Human erythrocyte membrane fluidity and calcium pump activity in primary combined hyperlipidaemia

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1. Human erythrocyte membrane cholesterol, fluidity and basal and calmodulin-stimulated calcium pump (Ca$^{2+}$-Mg$^{2+}$-ATPase) activities were compared in 24 patients with primary combined hyperlipidaemia and 20 age-matched normolipidaemic control subjects.

2. There was no correlation between serum and membrane cholesterol. Despite the differences in serum cholesterol levels between the two groups, membrane cholesterol levels were similar.

3. 1,6-Diphenyl-1,3,5-hexatriene anisotropy was lower in the hyperlipidaemic group, suggesting increased fluidity in the hydrocarbon core of the phospholipid membrane bilayer.

4. Basal calcium pump activity was lower in the hyperlipidaemic group with increased membrane fluidity.

5. These results suggest that membrane adaptive mechanisms can maintain membrane cholesterol within a narrow range, that serum triacylglycerol is more important than serum cholesterol in determining membrane fluidity and that increased membrane fluidity reduces basal calcium pump activity.

INTRODUCTION

The fluid nature of biological membranes is well documented and widely accepted [1]. In liposomes [2], erythrocytes [2] and platelets [3] increasing membrane cholesterol content reduces membrane fluidity and vice versa. Furthermore, in erythrocytes and platelets, in which lipid metabolism is minimal, serum and membrane lipids are in dynamic equilibrium [4]. Therefore, in clinical conditions in which lipid metabolism is abnormal, membrane fluidity should also be abnormal.

In the sarcoplasmic reticulum, an increase in membrane cholesterol content causes a reduction in calcium pump (Ca$^{2+}$-Mg$^{2+}$-ATPase enzyme) activity [5], although this has not been a consistent finding [6]. To our knowledge, no similar studies have been carried out on the erythrocyte. Adeoya et al. [7, 8] have shown the importance of membrane environment in determining erythrocyte calcium pump activity in rats and humans with hypertension. In intact membranes, calcium pump activity was lower in hypertensive than in normotensive subjects but, when the enzyme was extracted from the membrane, calcium pump activity was similar in the two groups.

The present study investigates the effect of primary combined hyperlipidaemia on erythrocyte membrane fluidity and calcium pump activity and examines the relationship between these parameters in this disorder.

MATERIALS AND METHODS

The study was approved by the Leicestershire Health Authority Ethics Committee, and all participants gave fully informed consent.

Subjects

Patients with combined hyperlipidaemia, age ≤65 years, were recruited from the Glenfield Hospital Lipid Clinic, and age-matched normolipidaemic control subjects were recruited from routine medical and surgical hospital admissions (n=16) and a small number of staff (n=4). Primary combined hyperlipidaemia was defined as serum total cholesterol ≥6.5 mmol/l and triacylglycerol ≥3.0 mmol/l, and normolipidaemia as serum total cholesterol <6.5 mmol/l and triacylglycerol <2.3 mmol/l after a 12 h fast in the absence of secondary causes of hyperlipidaemia such as renal, liver and thyroid dysfunction and diabetes mellitus.

Materials

Ammonium molybdate, butan-2-ol and trichloroacetic acid were purchased from Fisons (Loughborough, U.K.), 1,6-diphenyl-1,3,5-hexatriene (DPH) from Aldrich (Gillingham, Dorset, U.K.), 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) from Molecular Probes (Eugene, OR, U.S.A.), adenosine 5'-triphosphate (ATP), Hepes and Trizma base from Sigma (St Louis, MO, U.S.A.), [γ-32P]ATP from Amersham.

Key words: calcium pump, membrane cholesterol, membrane fluidity, triacylglycerol.

Abbreviations: Ca$^{2+}$-Mg$^{2+}$-ATPase, calcium-magnesium ATPase enzyme (or the calcium pump); CI, confidence interval; DPH, 1,6-diphenyl-1,3,5-hexatriene; IOV, inside-out vesicle; SLC, sodium-lithium countertransport; TMA-DPH, 1-[4-(trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene.

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Preparation of erythrocytes

After a 12-h fast, 10 ml of venous blood was collected into a plain tube for serum lipid estimation using Kodak Ektachem Clinical Chemistry Slides (Kodak Clinical Products, Rochester, NY, U.S.A.) and a further 10 ml into a tube containing lithium-heparin as anticoagulant. The lithium-heparin sample was immediately placed on ice and then centrifuged, within 20 min of collection, for 10 min at 1900 g, 4°C. The serum and buffy coat were discarded and the erythrocytes used to prepare ghost membranes and for the extraction of membrane cholesterol.

Preparation of calmodulin-deficient erythrocyte membranes

Ghost membranes were prepared by the method of Niggli et al. [9] with modification at the haemolysis stage where the washed cells were haemolysed in five volumes of buffer containing 2 mmol/l EDTA and 1 mmol/l Tris-HCl, pH 8.0, at 4°C and centrifuged for 10 min at 25 000 g, 4°C.

Extraction and estimation of membrane cholesterol

One millilitre of washed erythrocytes was used for membrane cholesterol extraction using a modified Rose and Oklander method [10] and the cholesterol extracts were stored at -20°C. Membrane cholesterol estimation of all the samples was performed in a single run. Five millilitres of the cholesterol extracts was dried at 50°C under oxygen-free nitrogen and reconstituted in 1 ml of propan-2-ol. Membrane cholesterol content was estimated using a cholesterol test combination kit (Boehringer Mannheim, Lewes, U.K.).

Estimation of membrane protein concentration

A 25-μl volume of membrane suspension was used for protein estimation by the method of Lowry et al. [11].

Determination of erythrocyte membrane fluidity

Freshly prepared 2 mmol/l stock solutions of DPH in tetrahydrofuran and TMA-DPH in dimethylformamide were diluted to working concentrations of 1 μmol/l in 10 mmol/l Tris-HCl buffer, pH 7.4. The microviscosity of erythrocyte ghost membranes was measured by incubating 3 ml of membrane suspension (100 μg/ml) with an equal volume of the 1 μmol/l DPH or TMA-DPH solution in a shaking waterbath at 37°C as described previously [12]. Intra-assay and inter-assay variabilities were 1.2% and 0.7%, respectively for DPH, and 1.8% and 0.1% respectively for TMA-DPH.

Assay for Ca²⁺-Mg²⁺-ATPase activity in ghost membranes

Basal and calmodulin-stimulated calcium pump activities were measured in triplicate under saturating conditions for all substrates, using an [γ-32P]ATP hydrolysis assay as described previously by Adeoya et al. [7]. The final concentrations of added ATP, calcium and calmodulin in the reaction mixture were 1.5 mmol/l, 20 μmol/l and 0.1 mol/l respectively. The released inorganic phosphate, 32P₁, was extracted as an acid-molybdate complex and counted in a toluene-based scintillant using the Packard LS 1500 counter (Pangbourne, Berks, U.K.). Intra-assay and inter-assay variabilities were 3.7% and 0.9%, respectively for basal activity, and 4.5% and 1.3% respectively for calmodulin-stimulated activity.

All experiments, except for membrane cholesterol estimation, were performed on the same day that the erythrocyte membranes were prepared.

Statistical analyses

Because most of the parameters were not normally distributed, the Mann-Whitney U-test was used to compare the two subject groups and Spearman’s rank correlation to assess the relationships between selected parameters. Results are expressed as median (range) and P < 0.05 was taken as significant.

RESULTS

Demography and clinical characteristics of the two groups, serum lipids and membrane anisotropy are shown in Table 1 and calcium pump activities in Fig. 1. There were no differences in age, body mass index and blood pressure profiles between the two groups. Basal calcium pump activity was reduced (Fig. 1) and membrane fluidity measured with DPH increased (reduced DPH anisotropy, Table 1) in the group with primary combined hyperlipidaemia compared with normolipidaemic subjects. TMA-DPH anisotropy, membrane cholesterol and calmodulin-stimulated calcium pump activities were similar in the two subject groups (Table 1, Fig. 1). Exogenous calmodulin stimulated the calcium pump 2.5-fold in the normal control subjects and 3-fold in the hyperlipidaemic group (Fig. 1).

Because the two groups were not well matched for sex, calcium pump activity was compared between genders in each group. No significant difference existed between the activity in females and males in both groups – differences between medians (95% confidence intervals, CI) were as follows: basal
Table I. Demography and clinical characteristics, serum lipids and membrane anisotropy in the two subject groups.
Results are expressed as median (range) and statistical analysis was by the Mann-Whitney U-test. Membrane microviscosity, presented as anisotropy (r), is inversely related to membrane fluidity. NS, non-significant.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (M/F)</td>
<td>20(9/11)</td>
<td>24(1/6)</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.2(20-22)</td>
<td>26.3(21.5-35.8)</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>87(65-116)</td>
<td>89(60-105)</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>140(110-219)</td>
<td>150(120-200)</td>
<td>0.07, NS</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>89(60-105)</td>
<td>82(66-107)</td>
<td>0.001</td>
</tr>
<tr>
<td>Serum total cholesterol (mmol/l)</td>
<td>7.1(6.2-4.4)</td>
<td>4.4(3.0-9.57)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum HDL-cholesterol (mmol/l)</td>
<td>7.1(6.2-4.4)</td>
<td>4.4(3.0-9.57)</td>
<td>NS</td>
</tr>
<tr>
<td>Serum triglycerol (mmol/l)</td>
<td>7.1(6.2-4.4)</td>
<td>4.4(3.0-9.57)</td>
<td>NS</td>
</tr>
<tr>
<td>Membrane anisotropy, DPH</td>
<td>0.26(0.262-0.272)</td>
<td>0.26(0.262-0.269)</td>
<td>NS</td>
</tr>
<tr>
<td>Membrane anisotropy, TMA-DPH</td>
<td>0.26(0.262-0.272)</td>
<td>0.26(0.262-0.269)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Fig. 1. Basal and calmodulin-stimulated calcium pump activities in normolipidaemic control subjects (NC) and in patients with primary combined hyperlipidaemia (PCH). Horizontal bars are median values. Basal activity was reduced in the group with PCH (P = 0.002).

activity, control group -0.19 (-0.72 to 0.10), hyperlipidaemic group 0.02 (-0.44 to 0.32); calmodulin-stimulated activity, -0.35 (-1.49 to 1.05) and 0.43 (-1.37 to 1.38) respectively.

In both groups, there were no correlations between serum cholesterol and membrane cholesterol of DPH anisotropy or between basal calcium pump activity and DPH anisotropy or serum triacylglycerol. There was no significant correlation between serum triacylglycerol and DPH anisotropy [normal controls, r = -0.3 (95% CI -0.7 to 0.1); hyperlipidaemic subjects, r = -0.2 (95% CI -0.6 to 0.2)].

DISCUSSION

DPH measures the fluidity of the hydrocarbon core of the membrane bilayer [4], and TMA-DPH measures that of the outer regions of the membrane (near the phospholipid–water interface) [13] where membrane cholesterol is located. In the present study there was increased fluidity of the hydrocarbon core of the membrane bilayer in the hyperlipidaemic group. Bharaj et al. [14] found no difference in both DPH and TMA-DPH anisotropies between patients with familial hypercholesterolaemia and age- and sex-matched control subjects. We have also found no differences in membrane cholesterol and both DPH and TMA-DPH anisotropies between patients with primary hypercholesterolaemia and normolipidaemic control subjects (S. I. Muzulu et al., unpublished work). Furthermore, a negative correlation between serum triacylglycerol and DPH anisotropy [15-17] has been described. These results suggest that serum triacylglycerol could be more important than serum cholesterol in determining membrane core fluidity in circulating cells.

As in the study of Bharaj et al. [14] and in our previous observations, the fluidity of the outer regions of the membrane of the hyperlipidaemic and normolipidaemic groups was similar. Consistent with this finding is the similarity in membrane cholesterol levels between these two groups despite differences in serum cholesterol values. This is most likely due to membrane adaptive mechanisms to maintain membrane cholesterol and fluidity within a narrow range.

The increased fluidity in the hydrocarbon core of the membranes of hyperlipidaemic subjects was associated with a reduction in basal calcium pump activity. Since membrane cholesterol and TMA-DPH anisotropy were similar to those in normolipidaemic control subjects the reduction in enzyme activity appears to be secondary to the effects of hypertriacylglycerolaemia. This is supported by the findings of Bharaj et al. [14] that hypercholesterolaemic patients and normal controls with similar and normal serum triacylglycerol levels also had similar membrane fluidity and calcium pump activity values. Further evidence that triacylglycerol affects membrane transport processes comes from the de-
scribed positive correlations between serum triacyl-
glycerol and sodium–lithium countertransport (SLC) activity [18–22]. Na⁺/K⁺ co-transport [20, 21] and Na⁺/H⁺ exchange [23]. The increased SLC activity may have been mediated by an increased membrane fluidity [24]. In the study of Brent et al. [22] SLC activity was increased in hypothyroidism and re-
duced in hyperthyroidism. When the underlying thyroid disorder was treated, the normalization of SLC activity was closely related to the changes in serum triacylglycerol. More recently, Carr et al. [25] showed that treating hyperlipidaemia normalized SLC activity in patients with combined hyper-
lipidaemia but not in isolated hypercholesterol-
eaemia, suggesting that serum triacylglycerols were more important than serum cholesterol in modifying SLC activity. It would be interesting to study the effect of lowering serum triacylglycerol on calcium pump activity in hypertriacylglycerolaemic patients.

Some of the effects of membrane lipids on calcium pump activity in the sarcoplasmic reticulum are mediated through the boundary lipids or phos-
pholipid annulus surrounding the enzyme, from which cholesterol is normally excluded [6]. It is possible that in hypertriacylglycerolaemia the fluidity of the boundary lipids is increased to a level above that necessary for optimal calcium pump activity resulting in the reduction in enzyme activity.

Calmodulin-stimulated calcium pump activity was similar in the two subject groups studied. It is possible that calmodulin overcomes the inhibitory effects of the membrane environment on enzyme activity. Adeoya et al. [26] found a similar effect of calmodulin on the accumulation of 45Ca²⁺ in inside-out vesicles (IOVs) from spontaneously hypertensive rats and Wistar–Kyoto normotensive control rats. The temperature at which half-maximal basal calcium pump activity occurred was signifi-
cantly different between the two groups. This inter-
group temperature difference was lost in the presence of calmodulin. It was proposed that cal-
modulin stabilizes the Ca²⁺–Mg²⁺-ATPase enzyme such that the effects of membrane environment on enzyme activity are lost [26].

A number of calcium-regulated processes in the arterial wall may be involved in the pathogenesis of atheroma. These include cell necrosis from intracellular calcium overload; synthesis of connective tissue matrix; lipid accumulation in the extracellular matrix; vascular smooth muscle proliferation and reactivity; and endothelial contraction and endothelium-derived relaxing factor synthesis and release [27, 28]. In animal models there is some evidence that calcium antagonists can retard athero-
genesis [27, 29]. Hence, pathological increases in intracellular calcium concentration as a result of altered calcium pump activity may contribute to atheroma and inappropriate vascular reactivity. Atheroma in combined hyperlipidaemia could be explained, in part, by impaired calcium pump activity secondary to an altered membrane en-
vironment.

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