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Effect of simvastatin therapy on cell membrane cholesterol content and membrane function as assessed by polymorphonuclear cell NADPH oxidase activity

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SUMMARY. Cell membrane cholesterol is an important determinant of membrane fluidity. Changes in fluidity have important consequences for membrane function. Treatment of hypercholesterolaemia could therefore affect membrane function by reducing cell membrane cholesterol levels. The aim of this study was to determine whether treatment with simvastatin affects membrane cholesterol and the activity of the polymorphonuclear cell membrane enzyme NADPH oxidase. Blood was obtained from 12 hypercholesterolaemic patients before, and 6 weeks after, treatment with simvastatin, and from 20 normolipidaemic subjects. Cell cholesterol was in the unesterified form indicating that it was membrane-associated. Pre-treatment mean cell cholesterol concentration in the hyperlipidaemics was higher ($P<0.05$) than in the normolipidaemics [4·19 fmol/cell, 95% confidence interval (CI) 3·38–5·05 versus 3·10 fmol/cell, 95% CI 2·58–3·61]. There was a strong correlation between cell cholesterol content and NADPH oxidase lag phase ($R_s=0.76$, $P<0.01$). Cell cholesterol fell to 3·52 fmol/cell (95% CI 2·77–4·28, $P<0.05$) following treatment and there was a correlation ($R_s=0.61$, $P<0.05$) between the reductions in cell cholesterol and lag phase.

Additional key phrases: hyperlipidaemia; hypercholesterolaemia; HMGCoA reductase inhibitors

Cholesterol is an essential component of the eukaryotic cell membrane, in which it intercalates in the phospholipid bilayer and modifies membrane fluidity.[1] Changes in membrane fluidity have consequences for the function of membrane associated proteins such as enzymes[2] and ion channels.[3] Cell membrane cholesterol is in dynamic equilibrium with both the extracellular and intracellular cholesterol pool,[1] membrane cholesterol exchanges with plasma lipoproteins,[1] and cells also have the capacity for intracellular cholesterol synthesis from acetyl CoA by a metabolic pathway involving the enzyme β-hydroxy β-methylglutaryl coenzyme A reductase (HMGCoA reductase).[4] The ubiquitous nature of HMGCoA reductase activity, as well as the complex cycle of lipoprotein metabolism[5] underlines the essential physiological role of cholesterol, which should not be overlooked even though hypercholesterolaemia is associated with increased risk of coronary artery disease.[6] There have been a few studies of cell membrane cholesterol levels and membrane function in hyperlipidaemia,[3,7–9] but the possibility that drug treatment of hypercholesterolaemia could modify cell membrane cholesterol levels and thereby impair function has received little attention.[10]

The polymorphonuclear cell (PMN) and macrophage plasma membrane-bound enzyme NADPH oxidase catalyses the production of the superoxide radical during the respiratory burst.[11] The development of catalytic activity by this complex enzyme depends upon activation of protein kinase C,[12] either as a result of membrane receptor stimulation[13] or by the direct action of phorbol esters. Activation of protein kinase C initiates an enzyme cascade involving

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the phosphorylation of cytosolic subunits, the activation of phospholipase A_2 and the translocation of at least three cytosolic proteins into the membrane.[14] These proteins aggregate with a flavoprotein-cytochrome *b* complex in the plasma membrane and initiate superoxide production.[15] At the same time a proton-conducting channel opens to provide charge compensation for the electrogenic transfer of an electron.[16] Several or all of these steps would be expected to be sensitive to changes in membrane fluidity and function, suggesting that the rate of assembly of the functional NADPH oxidase enzyme could act as a model for reporting changes in fluidity. There is evidence to support this; incubation of PMN cells *in vitro* in a cholesterol-rich medium results in incorporation of cholesterol into the membrane, a decrease in lateral membrane fluidity, and an increase in the lag phase for activation of NADPH oxidase by phorbol ester.[17]

The aim of this study was to determine whether lipid-lowering drug therapy with the HMGCoA reductase inhibitor simvastatin results in changes in cell membrane function, as reflected by changes in PMN NADPH oxidase activity, and to determine whether this is related to reduced cell membrane cholesterol content.

MATERIALS AND METHODS

Subjects

Patients (7 men and 5 women, mean age 49 years, range 30–62) with hypercholesterolaemia for whom lipid lowering drug therapy was clinically indicated were recruited. Nine were non-smokers and three were smokers. Mean serum total cholesterol concentration was 8·95 mmol/L (95% CI 8·29–9·61); two of the patients had mixed hyperlipidaemia (triglyceride >2·3 mmol/L) and the remainder had pure hypercholesterolaemia. All patients had previously received dietary advice and continued to follow this during the course of the study. Blood was taken after an overnight fast before, and 6 weeks after starting treatment with simvastatin 20 mg nocte (Merck Sharpe & Dohme Ltd, Hoddesdon, UK). No other lipid lowering drugs were taken during or for 3 months before the study. For comparison blood was also taken from 20 normolipidaemic subjects (10 men, 10 women, mean age 38 years, range 21–62). The study was approved by the United Bristol Healthcare Trust Research Ethics Committee.

Blood collection and polymorphonuclear cell isolation

Blood was taken into a plain glass container (6 mL) and into silicone coated plastic containers (12 mL) containing EDTA (Vacutainer Plus, Becton Dickinson, Meylan, France). Following centrifugation at 650 *g* for 10 min, serum total cholesterol was estimated by a standard enzymatic method[18] (cholesterol esterase/cholesterol oxidase) using a Hitachi 717 Analyser (Boehringer Mannheim UK (Diagnostic Systems), Lewes, UK). Polymorphonuclear leukocytes (PMNs) were isolated from the EDTA-anticoagulated blood by a double density Ficoll-Hypaque gradient technique[19] (Histopaque, Sigma Chemical Co, Poole, UK). Residual erythrocytes were removed by hypotonic lysis.[20] The PMNs were then washed twice in phosphate buffered saline before resuspension in a volume of 2 mL. Cell density was measured by an automated cell counter (Technicon H-2 System, Bayer Diagnostics, Basingstoke, UK). The mean proportion of PMNs in the final suspension was 97·8%; mean recovery was 64·2%. The cell suspension was stored at 4°C and PMN NADPH oxidase activity was measured within 3 h.

Polymorphonuclear cell NADPH oxidase assay

PMN NADPH oxidase activity was determined by following the rate of reduction of cytochrome *c* after stimulation with phorbol ester at 37°C using a Vmax microtitre plate reader (Molecular Devices, Menlo Park, CA, USA) enclosed in a custom built thermostatically controlled heated perspex box (University of Bristol Workshops, Bristol, UK).[21] Ferricytochrome *c* (Type VI, Sigma Chemical Co) was dissolved to a concentration of 99·5 μmol/L in phosphate buffered saline (138 mmol/L sodium chloride, 2·7 mmol/L potassium chloride, 8·1 mmol/L disodium hydrogen phosphate, 1·47 mmol/L potassium dihydrogen phosphate, pH 7·4) containing calcium chloride 0·9 mmol/L, magnesium chloride 0·5 mmol/L and glucose 7·5 mmol/L. Then 188·5 μL of the cytochrome *c* solution and either 5 μL water or 5 μL superoxide dismutase (SOD), 9000 units/mL (Sigma Chemical Co) were added alternately to each of eight adjacent wells of a microtitre plate (Falcon Microtest III, Becton Dickinson, Meylan, France). Fifty microlitres of cell suspension was added to each of the eight wells, mixed, and incubated for 3 min before the addition of 10 μL phorbol 12-myristate 13-acetate (8 μg/mL)

TABLE 1. *Serum total cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride concentrations; polymorphonuclear cell cholesterol content, NADPH oxidase lag phase and maximum rate (mean and 95% confidence interval) in hyperlipidaemic patients before and 6 weeks after starting treatment with simvastatin (n = 12) and in normolipidaemic subjects (n = 20)*

	Normal subjects	Hyperlipidaemic patients	
		Pre-treatment	On treatment
Total cholesterol (mmol/L)	5·05 (4·65–5·44)	8·95** (8·29–9·61)	6·55** (5·92–7·18)
HDL cholesterol (mmol/L)	1·31 (1·16–1·46)	1·20 (1·04–1·53)	1·38 (1·14–1·63)
Triglyceride (mmol/L)	0·91 (0·70–1·12)	2·18 (0·99–3·36)	1·86 (1·01–2·70)
Cell cholesterol (fmol/cell)	3·10 (2·58–3·61)	4·19* (3·38–5·05)	3·52* (2·77–4·28)
Maximum rate (nmol/10^7 cells/min)	40·0 (36·3–43·6)	45·6 (39·5–51·8)	44·4 (38·1–50·6)
Lag phase (s)	63·5 (58·4–101·1)	62·1 (42·3–81·9)	58·5 (41·6–75·4)

*$P < 0.05$, **$P < 0.001$ changes after treatment (Wilcoxon signed rank test)

in dimethyl sulphoxide and water (Sigma Chemical Co). Absorbance at 550 nm was measured at 8 s intervals for 6 min. The blank absorbance (in the presence of SOD) was subtracted from the test absorbance for each pair of wells, at each time point, to give a corrected rate of change of absorbance at 550 nm due specifically to production of cytochrome *c* reduced by superoxide. Maximum rate of superoxide production (V_{max}) was calculated using a molar extinction coefficient for reduced cytochrome *c* of 29·5 L cm^{-1} $mmol^{-1}$, and the lag phase was derived by extrapolation of a tangent to the reaction curve at the maximum rate of reaction to the baseline. The within-batch analytical coefficient of variation was 4·7% for rate and 8·0% for lag phase. The mean of four measurements of reaction rate and lag phase for each sample was calculated.

Polymorphonuclear cell cholesterol assay

The remainder of the cell suspension was used for the measurement of PMN cholesterol content by reversed-phase high performance liquid chromatography (LDC system, Milton Roy, Stone, UK).[22] Following centrifugation at 650 *g* for 10 min, 50 μL cholesterol heptadecanoate was added to the cell pellet as internal standard and cell cholesterol was extracted into 5 mL hexane-isopropanol (3:2 v/v). The extract was then evaporated to dryness under nitrogen and re-dissolved in 0·5 mL chloroform. Two millilitres of mobile phase (acetonitrile–isopropanol 1:1 v/v) was added to each sample immediately prior to injection onto a 5 μm Zorbax ODS column (4·4 mm × 25 cm, Du Pont, Chadds Ford, PA, USA). Cholesterol and cholesterol esters were detected by absorbance at 210 nm and quantitated by reference to the internal standard and an external standard of free cholesterol and cholesterol esters. The between-batch analytical coefficient of variation for free cholesterol was 6·2%.

Statistical analysis

Serum and cell cholesterol concentrations and the kinetics of PMN NADPH oxidase in the hyperlipidaemic patients and the normal subjects were compared by unpaired Wilcoxon signed rank tests. Changes in serum and cell cholesterol concentrations and the kinetics of PMN NADPH oxidase before and after treatment were compared by paired Wilcoxon signed rank tests. Correlations between the variables measured were tested for significance using the Spearman rank correlation method.

RESULTS

All patients complied with treatment with simvastatin and after 6 weeks there was a 29% ($P < 0.001$) reduction in mean serum cholesterol concentration to 6·55 mmol/L (95% CI 5·92–7·18). There was no significant change in serum high-density lipoprotein cholesterol or triglyceride concentrations (Table 1). Cell cholesterol was detected entirely as unesterified cholesterol (Fig. 1). Prior to treatment, mean cell cholesterol content was 4·19 fmol/cell (95% CI 3·38–5·05), which was significantly higher ($P < 0.05$) than the level in the normolipidaemic subjects (3·10 fmol/cell, 95% CI 2·58–3.61). There was no difference in PMN NADPH oxidase lag phase or V_{max} between patients and controls. However, in the patients, there was a strong correlation ($R_s = 0.76$, $P < 0.01$) between cell

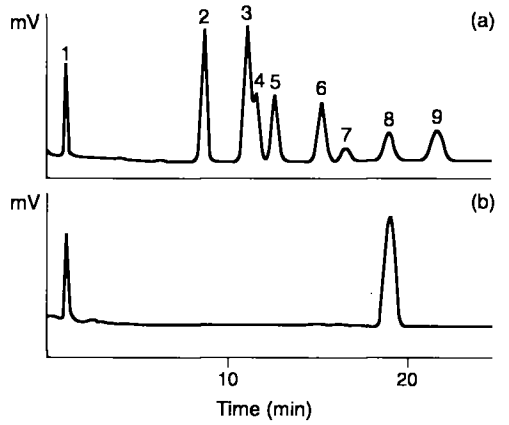

FIGURE 1. *High-performance liquid chromatography separation of (a), cholesterol and cholesterol ester standard mixture and (b), polymorphonuclear cell lipid extract. 1 = Unesterified cholesterol; 2 = cholesterol arachidonate; 3 = cholesterol linoleate; 4 = cholesterol palmitoleate; 5 = cholesterol myristate; 6 = cholesterol oleate; 7 = cholesterol palmitate; 8 = cholesterol heptadecanoate (internal standard), 9 = cholesterol stearate.*

cholesterol level and PMN NADPH oxidase lag phase (Fig. 2).

Treatment resulted in a 16% reduction ($P<0{\cdot}05$) in cell cholesterol to 3·52 fmol/cell (95% CI 2·77–4·28). There was a non-significant reduction in PMN NADPH oxidase lag phase of 6% and no change in V_{max} (Fig. 3, Table 1). In order to determine, however, whether for individual patients there was an association between the magnitude of changes in cell cholesterol content and NADPH oxidase activity, these parameters were compared directly (Fig. 4). The change in lag phase (mean reduction 3·59s, 95% CI −15.0–22·2) following treatment correlated ($R_s = 0{\cdot}608$, $P<0{\cdot}05$) with the change in cell cholesterol content (mean reduction 0·67 fmol/cell, 95% CI −0·14–1·47).

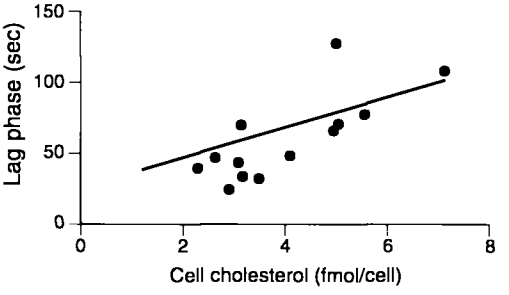

FIGURE 2. *Correlation between lag phase for stimulated polymorphonuclear (PMN) NADPH oxidase activity and PMN cell cholesterol content in hypercholesterolaemic patients (n = 12). $R_s = 0{\cdot}76$, $P<0.01$.*

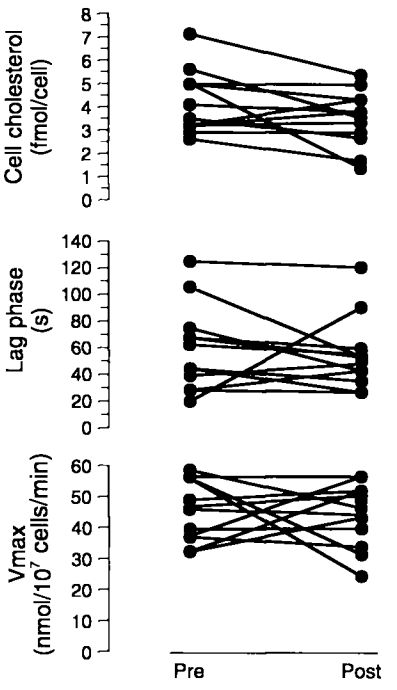

FIGURE 3. *Polymorphonuclear cell cholesterol, NADPH oxidase lag phase and V_{max} in hyperlipidaemic patients before and after treatment with simvastatin for 6 weeks.*

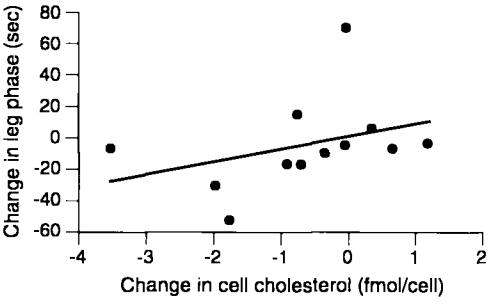

FIGURE 4. *Relationship between change in duration of lag phase and change in cell cholesterol content in hyperlipidaemic patients following treatment with simvastatin for 6 weeks. $R_s = 0{\cdot}608$, $P<0{\cdot}05$.*

DISCUSSION

Treatment of hypercholesterolaemia with an HMGCoA reductase inhibitor reduces the risk of coronary disease,[23,24] due largely to a

reduction in plasma low-density lipoprotein (LDL) concentration.[25] However, treatment may also have effects on cell membrane cholesterol composition. Membrane cholesterol, present almost entirely in the unesterified form,[22] is an important modulator of cell membrane fluidity.[26] This has been confirmed in biological membranes such as erythrocytes and PMN cells using fluorescence anisotropy to demonstrate increased lipid order and decreased lateral lipid mobility following *in vitro* enrichment of membrane cholesterol.[17] Maintenance of normal membrane fluidity is important for membrane structure and function, and consequences of alterations in membrane cholesterol content by *in vitro* enrichment include reduced erythrocyte deformability,[27] and an increase in the lag phase for stimulation of NADPH oxidase mediated superoxide production in PMN cells.[17] The results of this study indicate that this has physiological significance since there was a strong correlation between *in vivo* PMN cell cholesterol levels and PMN NADPH oxidase lag phase.

Membrane cholesterol is derived from a combination of direct exchange with extracellular lipoproteins, internalized LDL, and *de novo* synthesis.[1,4] Lipid lowering drug therapy with an HMGCoA inhibitor could interfere with all of these processes, with possible consequences for membrane function. Treatment with the HMGCoA reductase inhibitor lovastatin has been found to alter erythrocyte sodium-potassium pump activity but it is not known whether this was a direct effect of the cholesterol-lowering action of the drug.[28] In this study, simultaneous measurements of cell cholesterol and membrane function were made. It was not possible, given the relatively small number of PMN cells available for analysis, to fractionate and analyse the cell membrane, but the finding that PMN cholesterol was entirely unesterified indicates that it was of membrane origin. Simvastatin therapy reduced the elevated cell cholesterol levels in hyperlipidaemics towards those found in normolipidaemic subjects. One previous study[9] found no increase in membrane cholesterol levels in hyperlipidaemic patients; this may reflect differences between cell types or in the type of hyperlipidaemia under study.

PMN membrane function, as reported by the lag phase for NADPH oxidase, was influenced by cell membrane cholesterol content in untreated hyperlipidaemia, and cell membrane cholesterol content was reduced during simvastatin therapy. Although there was only a non-significant trend towards a reduction in NADPH oxidase lag phase as a result of treatment, possibly because of the small sample size and relative imprecision of measurement of this parameter, direct evidence for an effect of simvastatin on membrane function was provided by the observation that patients who exhibited the largest reductions in cell membrane cholesterol concentration following treatment also had the largest reduction in lag phase. Taken together, these results suggest that simvastatin exerts effects on membrane function which are due to changes in the lipid composition of the cell membrane. A contribution from other mechanisms, such as a direct effect on membrane function of the lipophilic drug simvastatin, however, cannot be excluded, and it would be of interest to study the effects of less lipophilic HMGCoA reductase inhibitors (e.g. pravastatin) in this context. There also remains the possibility of other cholesterol-independent effects of HMGCoA inhibition such as a reduction in the rate of synthesis of isoprenoids.[29] Some of the cytosolic subcomponents of NADPH oxidase are isoprenylated proteins,[30] and reduced prenylation would reduce solubility in the lipid bilayer.[31]

Although cell cholesterol concentrations were higher than normal in the untreated hyperlipidaemics, it is notable that pre-treatment NADPH oxidase activity was not affected. This could reflect adaptation to chronic hyperlipidaemia, and it would be of interest to study whether similar adaptation occurs during prolonged HMGCoA reductase inhibitor therapy.

PMN NADPH oxidase was chosen for study because it is a well characterized multi-component enzyme system whose function might be expected to be affected by the physical properties of the cell membrane. This enzyme is responsible for the generation of the superoxide radical,[11] a potential initiator of a cascade of oxidative reactions including lipid peroxidation,[32] an early step in atherogenesis.[33] Although it is unlikely that PMN superoxide generation is important in the pathogenesis of atheroma,[33] a reduction in the lag phase for PMN superoxide generation as a result of cholesterol lowering therapy would theoretically be a harmful side-effect of cholesterol lowering drug therapy.

Whatever the mechanism of the effect of HMGCoA reductase inhibitor therapy on cell membrane function, and regardless of whether

effects on PMN NADPH oxidase *per se* are of importance, these findings indicate that the role of cholesterol in the regulation of cell membrane function should not be forgotten in the drive to reduce serum cholesterol levels in order to reduce the risk of atherosclerosis.

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