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THE RELATION BETWEEN THE MEMBRANE CHOLESTEROL CONTENT AND ANION EXCHANGE IN THE ERYTHROCYTES OF PATIENTS WITH CHOLESTASIS

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The anion transport system was assessed in erythrocytes from fifteen patients with cholestasis and fifteen healthy subjects as the exchange of intracellular chloride for extracellular pyruvate. The rate constant of pyruvate-chloride exchange was significantly reduced in the patients compared with the controls. The membranes of the erythrocytes of the patients compared with the controls contained more cholesterol and phospholipid and had a higher molar ratio of cholesterol to phospholipid. The rate constant of pyruvate-chloride exchange was directly related to the cholesterol to phospholipid ratio of the membranes in the patients. In vitro loading with cholesterol of erythrocytes from healthy subjects reduced the rate constant of pyruvate-chloride exchange by an amount which was related to the increase in the cholesterol to phospholipid ratio of the cell membrane. The inhibitory effect of cholesterol loading on anion transport was similar to its previously reported inhibitory effect on the furosemide-sensitive sodium transport system in the erythrocytes.

Introduction

Proteins embedded in the lipid membrane bring about the transmembrane movement of ions, of metabolites such as sugars, amino acids and organic acids, and of the signals by which hormones unable to pass through the cell membrane, can influence intracellular metabolism. Changes in some of these functions of membrane proteins might contribute to some of the metabolic consequences and clinical features of liver disease.

Patients with liver disease have a reduced sodium flux out of their erythrocytes and this reduction is related to the raised cholesterol to phospholipid molar ratio of the membrane [1]. The reduction in sodium efflux is largely in the furosemide sensitive component of the sodium transport system [2], which has a maximum rate and is therefore probably based on an intra-membrane protein with receptor sites for sodium. There is a similar reduction in the furosemide sensitive sodium flux when normal erythrocytes are loaded with cholesterol 'in vitro' to the levels present in patients with cholestasis [2,3].

When normal cells are loaded to even greater cholesterol levels then the activity of the ($Na^+ + K^+$)-ATPase (the sodium pump) is reduced [2], just as it is when the cholesterol content of the environment of the isolated enzyme is increased [4,5]. However there is no significant inhibition of the sodium pump in patients with cholestasis.

The purpose of the present study was to establish whether an increase in the cholesterol-phospholipid ratio of the erythrocyte membrane seen in cholestasis affected another transport system based on a membrane protein, and the system we have investigated is the anion exchange system. The physiological role of this process is exchange of

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bicarbonate and chloride which is very rapid and difficult to measure. We chose to measure the slower exchange of pyruvate for chloride for two reasons. Unlike many carboxylic acids pyruvate shows very little non-ionic diffusion across the membrane [6,7], and at the high concentrations used in these experiments the exchange of pyruvate for chloride should be mediated predominantly by the inorganic anion exchange system [8] based on the band 3 protein [9], rather than the proposed monocarboxylate carrier [8,10].

Subjects and Methods

Anion transport and membrane cholesterol and phospholipid content were measured in erythrocytes from fifteen patients with chronic cholestasis and from fifteen healthy subjects (members of staff). The patients were ten men and five women aged 34–81 years, and the healthy subjects were ten men and five women aged 22–54 years.

The patients were chosen because they had the typical biochemical features of cholestasis. They had a raised plasma concentration of alkaline phosphatase (20–240 King Armstrong units per 100 ml; reference range 0–13), a raised total bilirubin (31–654 $\mu mol \cdot l^{-1}$; reference range 0–17), and a raised conjugated bilirubin (12–495 $\mu mol \cdot l^{-1}$). In ten patients there was radiological or operative evidence of extrahepatic biliary obstruction, but in the other five the cause of the cholestasis was not established.

Samples of venous blood (approx. 10 ml) were anticoagulated with lithium heparin, and centrifuged.

The erythrocytes were washed three times with cold buffer solution, and packed by centrifugation at $2200 \times g$ for 15 min at 4°C. The cholesterol and phospholipid content of the erythrocyte membranes was measured in 500 µl of washed cells, as previously described [2].

Pyruvate influx through the anion exchange mechanism was measured as the counter-efflux of chloride from the cells into the surrounding medium. An accurately measured volume of washed packed erythrocytes were cooled to 4°C. An equal volume of cold (4°C) iso-osmolar sodium pyruvate (160 $mmol \cdot l^{-1}$) was added to the cold cells, and the suspension was mixed immediately. A total of five samples of the suspension were removed at noted times during the following 45 min; the first sample was always taken one minute after mixing. Each sample of the suspension was centrifuged immediately in a Beckman microfuge at 4°C and the supernatant was stored. The cell suspension which remained after the five samples had been taken was warmed in a 37°C water bath for 10 min. A sample of the suspension was then centrifuged and the chloride content of the supernatant was taken as the equilibrium value.

The chloride content of each supernatant was measured with a Radiometer CMT 10 chloride meter.

During incubation the extracellular chloride concentration increased exponentially towards an equilibrium value. The logarithm of the difference between the chloride concentration at each time and the equilibrium value decreased linearly with time. The slope of this line was calculated by the least squares method of linear regression analysis and taken as the rate constant of pyruvate-chloride exchange. The relationship between chloride concentration (Cl^-), time (t) and rate constant (k) is given as:

$$\ln([Cl^-]_{t=\infty} - [Cl^-]_t) = \ln([Cl^-]_{t=\infty} - [Cl^-]_{t=0}) - kt$$

To study the effect of cholesterol loading in vitro on anion exchange, samples of washed erythrocytes from ten healthy subjects were divided into two portions, one was treated as a control and the other was loaded with cholesterol 'in vitro' as previously described [2]. The anion exchange and lipid content were measured in both samples.

The loading technique used pure diphosphatidylcholine and was essentially the same as that used recently [11] to demonstrate that the effects of cholesterol loading on anion transport are reversible and do not involve any loss of membrane protein. Also erythrocytes incubated with a cholesterol/phosphatidylcholine dispersion designed to leave the cholesterol to phospholipid ratio of the membrane unchanged showed no change in the rate constant for pyruvate-chloride exchange.

To define the characteristics of pyruvate exchange and the effects on it of cholesterol loading, we measured it in two pairs of samples at each of five pyruvate concentrations from 50 to 160 mmol

$\cdot 1^{-1}$. Each medium was made iso-osmolar by adding the appropriate volumes of iso-osmolar aqueous trisodium citrate which is not transported through the anion system [12]. In order to simplify this experiment we took advantage of the finding [13] that pyruvate and other anions exchange not only with intracellular chloride ions but also with intracellular hydroxyl ions. The appearance of hydroxyl ions (although quantitatively small) can be easily measured as a change in pH. The efflux of hydroxyl ions was measured by continuous monitoring of extracellular pH in 3 ml of an iso-osmolar solution of pyruvate (pH 7.40) to which 0.5 ml of washed, packed erythrocytes were added. There was an initial fall in extracellular pH due to a rapid but limited exchange of intracellular chloride for extracellular hydroxyl ions [13] and then the concentration of hydroxyl ions in the extracellular solution increased exponentially with time due to pyruvate-hydroxyl exchange until an equilibrium was reached (at about 3 min in normal cells at 25°C). The rate constant of pyruvate-hydroxyl exchange was calculated as described above for pyruvate-chloride exchange. The technique for pyruvate-hydroxyl exchange was simple and rapid enough to allow the measurement of pyruvate exchange at several different pyruvate concentrations on one batch of cells on the same day, an experiment which was not feasible using the chloride method.

The buffer used for both washing and incubation of cells contained (mmol $\cdot 1^{-1}$); NaCl 140, KCl 5, $MgSO_4$ 1, $CaCl_2$ 2, glucose 10, and bovine serum albumin 100 mg $\cdot 1^{-1}$. The iso-osmolar pyruvate solution contained 160 mmol $\cdot 1^{-1}$ of sodium pyruvate. The trisodium citrate solution was prepared to an osmolality of 290 mosmol $\cdot$ kg^{-1}, and all solutions were finally adjusted to an osmolality of 290 ± 5 mosmol $\cdot$ kg^{-1} and pH of 7.4 at 4°C.

Statistics

The results in each group are given as the mean and standard deviation. The significance of any difference between the mean values in the groups was assessed by Student's *t*-test. The correlation between two variables was assessed by the correlation coefficient. The best-fit relationships were calculated as the major axes of symmetry as described by Brace [14].

Results

The patients with cholestasis had a lower average rate constant for pyruvate-chloride exchange than the healthy subjects and a higher cholesterol to phospholipid molar ratio in the cell membrane (Table I). Fig. 1 shows that in the patients (closed circles) there was a significant inverse relationship between the rate constant of pyruvate-chloride exchange and the cholesterol phospholipid ratio ($r = -0.68$, $P < 0.01$) (Fig. 1).

When erythrocytes from healthy subjects were loaded with cholesterol in vitro, cholesterol to phospholipid ratios of between 1.00 and 1.84 were achieved (Fig. 1, closed squares). The rate constant of pyruvate-chloride exchange was significantly

TABLE I

THE CHOLESTEROL AND PHOSPHOLIPID CONTENT OF ERYTHROCYTE MEMBRANES AND THE RATIO BETWEEN THEM (C/P) AND THE RATE CONSTANT OF PYRUVATE-CHLORIDE EXCHANGE ($k_{P:Cl}$) IN 15 HEALTHY CONTROLS AND 15 PATIENTS WITH CHOLESTASIS

		Cholesterol (mmol $\cdot 1^{-1}$)	Phospholipid (mmol $\cdot 1^{-1}$)	C/P	$k_{P:Cl}$ (h^{-1})
Healthy controls	Mean	3.87	4.66	0.832	3.14
	S.D.	0.16	0.18	0.026	0.28
Patients	Mean	5.17	5.39	0.962	2.38
	S.D.	0.67	0.66	0.073	0.28
		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

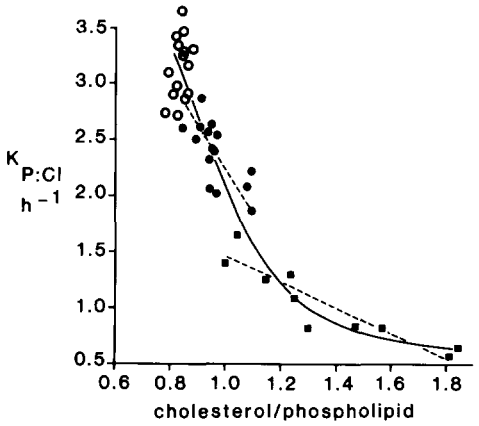

Fig. 1. The relationship between the rate constant of pyruvate-chloride exchange ($k_{P:Cl}$) and cholesterol/phospholipid ratio of eryhtrocyte membrane in healthy persons (open circles), in patients with cholestasis (closed circles) and in erythrocytes from healthy persons loaded with cholesterol in vitro (closed squares). The dotted lines are the separate linear relationship in the patient's and in the colesterol-loaded cells and the continuous line was drawn by eye through all the data.

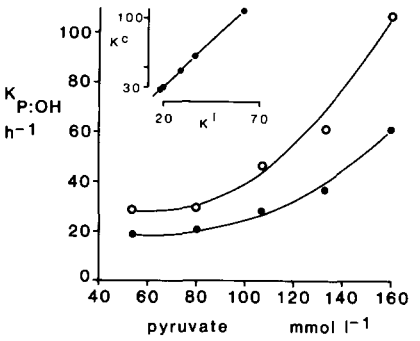

Fig. 2. The relationship between the extracellular pyruvate concentration and the rate constant for pyruvate-hydroxyl exchange ($k_{P:OH}$) in erythrocytes from healthy persons before (open circles) and after (closed circles) loading with cholesterol in vitro. The inset shows the relationship between the rate constants before and after loading (K^c and K^l, respectively).

lower in the loaded cells than in the unloaded cells (1.03 ± 0.35 compared with 2.67 ± 0.28 h^{-1}, $P < 0.001$) and in the loaded cells the rate constant was inversely related to the cholesterol to phospholipid ratio ($r = -0.90$; $P < 0.001$), (Fig. 1, closed squares).

When the results in the healthy controls, the patients, and the cholesterol loaded cells were examined together (Fig. 1) a continuous curvilinear relation between the rate constant for pyruvate-chloride exchange and the cholesterol to phospholipid ratio could be drawn through all the data which fitted well with the linear relationships calculated separately for the patients and for the cells from healthy subjects loaded with cholesterol in vitro.

Fig. 2 shows the relationship in unloaded and cholesterol loaded cells between extracellular pyruvate concentration and the rate constant of pyruvate-hydroxyl exchange. The rate constant for exchange increased with the extracellular pyruvate concentration but the relationship was not shaped like a rectangular hyperbola. The rate constant of pyruvate-hydroxyl exchange was lower in the loaded cells at all pyruvate concentrations studied and there was a linear relationship between the rate constant of the control erythrocytes and the rate constant of the same cells loaded with cholesterol (Fig. 2, inset).

A Lineweaver-Burk plot of the data in Fig. 2 was linear but gave a negative intercept of the axis for the rate of exchange confirming that Michaelis-Menten kinetics did not apply. Further attempts to define the kinetics using the Hill transform were not possible because an estimate of maximum velocity could not be obtained even from the rate constant at the highest concentration of pyruvate we could use in an iso-osmolar solution (Fig. 2).

Discussion

The experiments presented here demonstrate an inhibitory effect of cholesterol loading in vivo or in vitro on the anion transport mechanism as measured by pyruvate-chloride exchange. In patients with cholestasis the rate constant was reduced to 75% of normal and the most extreme in vitro loading reduced it to 20% of normal.

A previous report [11] has indicated that in vitro loading with cholesterol also inhibits sulphate exchange which is thought to occur through the inorganic anion exchange system based on the band 3 protein [9], but stimulates the exchange of lactate based on a more specific monocarboxylate carrier [10].

It would seem that since both pyruvate-chloride

and pyruvate-hydroxyl exchange are inhibited by excess cholesterol that we are dealing with changes in the inorganic anion exchange system. This view is supported by a previous report [8] that the exchange of pyruvate for chloride is sensitive to the band 3 specific inhibitor 4,4′-diisothiocyano-2,2′-stilbenedisulphonate (DIDS). Also pyruvate-hydroxyl exchange is sensitive to inhibition by DIDS in a very similar way to sulphate exchange (Jackson, P.A., unpublished observations).

We propose a curvilinear relationship between pyruvate-chloride exchange and the membrane cholesterol to phospholipid ratio. The difference in slopes of this relationship for the patient group and the erythrocytes loaded in vitro could also be explained by the change in the phospholipid pattern [1] associated with liver disease. This change involves an increase in phosphatidylcholine and a reduction in sphingomyelin which could accentuate the effects of cholesterol in the patients. A recent study of the changes in membrane lipids in liver disease has however indicated that excess cholesterol and not changes of phospholipids, is the major determinant of reduced membrane fluidity [15].

The shape of the relationship between pyruvate exchange for hydroxyl and pyruvate concentration in unloaded and cholesterol-loaded erythrocytes from healthy subjects was not hyperbolic.

Similar relationships have been observed for lactate and glycolate exchange in the bovine red cell [16], for phosphate and sulphate, and for pyruvate in the absence of other electrolytes in the erythrocyte ghost [7], as distinct from self exchange of anions such as chloride which show Michaelis-Menten kinetics [17].

The shape of the curve could be explained by the existence of multiple anion sites on each transport unit with some sort of interaction or co-operation between them [7,18].

The appearance of the curve could alternatively result from competition for binding sites from the citrate used to maintain tonicity. Citrate is impermeant; however it is an anion and it might bind to the anion carrier even if it was not transported. This explanation is unlikely for two reasons. Firstly, citrate has been successfully used to establish the K_m for phosphate exchange [12], were previous substitution with chloride, which is a competitor, had failed to allow saturation by phosphate. Secondly, citrate has been shown to stimulate rather than inhibit pyruvate influx into erythrocyte ghosts [7].

The rate of anion exchange is pH dependent [6,12]. The observed initial fall in pH differs at different concentrations of pyruvate. The initial rates of pyruvate-hydroxyl exchange might therefore be expected to differ. We have quantitated the exchange by determining a rate constant which is representative of the exchange, the rate of which is continuously changing with changing anion concentration and pH up to the equilibrium point. This does not, however, exclude the possibility that pH dependency is the cause of the unusual shape of the relationship between the rate constant and pyruvate concentration. Pyruvate net exchange [7] and pyruvate equilibrium efflux [16] however both show very little dependence on pH in the region pH 6 to 7.5 where all our measurements were made.

The linear relationship between the rate constant for pyruvate-hydroxyl exchange in normal cells and that in the same cells loaded with cholesterol however indicates that the shapes of the two kinetic curves are directly proportional to each other and that the reduction in anion exchange is probably a change in the amount rather than the nature of the process.

Explanations for such a change in amount could be a reduction in the number of functioning exchange units, or a reduction in their affinity for pyruvate or a reduction in their rate of turnover in the membrane caused by the known effects of excess cholesterol on membrane fluidity [15,19].

The effect of excess membrane chlolesterol on anion exchange as measured here is similar to the previously reported effects on furosemide-sensitive sodium efflux [2,3] and inorganic anion exchange [11].

Cholesterol loading also inhibits the sodium pump but this active process seems much less sensitive to such inhibition [2]. There is little effect of cholesterol loading in vivo or in vitro on the general membrane permeability to either sodium ions [2] or undissociated organic acids and non-electrolytes [20].

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