from 309 to 371 pmol of $[^{3}H]$palmitate incorporated/μmol of PE. These results suggest that the acylation of lyso-PE may account in part for the labeling of PE. Our previous studies (18) as well as the results in Table II show that phospholipase A$_{2}$ treatment has no significant effect on the extent of reaction of PE with TNBS.

Since $[^{3}H]$palmitate was incorporated into PE in an asymmetric manner, we tested $^{14}C$-labeled stearate, linoleate, and linolenate to see whether these fatty acids behave like palmitate. The data in Table IV show that palmitate, linoleate, and linolenate had a more rapid turnover in TNP-PE as compared to unreacted PE, whereas stearate did not. The

| Table I |
| --- |
| Asymmetric labeling of PE and PS by $[^{3}H]$palmitate in intact red cells as detected by reactivity to TNBS and FDNB |
| % Reaction of PE or PS with TNBS | pmol $[^{3}H]$palmitate incorporated/μmol of PE or PS |
| TNP-PE | Unreacted PE | 17.6 ± 0.6 | 4.9 | 180 ± 10 |
| TNP-PS | Unreacted PS | 3.1 ± 0.01 | 1.3 | 54 ± 1.9 |
| DNP-PE | Unreacted PE | 166 ± 11 | 45 | 155 ± 3 |
| DNP-PS | Unreacted PS | 51 ± 4 | 22 | 54 ± 2 |

Fig. 1. Asymmetric labeling of PE in the human red cell membrane. 0.5-ml aliquots of packed red cells were incubated for 15, 30, and 60 min at 37 °C in 20 ml of KRP buffer, pH 7.4, containing 5 mM glucose, 1% BSA, and 50 μCi of $[^{3}H]$palmitic acid. The cells were washed twice with buffer containing 1% BSA and resuspended in 20 ml of 120 mM NaCl and 40 mM NaHCO$_{3}$, pH 8.5, buffer containing 2 mM TNBS. The cells were reacted at 0 °C for 1 h. Ghosts were extracted by the Folch et al. procedure (14). PE and TNP-PE were separated by TLC and assayed for phosphatidylethanolamine Turnover in Red Cells.

Fig. 2. Time course of labeling of PE in the human red cell membrane by TNBS at 0 °C and 21 °C. 0.5-ml aliquots of packed red cells were reacted in 20 ml of 120 mM NaCl and 40 mM NaHCO$_{3}$ buffer, pH 8.5, containing 2 mM TNBS for different time periods. Ghosts were prepared, and the lipids were extracted and analyzed as explained in Fig. 1.
Effect of phospholipase A2 treatment on the content and labeling of red cell phospholipids

Twenty aliquots of 0.5 ml of packed red cells were incubated in 19.5 ml of KRP buffer, pH 7.4, containing 5 mM glucose and 1% BSA. Ten samples served as controls without phospholipase A2 treatment and 10 samples were treated with phospholipase A2 for 1 h at 37 °C. The cells were washed and resuspended in 20 ml of KRP buffer containing 50 μCi of [3H]palmitate and incubated for 1 h at 37 °C. The cells were washed twice with KRP buffer. The samples were suspended in 20 ml of 120 mM NaCl and 40 mM NaHCO3, pH 8.5, containing 2 mM TNBS and reacted for 1 h at 21 °C. The cells were washed once with KRP buffer, ghosts prepared, and lipids extracted and analyzed by TLC. Incorporation values are the mean ± S. D. for n = 5. The nanomole values are averages of duplicate lipid P analyses.

| Controls | Total | Phospholipase A2 |
|----------|-------|-----------------|
| pmol [3H]palmitate incorporated/μmol of PL | pmol [3H]palmitate incorporated/μmol of PL | pmol of PE |
| PC | 315 ± 12 | 337 ± 22 | 373 |
| PE | 137 ± 12 | 161 ± 3 | 402 |
| PS | 27 ± 3 | 33 ± 1 | 224 |

Effect of phospholipase A2 pretreatment on the asymmetric labeling of PE by [3H]palmitate in human red cells

Experimental details are given in Table II. Values are the mean ± S. D. for n = 5.

| nmol | % Reaction of PE with TNBS | pmol [3H]palmitate incorporated/μmol of PE |
|------|--------------------------|----------------------------------------|
| Control | | |
| TNP-PE | 18 ± 1.1 | 4.3 | 309 ± 10 |
| Unreacted PE | 416 ± 4 | 154 ± 1.8 |
| Phospholipase A2 | | |
| TNP-PE | 15 ± 0.1 | 3.6 | 371 ± 5 |
| Unreacted PE | 412 ± 3 | 153 ± 5 |

Asymmetric labeling of PE by [14C]palmitate, [14C]stearate, [14C]linoleate, and [14C]linolenate in intact red cells as assessed by the TNBS reaction at 0 °C

0.5 ml of packed red cells was pipetted into 18 tubes. 19.5 ml of KRP buffer, pH 7.4, containing 5 mM glucose and 1% BSA were added to each tube. Eleven μCi of [14C]palmitate (17.4 nmol), 11 μCi of [14C]stearate (196 nmol), 10 μCi of [14C]linoleate (192 nmol), and 11 μCi of [14C]linolenate (196 nmol) were added to two tubes each. All tubes were incubated at 37 °C for 3 h. The cells were centrifuged, washed twice with KRP buffer, and resuspended in 20 ml of 120 mM NaCl and 40 mM NaHCO3, pH 8.5, containing 2 mM TNBS and incubated at 0 °C for 1 h. The cells were washed once with KRP buffer, ghosts were prepared, and lipid extracted and analyzed by TLC. Incorporation values are the mean ± S. D. for n = 4.

| nmol | % Reaction of PE with TNBS | pmol of fatty acid incorporated/μmol of PE |
|------|--------------------------|----------------------------------------|
| [14C]Palmitate | 8.6 | 449 | 2.0 | 1495 ± 162 | 586 ± 7 |
| [14C]Stearate | 8.2 | 443 | 1.8 | 800 ± 64 | 985 ± 369 |
| [14C]Linoleate | 8.4 | 482 | 1.7 | 367 ± 55 | 164 ± 6 |
| [14C]Linolenate | 8.4 | 400 | 1.8 | 271 ± 85 | 165 ± 10 |

Discussion

The incorporation of fatty acids into red cell membrane phospholipids has been reported to occur in part by acylation of lysophospholipids (7,8). However, this is not the only pathway for incorporation of fatty acids into red cell phospholipids since exchange reactions can also occur (9). The asymmetric incorporation of fatty acids into PC on the inside of the membrane has been reported by Renooij et al. (7,8). There may be other processes which have to be considered in regard to the turnover and metabolism of red cell membrane phospholipids. Do certain phospholipids have a different asymmetric metabolism and are there discrete populations of phospholipids in every membrane or is the asymmetric labeling pattern in red cells due to white cell contamination?
molecules on the outer half of the membrane has a 4-fold ranged in the membrane. These enzymes which incorporate acids. The enzymes responsible for the incorporation of fatty acids into PE molecules. On the other hand, Renooij who found two ethanolamine-phospholipid fractions in human red cells which differed in their rates of incorporation of $^{32}$P and $[^1C]$acetate.

The significance of our findings to the origin and maintenance of the asymmetry of phospholipids in the red cell membrane can be only speculative at this time. PE has been shown to be asymmetrically distributed in the red cell membrane, being localized primarily on the inner half of the membrane (19). We find that a very small population of PE molecules on the outer half of the membrane has a 4-fold more rapid turnover of their fatty acids than the remaining PE molecules. On the other hand, Renooij et al. (7, 8) found that PC which is localized primarily on the outer half of the membrane has a population of molecules on the inner half of the membrane which has a more rapid turnover of their fatty acids. The enzymes responsible for the incorporation of fatty acids into these phospholipids may be asymmetrically arranged in the membrane. These enzymes which incorporate fatty acids into PE may show a preference for palmitic acid over stearic, linoleic, and linolenic acid. Whether PE molecules rich in palmitic acid have a different disposition in the membrane remains to be determined. The effect of fatty acid composition on the physical and biochemical properties of the various phospholipid classes in cell membranes is not well understood. Do those phospholipids with certain fatty acids interact differently with certain membrane proteins which somehow regulate their metabolism and transmembrane mobility? It is also possible that the fatty acids are incorporated into PE molecules on the inner surface of the membrane and that these newly synthesized molecules are rapidly translocated to the outer surface. If this is so, then it remains to be explained what mechanisms allow for the preferential translocation.

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