Reduced Na^+-K^+-ATPase Activity and Plasma Lysophosphatidylcholine Concentrations in Diabetic Patients

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A fraction from normal human plasma inhibiting Na^+-K^+ ATPase has been recently identified as lysophosphatidylcholine (LPC). The aim of this study was to investigate the existence of a relationship between the activity of the cellular membrane Na^+-K^+-ATPase and plasma LPC in human diabetes. We studied 10 patients with insulin-dependent diabetes mellitus (IDDM), 14 patients with non-insulin-dependent diabetes mellitus (NIDDM), and 10 sex- and age-matched control subjects. Plasma LPC concentrations were increased in both IDDM and NIDDM patients compared with control subjects. Na^+-K^+-ATPase activity was reduced in both groups of patients in erythrocyte and platelet membranes. There was a significant correlation between the concentrations of plasma LPC and Na^+-K^+-ATPase activity in both erythrocyte and platelet membranes ($P < 0.01$). To investigate the effect of LPC on the enzyme, Na^+-K^+-ATPase activity was determined in erythrocyte membranes obtained from six healthy subjects after in vitro incubation with increasing concentrations of LPC (1–10 μM). Enzymatic activity was significantly reduced by in vitro LPC at a concentration of 2.5 μM, with a further decrease at 5 μM. These data suggest that the decrease in Na^+-K^+-ATPase activity in diabetes might be due to increased LPC concentrations.
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A decrease in the enzymatic activity of the plasma membrane Na^+-K^+-ATPase has been described in various human diseases, such as hypertension (1) and diabetes (2). It has been hypothesized that the impaired active sodium transport might play a major role in the pathophysiology of the chronic complications of diabetes (retinopathy, nephropathy, neuropathy, and premature vascular disease) (2).

The molecular mechanism of the inhibition of the membrane Na^+-K^+-ATPase activity has been widely studied in hypertension, where the disturbances of ion transport have been attributed to plasma inhibitors (3) or to an intrinsic abnormality in the physicochemical structure of the cell membrane (4). Fractions from normal human plasma that inhibit Na^+-K^+-ATPase have been identified as nonesterified fatty acids (NEFA) and lysophosphatidylcholine (LPC) (5). Therefore, it has been suggested that these lipids might alter the sodium pump in disease states characterized by abnormalities in lipid metabolism (5).

The aim of this study is to investigate the relation between the activity of the cellular membrane Na^+-K^+-ATPase and the plasma concentrations of LPC in human diabetes, where both a decrease in sodium pump activity (2,6) and abnormal lipid metabolism (7) have been described.

RESEARCH DESIGN AND METHODS

We studied 10 patients with insulin-dependent diabetes mellitus (IDDM) (5 men, 5 women; 38 ± 9 years of age; duration of disease 13 ± 8 years; range of body mass index [BMI] 20.3–23.6 kg/m^2), 14 patients with non-insulin-dependent diabetes (NIDDM) (8 men, 6 women; 46 ± 7 years of age; duration of disease 7 ± 5 years; range of BMI 21.2–24.5 kg/m^2), and 10 sex- and age-matched healthy subjects (30–51 years of age; range of BMI 20.7–24.8 kg/m^2). All the subjects studied were normotensive and had no family history of hypertension. Two patients with IDDM and three with NIDDM had background retinopathy. No patient was taking lipid-lowering drugs. After overnight fasting, blood was drawn from a forearm vein for the determination of the following parameters: activity of the Na^+-K^+-ATPase of erythrocyte and platelet membranes, HbA_{1c}, plasma concentrations of glucose, triglycerides (TG), phospholipids (PL), total cholesterol (C), NEFA, phosphatidylcholine (PC), LPC, and sphingomyelin (SM). TG, PL, C, and NEFA were measured by enzymatic methods (8–11), and HbA_{1c} was measured by high-performance liquid chromatography (12).

The significance of differences was assessed by Student's *t* test for unpaired data. The relations between data were investigated by linear regression analysis.

Na^+-K^+-ATPase assay. Platelet plasma membranes were prepared as described previously (6). Erythrocyte membranes were obtained after lysis of erythrocytes in hypotonic phosphate buffer, as described by Burton et al. (13). In platelet and erythrocyte membranes, Na^+-K^+-ATPase activity was determined as described previously (6,14) by incubating membranes at 37°C in 1 ml of medium ($MgCl_2$ 5 mM, NaCl 140 mM, KCl 14 mM, in Tris-HCl 40 mM, pH 7.7). The ATPase reaction was started by the addition of 3 mM Na_2ATP and stopped 20 min later by the addition of 1 ml of trichloroacetic acid 15%. Inorganic phosphorus (P_i) hydrolyzed from the reaction was measured by the method of Fiske and Subbarow (15). ATPase activity assayed in the presence of 10 mM ouabain was subtracted from the total Mg^{2+}-dependent ATPase activity to calculate the activity of the ouabain-sensitive Na^+-K^+-ATPase. The results have been expressed as micromoles of P_i per milligrams of membrane protein per hour. Protein concentration was determined by the method of Lowry et al. (16), using albumin as a standard.

Plasma LPC assay. Lipids were extracted from total plasma by the method of Folch et al. (17) and separated by thin-layer chromatography

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NEFA, nonesterified fatty acids; LPC, lysophosphatidylcholine; IDDM, insulin-dependent diabetes mellitus; BMI, body mass index; NIDDM, non-insulin-dependent diabetes mellitus; TG, triglyceride; PL, phospholipid; C, total cholesterol; PC, phosphatidylcholine; SM, sphingomyelin; BSA, bovine serum albumin; P_i, inorganic phosphorus.

TABLE 1
Fasting glycemia, HbA_{1c}, and plasma concentrations of TGs, C, PLs, and NEFAs in normal subjects and in patients affected by IDDM and NIDDM

	Control subjects	IDDM subjects	NIDDM subjects
n	10	10	14
Fasting glycemia (mM)	4.3 ± 0.3	9.0 ± 2.9*	8.1 ± 2.0*
HbA_{1c} (%)	4.7 ± 1.3	8.6 ± 1.2*	8.1 ± 1.3*
Plasma TG (mM)	1.09 ± 0.31	1.41 ± 0.25†	1.69 ± 0.49*
Plasma C (mM)	4.59 ± 0.47	5.18 ± 0.43†	5.41 ± 0.87†
Plasma PL (mM)	2.42 ± 0.32	3.01 ± 0.69	3.13 ± 0.69
Plasma NEFA (mM)	0.62 ± 0.32	0.63 ± 0.29	0.94 ± 0.38†

Data are means ± SD. Comparisons are made between normal subjects and diabetic patients. *$P < 0.01$; †$P < 0.05$.

on silica gel 60 plates with chloroform:methanol:7 N ammonia (76:30:5, vol/vol). The lipids were visualized by iodine vapor, scraped, and analyzed for P_i by the method of Ames (18). The PLs were identified by comigration with standards. PC, LPC, and SM concentrations were expressed as micromoles of P_i per liter of plasma (μmol P_i/l).

In vitro experiments. To test directly the effect of LPC on the plasma membrane, erythrocyte membranes from six healthy subjects and five patients affected by IDDM were incubated for 2 h at 37°C with LPC (1-palmitoyl-glycerol-3-phosphorylcholine, Sigma) at increasing concentrations (0, 1, 2.5, 5, 10 μM). Solutions containing 1,000 times the final LPC concentrations were prepared in 100% ethanol and further diluted in the incubation medium. Tubes without LPC had an identical amount of 100% ethanol added. An incubation of the membranes with different LPC levels (0, 10, 50, 100, 150, 200, 250 μM) was also performed under the same conditions in the incubation medium supplemented with 4% bovine serum albumin (BSA) (i.e., albumin levels in the range of the normal plasma concentrations). The erythrocyte membranes used for the incubation experiments were prepared according to the method of Burton et al. (13), including repeated washing in isotonic saline and in phosphate buffers of decreasing molarity, so that the presence of any residual amount of plasma LPC could be excluded. After incubation, Na^+-K^+-ATPase activity was determined in the membranes by the method described above.

RESULTS

The patients with IDDM and NIDDM did not have significantly different plasma PL concentrations compared with control subjects, but they had significantly higher concentrations of plasma C and TG, fasting glycemia, and HbA_{1c} (Table 1). The plasma concentrations of NEFA were significantly increased in the patients with NIDDM compared with healthy subjects (Table 1; $P < 0.05$) but were unchanged in IDDM subjects (Table 1).

The composition of plasma PLs was altered in the diabetic subjects (Table 2). Plasma LPC concentrations were increased in both the patients with IDDM and NIDDM compared with healthy subjects ($P < 0.01$) (Table 2). The concentrations of plasma PC and SM were not different among the three groups studied (Table 2).

TABLE 2
Plasma LPC, SM, and PC in normal subjects and in patients affected by IDDM and NIDDM

	Control subjects	IDDM subjects	NIDDM subjects
n	10	10	14
LPC (μmol P_i/l)	143 ± 22	237 ± 45*	208 ± 41*
SM (μmol P_i/l)	257 ± 63	299 ± 74	284 ± 61
PC (μmol P_i/l)	752 ± 69	741 ± 83	703 ± 74

Data are means ± SD. Comparisons are made between normal subjects and diabetic patients. *$P < 0.01$.

TABLE 3
Na^+-K^+-ATPase activity in the erythrocyte membranes and in the platelet membranes from normal subjects and from patients affected by IDDM and NIDDM

	Control subjects	IDDM subjects	NIDDM subjects
n	10	10	14
Erythrocyte Na^+-K^+-ATPase (μmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$)	1.51 ± 0.18	0.98 ± 0.23*	1.07 ± 0.15*
Platelet Na^+-K^+-ATPase (μmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$)	1.55 ± 0.21	1.02 ± 0.17*	1.10 ± 0.12*

Data are means ± SD. Comparisons are made between normal subjects and diabetic patients. *$P < 0.01$.

Na^+-K^+-ATPase activity was lower in both groups of patients compared with control subjects in the erythrocyte and platelet membranes ($P < 0.01$) (Table 3). The linear regression analysis showed a significant correlation between Na^+-K^+-ATPase activity in platelet and erythrocyte membranes (data not shown; $r = 0.81$, $P < 0.01$). Non-ouabain-inhibited ATPase activity was not significantly different in diabetic subjects compared with control subjects (erythrocyte membranes: controls = 1.81 ± 0.32, IDDM = 1.92 ± 0.29, NIDDM = 1.89 ± 0.27 μmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$; platelet membranes: controls = 2.05 ± 0.39, IDDM = 2.12 ± 0.27, NIDDM = 1.96 ± 0.31 μmol $P_i \cdot mg\ protein^{-1} \cdot h^{-1}$).

There were significant correlations of the concentrations of plasma LPC with the Na^+-K^+-ATPase activities in erythrocyte ($r = 0.78$; $P < 0.001$; Fig. 1) and platelet membranes ($r = 0.71$; $P < 0.01$; Fig. 2). No significant relation was found between Na^+-K^+-ATPase activity in platelet and erythrocyte membranes and fasting blood glucose concentrations, HbA_{1c}, plasma NEFA, TG, C, PL, SM, or PC concentrations.

The susceptibility of the Na^+-K^+-ATPase of erythrocyte membranes from healthy subjects to inhibition by LPC in vitro without BSA supplementation is shown in Fig. 3.

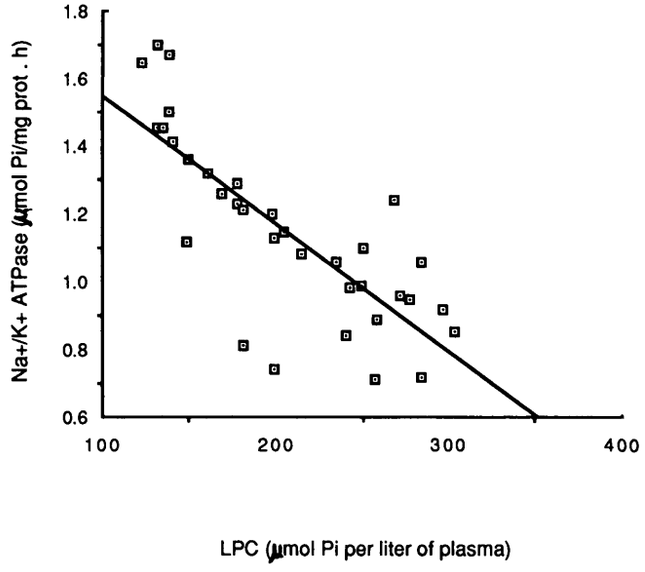

FIG. 1. Linear regression analysis of the relationship between plasma LPC concentrations and the enzymatic activity of the Na^+-K^+-ATPase of the erythrocyte membrane. The regression equation was $y = 1.926 - 0.004x$. $r = 0.78$; $P < 0.001$.

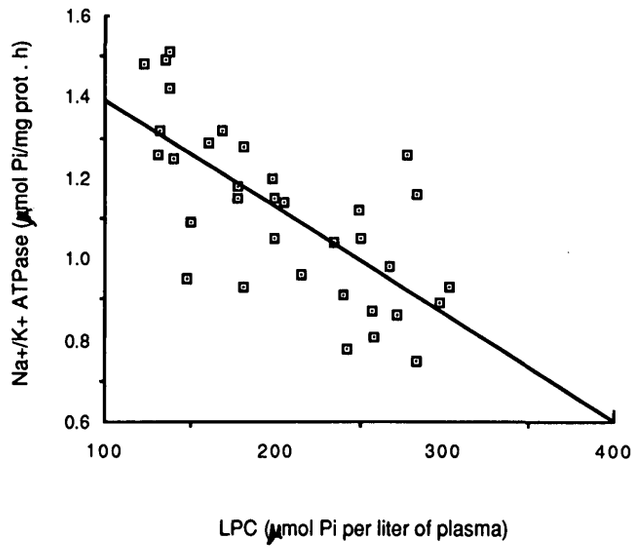

FIG. 2. Linear regression analysis of the relationship between plasma LPC concentrations and the enzymatic activity of the Na^+-K^+-ATPase of the platelet membrane. The regression equation was $y = 1.659 - 0.003\times$. $r = 0.71$; $P < 0.01$.

Inhibition of the enzyme was ~50% at 2.5 μM, with a further decrease in the Na^+-K^+-ATPase activity at a concentration of 5 μM LPC. In the presence of BSA, we observed no inhibition of the Na^+-K^+-ATPase activity of membranes from healthy subjects at 10, 50, 100, and 150 μM LPC, but a 40% inhibition was found at 200 μM LPC with a further decrease at 250 μM LPC (60% inhibition). In the membranes obtained from IDDM subjects, the basally reduced Na^+-K^+-ATPase activity was not inhibited further at any of the LPC concentrations tested, either in the presence or in the absence of BSA (data not shown).

DISCUSSION

The role of Na^+-K^+-ATPase inhibition in the pathogenesis of the chronic complications of diabetes has been the object of numerous studies (2,6). Our results show that both erythrocyte and platelet membranes from diabetic patients have lower Na^+-K^+-ATPase activity than do membranes obtained from healthy control subjects, which confirms data previously reported by our group (6,19). Moreover, we found an increase in the plasma LPC concentrations in patients with IDDM and NIDDM, without changes in the plasma PC and SM concentrations, and a significant negative correlation between the Na^+-K^+-ATPase activity of platelet and erythrocyte membranes and the plasma concentrations of LPC. Finally, in vitro incubation with LPC caused inhibition of Na^+-K^+-ATPase activity in erythrocyte membranes from healthy subjects.

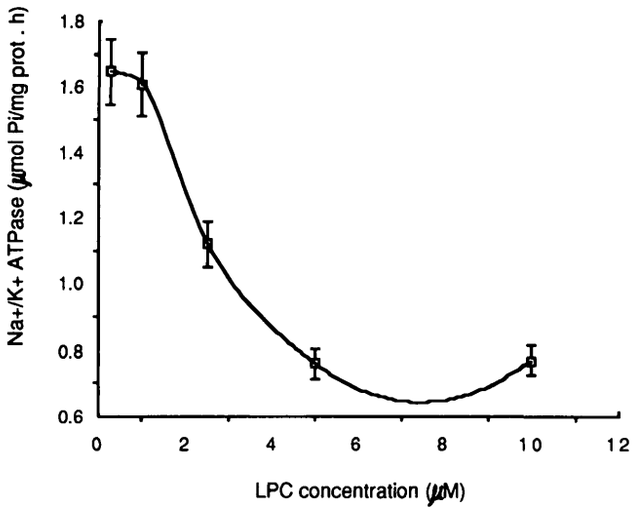

FIG. 3. The effect of increasing concentrations of LPC on Na^+-K^+-ATPase activity in erythrocyte membranes from healthy subjects without albumin addition. Data are the means ± SD of six experiments.

Erythrocytes and platelets showed, in our work, a similar reduction in Na^+-K^+-ATPase activity in diabetic subjects, with a significant correlation between the enzymatic activity in their membranes. Both cellular types lack nuclei with synthetic machinery and are strictly dependent on the plasma lipid composition for changes in the membrane lipid content. A different behavior of erythrocyte and platelet Na^+-K^+-ATPase has been observed in thyrotoxicosis: reduced enzymatic activity in erythrocytes (20) and increased activity in platelets (21). The hypothesis trying to explain such contrasting observations suggests that thyroid hormones might accelerate the degradation of membrane proteins, including Na^+-K^+-ATPase, in the circulation during erythrocyte aging (20,22), but in platelets, because of their shorter lifespan, only the stimulatory effect on the protein synthesis might be detectable. Our previous work (23) demonstrated that in diabetes, Na^+-K^+-ATPase activity shows an uncompetitive inhibition by a mechanism completely different from the hypothesized effect of thyroid hormones, acting on the synthesis and degradation of protein molecules (20,22). Moreover, in diabetes, the reduction in Na^+-K^+-ATPase activity seems to be a ubiquitous alteration, as it has been widely described in various cellular types (2,6).

The reduced Na^+-K^+-ATPase activity in diabetes apparently contrasts with the stimulatory effect of glucose ingestion and hyperglycemia in normal subjects (24). However, the effect of hyperglycemia during glucose load in healthy subjects might be mediated by the increase in insulin levels, as the stimulatory action of insulin on Na^+-K^+-ATPase is well known.

Elevated plasma NEFA concentrations were found in this study in patients with NIDDM, but normal NEFA levels were observed in subjects affected by IDDM with similar glycemic control. This finding is consistent with previous studies that suggest that the raised NEFA plasma levels in NIDDM in the fasting state might be due to the resistance to the antilipolytic effect of insulin (26). The normal NEFA levels in the IDDM patients studied here might be related to persistent low levels of plasma insulin because of the exogenous hormone given subcutaneously 12 h before the collection of blood samples.

Ng and Hockaday (24) have shown a correlation between plasma NEFA levels and the leucocyte ouabain-sensitive sodium efflux in nondiabetic subjects both in the fasting state and after oral glucose load. However, Poston et al. (27) disagreed with these data, reporting the lack of correlation between glycoside-sensitive sodium efflux rate constant of leucocytes and the serum NEFA concentration. Our results give further support to the latter work (27), as we did not observe any relation between platelet and erythrocyte Na^+-K^+-ATPase and plasma NEFA levels in healthy subjects and in patients affected by IDDM and NIDDM.

On the contrary, our results suggest that raised plasma

LPC concentrations may be involved in the changes in Na^+-K^+-ATPase activity observed in diabetes. Previous results support such a hypothesis. Kelly et al. (5) showed that a plasma fraction with Na^+-K^+-ATPase inhibitory activity contained LPC and that LPC inhibited in vitro both Na^+-K^+-ATPase activity and ouabain-binding to the enzyme isolated from experimental animals. LPC has also been reported to inhibit Na^+-K^+-ATPase activity in cardiac sarcolemma, which suggests a role of LPC accumulation during myocardial ischemia in tissue dysfunction (28). The in vitro Na^+-K^+-ATPase inhibition occurred at LPC concentrations lower than the plasma LPC levels observed both in normal and diabetic subjects (in vitro 2.5–10 μM, corresponding to an identical molarity of P_i, versus 100–300 μM P_i, which is the range of plasma LPC concentrations observed in vivo). The difference between plasma LPC levels and the in vitro concentrations of LPC inhibiting Na^+-K^+-ATPase activity might be due to the limited interaction between plasma LPC—partly bound to albumin and partly incorporated in lipoproteins—and the cellular membranes, determining an actual reduction in plasma free LPC availability. The relevance of protein binding is demonstrated by the observation that, in the presence of BSA, only LPC concentrations in the range of the plasma levels detected in diabetic subjects were able to inhibit Na^+-K^+-ATPase activity in normal membranes. The absence of inhibition observed in the experiments performed on membranes from IDDM patients might be explained by the existence of a basal inhibition because of the same mechanism of action.

The experimental model used for the in vitro study used plasma membranes instead of intact cells to avoid the influence on Na^+-K^+-ATPase of variations in cytoplasmic ion levels caused by the contemporary LPC action on other transport systems. In fact, it has recently been reported that the addition of LPC to intact cells can induce an increase in cytoplasmic Ca^{2+}, probably by mobilization from IP_3-sensitive intracellular stores (28,29), and calcium cytoplasmic concentrations are known to affect Na^+-K^+-ATPase activity (30). However, the study of the physiological regulation of Na^+-K^+-ATPase activity in situ is critically relevant to our hypothesis and deserves future investigations.

LPC is an amphiphilic molecule that is rapidly incorporated into plasma membranes. Its action on Na^+-K^+-ATPase might be mediated indirectly by a modification of the physicochemical properties of the membrane, such as fluidity, or by an alteration in the boundary lipid environment of the enzyme. In any case, this action should determine conformational changes of the protein, in agreement with our previous studies on diabetic subjects, which demonstrated the existance of an uncompetitive inhibition of the erythrocyte membrane Na^+-K^+-ATPase activity (23). The hypothesis of an indirect action of circulating lipids on the Na^+-K^+-ATPase activity, mediated through an effect on membrane fluidity, is supported by the finding of a significant relation between Na^+-K^+-ATPase activity and membrane fluidity in diabetic patients (31).

Increased plasma LPC concentrations might be caused by oxidative degradation of plasma lipids, with activation of previously masked phospholipase A2 resulting in the release of lysophosphatides and oxidized fatty acids (32). Diabetic patients present increased superoxide production (33) and enhanced levels of lipid peroxidation products, both in the plasma membranes (34) and in low-density lipoproteins (35). Lipid peroxidation, therefore, might determine the increased plasma LPC concentrations observed in diabetic patients. Further studies are needed to clarify the relationship between the Na^+-K^+-ATPase activity and the altered lipid metabolism in diabetes.

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