Decreased Acylation of Phosphatidylcholine in Diabetic Rat Erythrocytes

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We investigated the effects of streptozocin-induced diabetes on composition and metabolism of rat erythrocyte lipids. Diabetes produced no change in contents of cholesterol, total phospholipids, and proportions of phosphatidylcholine and phosphatidylethanolamine in phospholipids. The acylation of total phospholipids with palmitic, oleic, or arachidonic acids was decreased ($P < .01$) in intact erythrocytes from diabetic versus control animals. This anomaly was underlaid by a decrease ($P < .01$) in acylation of phosphatidylcholine, whereas phosphatidylethanolamine was unaffected. The impaired acylation of phosphatidylcholine was unchanged in vitro by insulin or coenzyme A but was restored to control values by ATP and by insulin treatment of the diabetic rats. We conclude that diabetes specifically alters the acylation of at least phosphatidylcholine in rat erythrocyte, an effect that might modify the remodeling of erythrocyte phospholipids and thereby the membrane function.
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Diabetes mellitus extensively alters lipid metabolism. Much work in this area has focused on alterations in composition of the lipid matrix of the cell membrane, particularly in the fatty acid composition of phospholipids. Such diabetes-induced alterations have been described in microsomes (1), mitochondria (2), and plasma membranes (3) of liver cells; in renal plasma membranes (4); and in total phospholipids of liver, plasma, and heart (5). Decreases in the degree of fatty acid unsaturation have usually been described, without modification of the phospholipid class distribution. Changes in the membrane cholesterol content and cholesterol-to-phospholipid ratio have also been reported (6,7). These diabetes-induced compositional anomalies have been associated with enzymatic changes (1,2,4,8,9) and/or alterations in the physical properties of the membranes (7).

Studies on the effect of diabetes on lipids of erythrocyte membranes are scarce. Erythrocytes of streptozocin-induced diabetic (STZ-D) rats have been shown to have compositional anomalies, e.g., decreased amounts of cholesterol (3) and arachidonate-rich phospholipids (5), in agreement with reports in other tissues (10). Alterations in phospholipid metabolism are not as well established. To our knowledge, the influence of diabetes on the rate of fatty acid turnover in erythrocyte phospholipids has not been investigated. Because this turnover rate may be an important factor in the regulation of membrane function (11), we examined the effect of STZ-D in rats, and its repair with insulin, on the incorporation of various ^{3}H-labeled fatty acids in two main classes of phospholipids of the erythrocyte membrane, i.e., phosphatidylcholine (PC) and phosphatidylethanolamine (PE).

MATERIALS AND METHODS

Animals. Adult male Sprague-Dawley rats (IFFA-CREDO, L'Arbresle, France) weighing ~300 g were housed at 22°C on an 0800–2000 h light schedule. They were fed a standard rat diet and water ad libitum. Diabetes was produced by tail vein injection with 75 mg STZ/kg body wt i.v. dissolved in 50 mM sodium citrate buffer (pH 4.5); control rats were injected with the vehicle only. Forty-eight hours after injection, blood was taken from the tail vein of the STZ-injected animals for glucose determination (Haemo-Glucotest, Boehringer Mannheim, Meylan, France), and only rats with nonfasting serum glucose concentrations >400 mg/dl were considered diabetic. In additional experiments, 48 h after the STZ injection, diabetic rats were injected subcutaneously with MC insulin (Novo, Paris) in the morning at doses of 3 U/day per rat for the first 3 days and then with 4 U/day for the subsequent 16–18 days. The diabetic control group was injected with normal saline only. Blood was taken for glucose determination before the animals were killed, again only rats

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with blood glucose >400 mg/dl were considered diabetic; 7 of 41 rats from this group died during the experiments. Only rats that showed a blood glucose value <180 mg/dl before death were included in the insulin-treated diabetic group. All rats were decapitated at 0900 h under light anesthesia (25 mg/kg i.p. pentobarbital sodium). In each experiment, control and experimental animals were processed concomitantly.

Blood samples. Blood samples were received on citrate (0.13 M) and immediately processed. A platelet- and leukocyte-free suspension of erythrocytes was prepared from each sample by filtration on cellulose as described by Beutler et al. (12). The purified erythrocytes were centrifuged at 200 × *g* for 5 min and washed twice with buffer A, which contained 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Tris, and 5 mM glucose (pH 7.4); they were resuspended in buffer A to 25% hematocrit. Erythrocyte counts, mean cellular volume (MCV), and mean cellular hemoglobin content (MCH) were determined in a Coulter counter model S (Coultronics, Margency, France). There was no detectable hemolysis at any step of the erythrocyte purification.

Cell incubation. Erythrocytes were incubated in triplicate for 90 min at 37°C in a medium made of 100 μl of erythrocyte suspension (2 to 4 × 10^8 cells), 100 μl medium B (medium A plus 1 mM $CaCl_2$), and 100 μl of a substrate mixture containing defatted albumin (0.16%, wt/vol) and the ^{3}H-labeled fatty acid (2 × 10^6 counts/min) in medium B at a final pH of 7.4. Before incubation, the substrates (final concn 3.6 μM) were sonicated for 1 min with a Branson sonicator (model B 12; Heat System Ultrasonics, Plainview, TX) at setting 4. After incubation, erythrocytes were washed three times with 40 vol of ice-cold medium A containing defatted albumin (2%, wt/vol) to remove excess fatty acids; then, they were lysed with 10 vol of ice-cold water. Membrane lipids were extracted by the procedure of Ways and Hanahan (13), as modified by Dise et al. (14).

Isolation of phospholipids. The lipid extracts were concentrated under nitrogen and submitted to thin-layer chromatography (TLC) on silica gel 60 plates (Merck, Darmstadt, FRG) developed in petroleum ether (40–60°C)–diethyl ether–acetic acid (80:20:1, vol/vol/vol) to separate neutral lipids from phospholipids. PC and PE were further separated on TLC plates developed in chloroform-methanol-water (75:25:3, vol/vol/vol). In this system, sphingomyelin (SM), phosphatidylserine (PS), and phosphatidylinositol (PI) were not completely separable; if used, they were coeluted and quantitated as a sum, referred to as SM-PS-PI. The lipid spots, located with iodine vapor, were identified with reference standards, scraped off, and analyzed for phospholipid phosphorus (PL-P) (15) and radioactivity content. The mean recovery of PL-P was 71 ± 8% (n = 6).

Incorporation rates were evaluated from the specific activity of the exogenous ^{3}H-labeled fatty acid, which was assumed to be constant during incubation, because intact erythrocytes do not spontaneously release fatty acids into the medium. Total cholesterol was measured via enzymatic assay (Merck). Results are expressed as means ± SD. Data from treated and control animals were compared via the unpaired t test, taking a value of $P < .05$ as significant.

Materials. [9,10-^{3}H(N)]palmitic acid (16:0, 23.5 Ci/mmol) and [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid (20:4, 83.8 Ci/mmol) were purchased from New England Nuclear (Boston, MA), and [9,10-^{3}H(N)]oleic acid (18:1, 4.8 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Unlabeled fatty acids were obtained from Sigma (St. Louis, MO). Defatted albumin was from Calbiochem-Behring (La Jolla, CA).

RESULTS

All the untreated STZ-D rats had polydipsia and polyuria and weighed significantly less than control rats (306 ± 29 vs. 441 ± 13 g, $P < .001$). They had overtly elevated plasma glucose levels (>400 mg/dl) compared with control rats (<120 mg/dl) 18–21 days after induction of diabetes. The diabetic rats treated with 3–4 U protamine zinc insulin daily for 16–18 days had only a 7% decrease in body weight and their plasma glucose levels were lower (<180 mg/dl) than those of the untreated diabetic animals.

The mean values of MCV (54 ± 1.5 fl) and MCH (18.5 ± 0.6 pg) of erythrocytes from diabetic rats were not statistically different from those of control rats (53 ± 1.0 fl and 19 ± 0.5 pg, respectively). We found no compositional anomaly in the main classes of components of the lipid matrix of the erythrocyte membrane from STZ-D rats. The main contents in cholesterol were (in μmol/10^{10} cells) 1.81 ± 0.4 vs. 1.80 ± 0.2 in diabetic vs. control rats, whereas those in total phospholipids were 2.42 ± 0.3 vs. 2.40 ± 0.3 in diabetic vs. control rats. The diabetic state did not modify the propor-

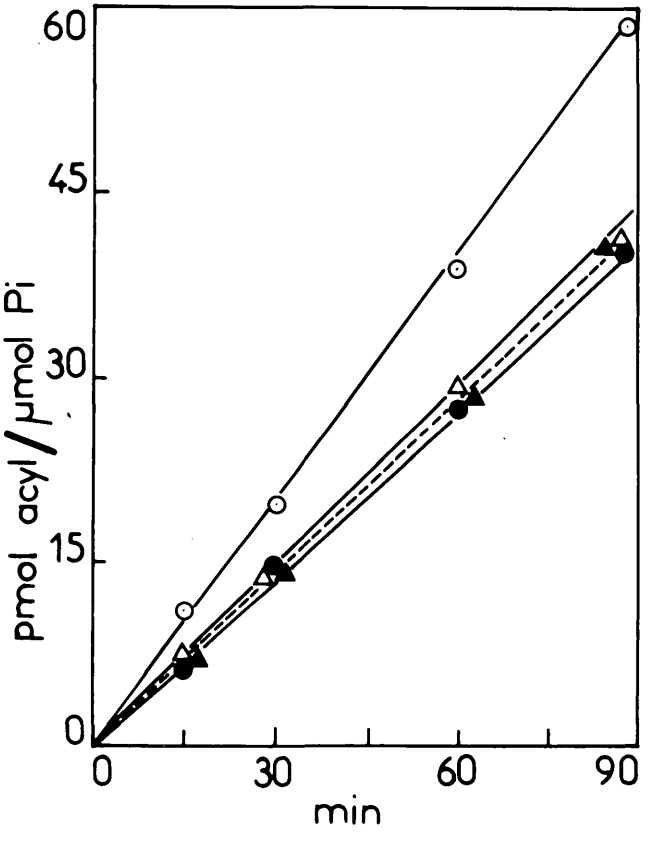

FIG. 1. Time course of acylation of phosphatidylcholine (*circles*) and phosphatidylethanolamine (*triangles*) with [^{3}H]oleic acid (3.6 μM) in intact erythrocytes from control (*open symbols*) and streptozocin-induced diabetic (*closed symbols*) rats. Three assays contributed to each point; values agreed within 8%.

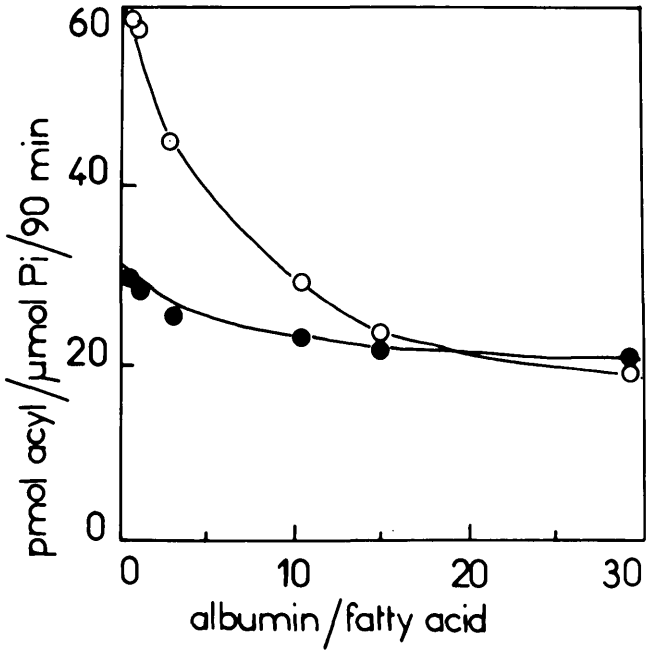

FIG. 2. Effects of variation in albumin-to–fatty acid ratio (mol/mol) on rates of acylation of phosphatidylcholine (○) and phosphatidylethanolamine (●) in intact erythrocytes from control rats. Concentration of [^{3}H]oleic acid was kept constant at 3.6 μM. Three assays contributed to each point; values agreed within 11%.

tional distribution (mol/dl) of PC and PE relative to total phospholipids in the erythrocyte membrane. In control rats, PC accounted for 46.5 ± 3.5 and PE for 28.5 ± 2.0% vs. 45.5 ± 4.2 and 31.7 ± 1.5%, respectively, in diabetic rats. SM-PS-PI accounted for the remainder.

Analytical experiments were performed with [^{3}H]18:1, the most abundant fatty acid found in association with albumin (16). In intact erythrocytes from control and diabetic rats, PC and PE were acylated according to zero-order kinetics for at least 90 min (Fig. 1). Acylation rates were proportional to the number of erythrocytes within the range 0.5 to 4.0 × 10^8 cells. As shown in Fig. 2, increasing albumin concentration from 3 to 300 μM in the incubation medium decreased the amounts of [^{3}H]18:1 found in PC and PE by 66 and 37%, respectively; >150 μM, a concentration corresponding to an albumin-to–fatty acid molar ratio of 15, there was no difference between the labeling of PC and PE.

As shown in Table 1, STZ-D decreased the entry of 16:0, 18:1, and 20:4 into PC by 30, 41, and 32%, respectively, without significant difference ($P > .05$) between the three acids. No change in acylation affected PE or SM-PS-PI as a whole. The decreased acylation rates of PC most probably accounted for the decreases in incorporation rates concomitantly measured in total phospholipids, which amounted to 26% ($P < .001$) for 16:0, 22% ($P < .02$) for 18:1, and 18% ($P < .01$) for 20:4 (data not shown). In one severely diabetic rat showing a 53% loss in body weight 21 days after STZ injection, the acylation of PC with [^{3}H]18:1 was decreased by 80%, with negligible (<5%) change in PE.

The dependence of phospholipid acylation on various compounds is shown in Table 2. ATP stimulated the acylation of PC in controls 1.9-fold, compared to 3.2-fold in diabetic rats, where acylation rates attained 110% of corresponding control values. ATP had no effect on the acylation of PE in diabetic or in control rats. The entry of 18:1 in PC or PE showed no dependence on CoA either alone or in association with ATP. Likewise, the preincubation for 15 min at 37°C of erythrocytes from diabetic rats with insulin produced no change.

Further studies were carried out to determine if the decreased acylation rates of PC in STZ-D rats returned to normal on in vivo treatment with insulin. As shown in Table 3, 16–18 days of insulin treatment returned the acylation rates to 96% of control values, without significant change in PE. ATP stimulated the acylation of PC similarly in insulin-treated and control rats.

DISCUSSION

Intact erythrocytes submitted to short-term incubation at 37°C incorporated ^{3}H-labeled fatty acids into membrane phospholipids, the specific activities of which increased according to zero-order kinetics. The decreasing effect of albumin on acylation rates probably reflects the binding of the fatty acid to albumin and its lesser availability in the free state for cellular uptake (17). The acylation process occurring at high albumin concentrations might involve a mechanism similar to that described in liver cells, where receptors for albumin appear to facilitate the entry of albumin-bound fatty acids (18). In any case, albumin-dependent uptake processes appear to occur at values of the albumin-to–fatty acid molar ratio >15 (Fig. 2). We used a value of 3 herein, which was well within the physiologic range of 1.25–5.0 found in the rat.

We found that STZ-D rats show a marked and apparently specific impairment in the acylation of PC. In control rats, fatty acid incorporation into total phospholipids was maximum with 20:4 > 16:0 > 18:1, and this decreasing order was maintained in diabetic animals. The three ^{3}H-labeled

TABLE 1
Effect of streptozocin-induced diabetes on incorporation of ^{3}H-labeled fatty acids into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of erythrocytes

	Palmitic acid		Oleic acid		Arachidonic acid	
Rats	PC	PE	PC	PE	PC	PE
Control	282 ± 34	34 ± 9.2	55 ± 9.5	30 ± 5.3	285 ± 29	103 ± 9.9
Diabetic	196 ± 12	31 ± 2.0	32 ± 3.5	27 ± 2.1	194 ± 33	106 ± 9.8
P	<.01	NS	<.01	NS	<.01	NS

Intact purified erythrocytes were incubated for 90 min at 37°C with indicated ^{3}H-labeled fatty acids (3.6 μM, ~4 × 10^6 dpm). Incorporation rates were evaluated from specific activity of ^{3}H-labeled fatty acids (see MATERIALS AND METHODS). Values are in pmol fatty acid · μmol^{-1} phospholipid phosphorus · 90 min^{-1} (means ± SD for 12 rats; assays in triplicate), obtained in 2 separate experiments.

TABLE 2
Incorporation of [^{3}H]oleic acid into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of erythrocytes from control and streptozocin-induced diabetic rats under various experimental conditions

Addition to incubation medium	n	PC		PE	
		Control	Diabetic	Control	Diabetic
Control	9	61.0 ± 4.9	40 ± 4.8†	28.7 ± 2.6	31.1 ± 3.8
Insulin* (500 μU/ml)	3	62.9 ± 6.6	42 ± 6.0†	27.2 ± 2.3	29.7 ± 4.3
CoA (5 μM)	3	57.1 ± 4.0	44 ± 4.3†	29.8 ± 4.3	29.6 ± 3.4
ATP (1.5 mM)	6	117 ± 9.1‡	129 ± 8.3‡	32.9 ± 4.0	27.7 ± 4.6
ATP + CoA	3	121 ± 9.4‡	114 ± 6.0‡	29.8 ± 4.8	32.8 ± 2.8

Basal experimental conditions as in Table 1. Additives were present in media during 90-min incubation. Dithiothreitol (15 μM) was added in incubation media containing CoA. *n* = Number of animals with determinations in triplicate. Results are in pmol oleic acid · μmol^{-1} phospholipid phosphorus · 90 min^{-1} (means ± SD).
*Insulin was added for a 15-min preincubation period before introducing the substrate.
†$P < .01$ for diabetic vs. control rats.
‡$P < .001$ for values in presence of additives vs. controls for same animal group.

fatty acids entered into PC and PE of erythrocytes from control and diabetic rats according to a roughly similar profile: both PC and PE showed the highest specific activities with 20:4 and the lowest with 18:1. An exception was the relatively high rate of acylation of PC with 16:0 compared with that of PE (per mol PL-P): 16:0 acylated 8.3- and 6.3-fold more PC than PE in control and treated rats, respectively (cf. Tables 1 and 2). These higher rates of entry of 16:0 into PC are consonant with a higher content of disaturated subclasses in PC (19); they confirm previous results of our group (see Table 3 in ref. 20).

Previous studies have shown that erythrocytes cannot biosynthesize phospholipids by a de novo pathway, whereas they can take up and incorporate long-chain fatty acids into phospholipids (21). On uptake, fatty acids are first activated to acyl-CoA by an acyl-CoA ligase in the presence of ATP. This activation is mandatory for the subsequent esterification of the acyl chain; ATP-depleted erythrocytes take up less fatty acids than intact cells (22). After activation, fatty acid metabolism in erythrocytes is limited to deacylation-reacylation reactions, which are catalyzed by phospholipases A and acyl-CoA–lysophosphoglyceride acyltransferases, respectively (23). Theoretically, the diabetes-induced decrease in rates of acylation of PC could result from decreases in rates of either deacylation and/or reacylation. Our study does not clearly discriminate between these possibilities. There is no evidence that deacylation might be impaired in diabetic erythrocytes, although decreased levels of phospholipase A_2 activities have been found in other tissues, e.g., in plasma and liver from uncontrolled STZ-D rats (24). Rather, the acylation defect might at least partly result from a decreased capacity of reacylation possibly due to the impaired ATP-dependent activation of fatty acids because *1*) the acylation defect is fully restored by ATP, *2*) CoA has no effect, and *3*) energy-dependent processes are probably altered in uncontrolled diabetes because of the prominent effect of insulin on oxidative phosphorylation and ATP generation. A net accumulation of fatty acids, seemingly due to decreased rates of reacylation, has been found in response to ATP depletion in bone marrow macrophages (25) and in cultured myocardial cells (26). Also, the fact that normal mature erythrocytes rapidly lose their acylating activity on washing and incubation in vitro (27) may explain the stimulating effect of ATP on the acylation process in control cells (Tables 2 and 3). However, extracellular ATP does not seem to freely cross the plasma membrane of viable cells and may influence many biological processes, including the permeability of the erythrocyte membrane, on binding to the external cell surface (28). Work is in progress to check the validity of these biochemical mechanisms.

In control rats, fatty acids entered preferentially into PC, consonant with the fact that PC is the major phospholipid substrate for fatty acid entry in normal mature erythrocytes (29). This might be one of the reasons the diabetes-induced acylation impairment primarily (if not uniquely) affects PC. However, the fact that PE is not altered is surprising because in normal erythrocytes, the acylation rates of PE are ~70% those of PC (see Fig. 1 and ref. 22). That PC may be specifically affected in diabetes is nevertheless supported by the fact that in compositional studies, erythrocytes from

TABLE 3
Effect of treatment with insulin on incorporation of exogenous [^{3}H]oleic acid into phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of erythrocytes from streptozocin-induced diabetic rats

Addition to incubation medium	PC			PE		
	Control	Diabetic	Insulin-treated diabetic	Control	Diabetic	Insulin-treated diabetic
None	66.6 ± 4.1	44.2 ± 3.9	63.9 ± 6.7	30.2 ± 2.5	32.9 ± 4.2	25.6 ± 3.4
ATP (1.5 mM)	107 ± 9.3	132 ± 11	96.0 ± 10	29.9 ± 3.1	35.6 ± 4.3	24.8 ± 3.9

Experimental conditions as in Table 2. Values are in pmol oleic acid · μmol^{-1} phospholipid phosphorus · 90 min^{-1} (means ± SD of 6 animals in each group; determinations in triplicate). Only animals with plasma glucose >400 and <180 mg/dl at 21 days were selected as diabetic and insulin-treated diabetic groups, respectively.

STZ-D rats showed decreased proportions of 20:4, 18:1, and 16:0 in PC and not in PE (5). Taken together, these findings suggest that *1*) decreased acylation rates reflect not only a reduced turnover of acyl chains but are also associated with net changes in the fatty acid content of PC, and *2*) an acylation defect may contribute to the reported decrease in the proportion of 20:4 in PC of liver membranes from diabetic rats in addition to the invoked impairment in fatty acid desaturating activity (30).

The PC defect is reminiscent of decreases in acylation rates that occur in association with ATP depletion in the course of erythrocyte aging (31). In this case, however, acylation rates decline to about the same extent in both PC and PE (32). Because PC, and not PE, is affected in diabetes, insulin deprivation could be associated with a more specific anomaly of PC metabolism, perhaps related to the alteration of a PC-specific enzyme of the reacylation pathway. Whatever its mechanism, this anomaly appears to be a direct consequence of insulin deprivation and not a cytotoxic effect of STZ, because administration of insulin to the STZ-D animals reverses the observed change.

Recent studies in various biological systems including erythrocytes have shown that changes in fatty acid turnover might modify the continuous remodeling of phospholipid fatty acid moieties and thereby the membrane function (11). Whether the modifications described in the rat model relate to altered functional properties of erythrocytes in human diabetic patients remains to be proven. For example, alterations in rheological properties (33) appear to be rapidly corrected by addition of insulin in vitro (34), whereas we show here that the diabetes-induced impairment in PC acylation is unchanged by exposure of erythrocytes to insulin.

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