Statins have beneficial effects on platelet free radical activity and intracellular distribution of GTPases in hyperlipidaemia

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ABSTRACT
In addition to lowering cholesterol, statins may alter endothelial release of the vasodilator NO and harmful superoxide free radicals. Statins also reduce cholesterol intermediates including isoprenoids. These are important for post-translational modification of substances including the GTPases Rho and Rac. By altering the membrane association of these molecules, statins affect intracellular positioning and hence activity of a multitude of substances. These include eNOS (endothelial NO synthase), which produces NO (inhibited by Rho), and NADPH oxidase, which produces superoxide (dependent on Rac). Statins may improve endothelial function by enhancing production of NO while decreasing superoxide production. A total of 40 hypercholesterolaemic patients were randomized to treatment with either atorvastatin or placebo; 20 normolipid-aemic patients were also studied. Platelet nitrite, NO and superoxide were examined as was the cellular distribution of the GTPases Rho and Rac at baseline and after 8 weeks of treatment. Following atorvastatin therapy, platelet NO was increased (3.2 pmol/10⁸ platelets) and superoxide output was attenuated −3.4 pmol · min⁻¹ · (10⁸ platelets)⁻¹ when compared with placebo. The detection of both Rho and Rac was significantly reduced in the membranes of platelets, implying reduced activity. In conclusion, the results of the present study show altered NO/superoxide production following statin therapy. A potential mechanism for this is the change in the distribution of intracellular GTPases, which was considered to be secondary to decreases in isoprenoid intermediates, suggesting that the activity of the former had been affected by atorvastatin.

INTRODUCTION
Statins [HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitors] reduce cardiovascular mortality and morbidity [1,2]. The chief mechanism by which they produce benefit is by lowering LDL (low-density lipoprotein)-cholesterol; however analysis of several randomized trials [1,2] and a wealth of human and animal cellular findings [3,4] suggest that statins have additional ‘pleiotropic’ activity.
One of the earliest indicators of vascular disease is the development of ED (endothelial dysfunction) that acts as the substrate for the accelerated development of atherosclerosis. A principle factor in the development of ED is a decrease in the bioactivity of NO. This may be because of decreased production or enhanced degradation
by ROS (reactive oxygen species), most importantly \( \text{O}_2^{\bullet-} \) (superoxide radical) [5,6]. Statins not only lower LDL-cholesterol [7], but improve endothelial function, and this may contribute to the improved clinical outcomes associated with administration of these drugs [8].

Statins decrease intracellular levels of all of the intermediates of the intrinsic lipid production pathway, including cholesterol [9]. Several of these intermediates, particularly the isoprenoids FPP (farnesyl pyrophosphate) and GGPP (geranylgeranyl pyrophosphate), are thought to have significant biological activity [10]. Their actions have been well established, particularly with respect to intracellular trafficking and signalling [11]. The molecules have been shown to adhere to cellular proteins at a post-translational stage and facilitate the interaction with intracellular organelles or the cell membrane resulting in alteration of activity.

The GTPases Rac and Rho are of particular importance in relation to endothelial function as they can influence the production of NO and \( \text{O}_2^{\bullet-} \). NO production by eNOS (endothelial NO synthase) has been shown to be reduced by Rho in several models [12], whereas the primary source of \( \text{O}_2^{\bullet-} \), namely NADPH oxidase, depends on Rac for conformational change and subsequent activation [13,14].

The aims of the present study were to examine the release of NO, nitrite and \( \text{O}_2^{\bullet-} \) from platelets (used as a compartmentalized tissue model for endothelial cells) in patients with hypercholesterolaemia and control subjects, and to determine the effects of atorvastatin administration. This model permitted the study of the relative concentration of Rho and Rac GTPases in the membrane (where activity is enhanced) and cytosol (where activity is decreased) of platelet samples at baseline and after treatment.

MATERIALS AND METHODS

Patient recruitment and trial design
A total of 40 patients with hyperlipidaemia and 20 normolipidaemic age- and gender-matched controls were recruited. Hyperlipidaemic patients were aged between 30 and 70 years, and had a total cholesterol level of at least 6.5 mmol/l. Individuals were excluded if they had any history of CVD (cardiovascular disease), hypertension [or BP (blood pressure) >140/80 mmHg] and diabetes mellitus (or fasting plasma glucose >7.0 mmol/l), or if they were taking anti-platelet, anti-hypertensive or anti-anginal medication. Patients with marked hypertriglyceridaemia (>2.5 mmol/l) were also excluded, as were those treated with a statin in the 6 weeks prior to the study. A total of 98% of patients were statin-naïve at the time of enrolment. All patients received written information and signed consent forms. The study was approved by the Queens University Belfast Research Ethics committee and the investigation conformed with the principles outlined in the Declaration of Helsinki.

Blood sampling and ultrasound investigations were performed in the morning in a temperature-controlled laboratory. Subjects fasted for at least 12 h and refrained from caffeine, alcohol, cigarettes and over-the-counter drug preparations for 24 h prior to study. Blood was drawn with minimal agitation and was separated into whole-blood (for lipid and glucose) and platelet (NO, \( \text{O}_2^{\bullet-} \) and GTPases) samples.

Patients were randomized in a double-blind manner to receive atorvastatin initially at 10 mg/day increasing to 20 mg/day after 4 weeks, or placebo for an 8-week period (20 patients per group). The atorvastatin and placebo medication were kindly donated to the study by Pfizer UK. Studies were repeated after 8 weeks of treatment. Control subjects (cholesterol <5.2 mmol/l) underwent a baseline study only.

Measurement of biochemical parameters
Total cholesterol, triacylglycerols (triglycerides) and HDL (high-density lipoprotein)-cholesterol were measured using a Roche/Hitachi 911 analyser. Total cholesterol was quantified enzymatically using the Trinder reaction. The LDL-cholesterol concentration was calculated using the Friedwald formula.

Platelet preparation
Platelets were prepared for analysis as described previously [15]. Collection tubes were centrifuged at 160 g for 17 min at room temperature (22°C). The platelet-rich plasma fraction was removed and centrifuged at 500 g for 10 min at room temperature. It was passed through a 17 mm × 50 mm Sepharose-2B column equilibrated with albumin-free Tyrodes/Hepes-buffered saline to create gel-filtered platelet fractions. Platelet counts were calculated in an automated counter. The fresh platelet fractions were used immediately for NO and \( \text{O}_2^{\bullet-} \) detection.

NO analysis
Release of NO from biological systems is most sensitively examined using an electrochemical microprobe as first described by Shibuki [16] and later adapted for platelet NO measurement [17]. NO diffuses through a gas-permeable membrane on the probe and is oxidized by the metallic electrode resulting in an electrical current.

Measurement of platelet NO release
The release of NO was measured using an NO meter and am NO-700 NO-selective micro-electrode (Innovative Instruments). The inter-day variation in our laboratory was less than 7%. Methods utilized in our laboratory have been fully described previously [15]. In brief, to each platelet sample was added 20 μl each of 120 μmol/l calcium and magnesium. The first sample of platelets was
not activated. The second ‘native’ sample was tested for absolute NO determination following activation. A total of 50 μl of 1 mmol/l of the eNOS inhibitor L-NAME (Nω-nitro-L-arginine methyl ester) was added to the third sample and 20 μl of 100 μmol/l SOD (superoxide dismutase) was added to the fourth tube. An electrical current was then displayed digitally on the attached computer and allowed to stabilize. Platelet activation and stimulated NO release was produced by the addition of 24 μl of 200 μmol/l ADP and 36 μl of fibrinogen (10 μg/μl), and the change in current was measured for a 15 min period. Calculation of platelet NO release was quantified by measuring the height of the NO peak post-platelet stimulation in pA in comparison with the calibration standard for that day and is expressed as the NO release corrected for platelet content in pmol/10⁸ platelets. An aliquot (250 μl) of test sample was mixed with 250 μl of 6 mol/l Tris and frozen for nitrite analysis.

**Measurement of platelet nitrite**

Owing to the short half-life of NO and the relative difficulty of analysis, platelet nitrite was also quantified to ensure reliability of the results. Nitrite is an indirect measure of NO release and is a more stable oxidized form. Total platelet nitrite was quantified in samples frozen after NO analysis. Frozen samples were thawed and nitrite was reduced to NO using 1.5 ml of sulfuric acid and potassium iodide solution (0.5% w/v). A cadmium reducing wire was placed in the platelet samples which were mixed for 5 min and equilibrated at 37 °C. Nitrite was then detected as for real-time NO release.

**O₂•− analysis**

Platelet release of O₂•− was measured using lucigenin-enhanced chemiluminescence [18]. Earlier criticism of this method centred on the discovery that lucigenin itself can produce O₂•−, whereas later studies in our own and other laboratories have confirmed that the lower doses of lucigenin (i.e. 20 μmol/l) employed are not associated with redox cycling [19].

**Measurement of platelet O₂•− release**

Samples of gel-filtered platelets were diluted and 10 μl of 1.8 mmol/l lucigenin was added, yielding a solution concentration of 20 μmol/l. The sample was given time to dark adapt and was placed in a Sirius Luminometer (Berthold Detection Systems). Inter-day variation in the assay in our laboratory was 8.4 %.

The platelet sample was activated using 100 μl of 10 μmol/l PMA. Basal release of superoxide, determined as chemiluminescence units/min, was recorded for up to 20 min to achieve a steady state. This method was used to determine O₂•− release from platelet samples containing diluent and lucigenin (Native sample), 5 μl of 500 μmol/l L-NAME (L-NAME sample) and from a sample containing 10 μl of 100 μM DPI (diphenyleneiodonium) and 1 mmol/l quinacrine (DPI/Quinacrine sample) [14, 15]. The plateau height of the DPI/Quinacrine curve was subtracted from the native and the L-NAME curves. Areas under the curve were calculated and compared with the daily calibration curves adjusted for the known efficiency of the reaction, which is 30%. These values are then expressed as pmol of O₂•−·min⁻¹·(10⁸ platelets)⁻¹.

**Determination of cytosolic and membrane-associated Rac and Rho in platelet samples**

Platelet samples were treated fresh to obtain separate membrane and cytosolic fractions. Methods used were as employed previously in our laboratory and in accordance with those described in the literature [20,21]. The platelet pellet was isolated by centrifugation and the purity of each collection confirmed by flow cytometry. The platelet pellet was washed, sonicated (3 × 10 s at 25 W on ice using Vibra cell VC50 tip sonicator) and centrifuged (100,000 g for 30 min at 4 °C). The supernatant was transferred to each of two cryovials and stored at −80 °C. The pellet was washed and buffer [20 mmol/l Tris/HCl (pH 7.5), 0.25 mmol/l sucrose, 10 mmol/l EGTA, 2 mmol/l EDTA, 1 mmol/l dithiothreitol, 0.1% leupeptin, 0.1% aprotinin, 0.2 mmol/l orthovanadate, 1 mmol/l PMSF and 0.06 mmol/l n-octylglucoside] was added. The sample was incubated on ice for 15 min and centrifuged (100,000 g for 30 min at 4 °C). The supernatant (55 μl) was transferred to each of two cryovials and stored at −80 °C.

**Western blotting and measurement of Rac and Rho distribution**

A standard amount (10 μg) of each fraction (cytosol or membrane) was diluted with 2 μl of mercaptoethanol/loading buffer (1:1, v/v) and, in addition, a protein molecular-mass marker was loaded on to a 12 % (w/v) polyacrylamide gel. The proteins were separated by molecular mass using SDS/PAGE. Following protein separation, the proteins within the gel were transferred on to a PVDF membrane (Immobilon P; Millipore).

After washing and incubation with blocking buffer (Tris-buffered saline (pH 7.4) containing 0.1% Tween 20 with the addition on 5% (v/v) milk protein), the samples were incubated with primary antibody (anti-RhoA and anti-Rac1; Santa Cruz Biotechnology). The membrane was incubated for 1 h at room temperature with a 1:10,000 dilution of HRP (horseradish peroxidase)-labelled secondary antibody (Bio-Rad Laboratories). The labelled protein was detected using enhanced chemiluminescence (ECL® Plus; Amersham Biosciences) and exposed to X-ray film (Fotochemische) in a dark room. Densitometry was used to quantify each band (Analytical Image Systems; Imaging Research) [20,21].
Table 1  Baseline characteristics of the patients and controls
Values are medians (range). *P < 0.05 compared with the placebo group.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Atorvastatin group</th>
<th>Placebo group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49.7 (34–64)</td>
<td>50.7 (37–69)</td>
<td>49.7 (43–63)</td>
</tr>
<tr>
<td>Gender (n) (male/female)</td>
<td>10/10</td>
<td>11/9</td>
<td>10/10</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.2 (24.1–27.6)</td>
<td>25.4 (24.6–26.3)</td>
<td>25.3 (23.9–27.2)</td>
</tr>
<tr>
<td>Cigarette smokers (n)</td>
<td>6 out of 20*</td>
<td>3 out of 20</td>
<td>4 out of 20</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>123 (118–129)</td>
<td>124 (119–130)</td>
<td>122 (116–128)</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>72 (68–76)</td>
<td>74 (70–77)</td>
<td>71 (67–74)</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.2 (4–4.4)</td>
<td>4.2 (4–4.4)</td>
<td>4.3 (4.1–4.5)</td>
</tr>
</tbody>
</table>

Statistical analysis
Analysis of parameters was performed using SPSS version 12.0.1 for Windows. Results are expressed as means ± 95 % CIs (confidence intervals) throughout, unless otherwise stated. Normally distributed variables were compared using Student’s t tests. Variables not normally distributed were analysed by non-parametric means.

The distribution of both GTPases was studied in the cell membrane and cytosol for patients with hyperlipidaemia both before and after atorvastatin and placebo. Owing to the skewed distribution, medians [first and third IQR (interquartile range)] are used in preference to means ± 95 % CI. Correlations between the change in serum cholesterol and NO and O₂⁻⁻ were made using Spearman’s rank correlation test.

RESULTS

Baseline characteristics of the patients and controls
Patient and control characteristics are summarized in Table 1. The patients were well matched for age and gender. There were no significant differences in weight, BMI (body mass index) or baseline serum glucose between groups. The patient groups were also well matched for BP. There were more smokers in the atorvastatin group compared with the placebo group. There was a highly significant statistical difference between the hypercholesterolaemic patient groups and control subjects in relation to baseline total cholesterol, LDL-cholesterol and cholesterol/HDL-cholesterol ratio (Table 2). No significant difference in HDL-cholesterol was detected between the three groups. There was also a significant difference between triacylglycerol levels in the statin-treated and placebo groups compared with controls (Table 2).

Changes in lipid parameters
Changes in lipid profile over the study period are shown in Table 2. Treatment with atorvastatin resulted in a significant reduction in total cholesterol, the LDL-cholesterol, cholesterol/HDL-cholesterol ratio and triacylglycerol. No difference in HDL-cholesterol was observed. In the placebo group, there was no significant change in any of the lipid parameters over the study period.

Platelet NO release
Changes in platelet NO release are shown in Table 2. Treatment with atorvastatin resulted in a significant (P = 0.05) increase in NO release of 3.2 (95 % CI, 0.55–7.83) pmol/10⁸ platelets (Figure 1A). There was no significant change in NO release in the placebo group. L-NAME inhibited the production of NO in all samples with no statistically significant differences in any group. SOD was associated with an increase in NO detection. No statistically significant correlation was found between changes in platelet NO and alterations in total cholesterol at the trial end.

Platelet nitrite content
Treatment with atorvastatin resulted in a significant (P = 0.002) increase in platelet nitrite of 26.4 pmol/10⁸ platelets (95 % CI, 9.6–43.2), whereas treatment with placebo resulted in a non-significant decrease of 6.4 pmol/10⁸ platelets (95 % CI, −17.01 to 4.19), confirming the changes observed in NO.

Platelet O₂⁻⁻ release
Changes in O₂⁻⁻ release are also shown in Table 2. Baseline O₂⁻⁻ levels were significantly (P = 0.04) lower in the control group compared with the patient groups. Treatment with atorvastatin resulted in a significant (P = 0.001) reduction in platelet O₂⁻⁻ release of 3.4 (95 % CI, 1.4–4.9) pmol · min⁻¹ · (10⁸ platelets)⁻¹ (Figure 1B). No significant change was observed with placebo. There was no correlation between the change in platelet O₂⁻⁻ and change in total cholesterol.

Rho GTPase
There was no statistically significant difference in the quantification of Rho in either the membrane or cytosol at baseline. Treatment with atorvastatin resulted in
### Table 2

Alterations in biochemical parameters in patients and controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Atorvastatin Group</th>
<th>Placebo Group</th>
<th>Control Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood lipid</td>
<td></td>
<td></td>
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<tr>
<td>Cholesterol (mmol/l)</td>
<td>7.4 (6.9–7.9)</td>
<td>5.0 (4.8–5.5)</td>
<td>5.0 (4.8–5.5)</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>5.0 (4.6–5.5)</td>
<td>2.8 (2.5–3.1)</td>
<td>2.8 (2.5–3.1)</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.4 (1.3–1.4)</td>
<td>1.4 (1.3–1.4)</td>
<td>1.4 (1.3–1.4)</td>
</tr>
<tr>
<td>Triacylglycerol (mmol/l)</td>
<td>2.2 (1.9–2.6)</td>
<td>1.8 (1.5–2.2)</td>
<td>1.8 (1.5–2.2)</td>
</tr>
<tr>
<td>Total cholesterol/HDL-cholesterol ratio</td>
<td>5.6 (4.9–6.3)</td>
<td>3.8 (3.3–4.3)</td>
<td>3.8 (3.3–4.3)</td>
</tr>
<tr>
<td>Platelet NO release (pmol/10^8 platelets)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>6.05 (3.8–8.35)</td>
<td>6.4 (3.9–8.8)</td>
<td>6.55 (3.9–8.8)</td>
</tr>
<tr>
<td>L-NAME</td>
<td>1.5 (1.05–1.95)</td>
<td>1.3 (0.95–1.6)</td>
<td>1.3 (0.95–1.6)</td>
</tr>
<tr>
<td>SOD</td>
<td>7.0 (6.2–8.9)</td>
<td>7.0 (6.2–8.9)</td>
<td>7.0 (6.2–8.9)</td>
</tr>
<tr>
<td>Platelet nitrite content (pmol/10^8 platelets)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>37.39 (27.03–47.75)</td>
<td>36.79 (24.36–83.82)</td>
<td>43.05 (34.54–51.58)</td>
</tr>
<tr>
<td>L-NAME</td>
<td>14.5 (12.4–18.0)</td>
<td>14.5 (12.4–18.0)</td>
<td>14.5 (12.4–18.0)</td>
</tr>
<tr>
<td>DPI/Quinacrine</td>
<td>6.6 (4.5–8.9)</td>
<td>6.6 (4.5–8.9)</td>
<td>6.6 (4.5–8.9)</td>
</tr>
<tr>
<td>Native − DPI</td>
<td>8.8 (6.0–13.95)</td>
<td>8.8 (6.0–13.95)</td>
<td>8.8 (6.0–13.95)</td>
</tr>
<tr>
<td>Platelet O2/H2O release [pmol·min⁻¹·(10^8 platelets)⁻₁]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
<td>9.3 (6.7–11.9)</td>
<td>7.8 (5.7–10.0)</td>
<td>7.8 (5.7–10.0)</td>
</tr>
<tr>
<td>L-NAME</td>
<td>8.0 (5.6–10.5)</td>
<td>7.3 (5.3–9.3)</td>
<td>7.3 (5.3–9.3)</td>
</tr>
<tr>
<td>DPI/Quinacrine</td>
<td>1.8 (1.4–2.2)</td>
<td>1.5 (1.2–1.8)</td>
<td>1.5 (1.2–1.8)</td>
</tr>
<tr>
<td>Native − DPI</td>
<td>7.5 (5.1–9.9)</td>
<td>6.3 (4.3–8.4)</td>
<td>6.3 (4.3–8.4)</td>
</tr>
</tbody>
</table>
Figure 1  Platelet NO (A) and O$_2^*$ (B) in controls, and patients pre- and post-atorvastatin and placebo

*P = 0.05 and †P = 0.001.

a statistically significant (P < 0.001) decrease in Rho quantification in the platelet cell membranes [1380 (IQR, 1090–1825) DU (densitometry units) at the trial start and 641 (IQR, 106–800) DU at the trial end] (Figure 2A). There was a significant (P = 0.02) increase in Rho in the platelet cell membrane in the placebo group [1365 (IQR, 1107–1911) DU at the trial start and 1740 (IQR, 1464–1923) DU at the trial end]. Treatment with atorvastatin also resulted in a statistically significant (P = 0.04) increase in the amount of Rho found in the cell cytosol [1105 (IQR, 710–1273) DU at the trial start and 1445 (IQR, 1062–2003) DU at the trial end]. The amount of Rho in the cell cytosol was not affected by treatment with placebo [1222 (IQR, 891–1868) DU at the trial start and 1129 (IQR, 946–1418) DU at the trial end] (Figure 3).

**Rac GTPase**

No significant difference was found at baseline between groups when comparing the relative distribution of Rac GTPase in the platelet membranes and cytosol. Treatment with atorvastatin resulted in a statistically significant (P = 0.04) increase in cell membrane Rac [2592 (IQR, 1757–3700) DU at the trial start and 1941 (IQR, 76–2796) DU at the trial end] compared with placebo [2775 (IQR, 2094–3507) DU at the trial start and 2740 (IQR, 2490–3689) DU at the trial end], whereas no significant alteration in membrane Rac was found (Figure 2B). Cytosolic Rac GTPase showed a trend towards an increase with atorvastatin [1568 (IQR, 1288–1880) DU at the trial start and 1888 (IQR, 1387–2372) DU at the trial end], but this did not reach significance. Platelet samples from the placebo group had no change in cytosolic Rac [1793 (IQR, 1584–2833) DU at the trial start and 1675 (IQR, 1363–2136) DU at the trial end] (Figure 4).

**DISCUSSION**

The present study is, to our knowledge, the first human study demonstrating clear changes in NO and O$_2^*$ release in conjunction with altered Rho and Rac
localization in samples from patients following treatment with atorvastatin at a commonly prescribed dose.

ED is now considered a very early stage in the development of CVD, and attenuated NO bioavailability has been shown to occur with a number of CVD risk factors including hyperlipidaemia [22]. Other investigators have shown previously that platelets contain the same pathways for NO production that are found in endothelial cells [23,24]. Platelet-derived NO is also thought to be diminished in the presence of certain CVD risk factors [25], and the actions of platelet NO have been identified as complementary to endothelial cell NO [26]. Statins have been associated with enhanced NO bioactivity both in endothelial cells [27] and platelets [28].

Patients with hyperlipidaemia had a marked attenuation of NO release that was significantly improved following 8 weeks of treatment with atorvastatin, and this was accompanied by a significant increase in nitrite, a stable NO metabolite. No change in NO release was associated with placebo, suggesting that the alteration in detectable NO was related to statin administration. No relationship was demonstrated between the magnitude of total cholesterol/LDL-cholesterol reduction and enhanced NO release.

A significant change was identified in membrane association of the eNOS-inhibitory factor Rho GTPase with statin therapy. This implies that atorvastatin also altered the environment within the platelets to influence Rho membrane attachment and free radical production. Previous studies have shown that membrane association of Rho relate to isoprenoid levels, in particular GGPP [12]. Atorvastatin may not only decrease intracellular cholesterol, but also intermediates such as GGPP that alter proteins which depend on isoprenoid molecules for their trafficking and ultimate activity.

A marked increase in platelet O$_2^{•-}$ was found in patients with hyperlipidaemia which was suppressed after treatment with atorvastatin. This may be secondary to decreased activation of NADPH oxidase as demonstrated previously by our group [14,15] and others [29]. Although previous studies have shown that lipid-lowering alone can produce this improvement in ROS levels [29], we found no significant relationship between lipid alteration and decreased levels of oxidative stress in our present study.

The detection of Rac GTPase in the membranes of platelet samples was markedly reduced following statin treatment. Rac is an integral part of the NADPH oxidase system, being crucial to the conformational changes that are necessary following activation and hence O$_2^{•-}$ release [13,14]. Therefore the decrease in Rac detected in the cell membranes may have lead to a reduction in the ability of NADPH oxidase to form O$_2^{•-}$ with statin therapy.

In these and previous studies, our group has employed platelets as an easily accessible ex vivo compartmentalized model for endothelial cells. Platelets represent an excellent source for this purpose, given the fact that platelets and endothelial cells share identical eNOS [23,24] and NADPH oxidase subtype, in contrast with alternative models, such as neutrophils, which differ significantly particularly with respect to NADPH oxidase [14,15].

The results of the present study suggest that mechanisms in addition to the lipid-altering properties of atorvastatin may be important in humans. There are a number of limitations within the present study worthy of mention. Although the implication is that Rho and Rac alterations are secondary to changes in isoprenylation, this was not specifically tested in the present study. Furthermore, there was no alternative lipid-lowering arm to test further the hypothesis that the described actions occur secondary to intrinsic statin properties. However, other groups have described alterations in various factors independently of lipid-lowering with statins not found with other hypolipidaemic medication [30]. Despite these limitations, the results of the present study suggest that statin therapy can alter not only effectors of endothelial function, but intracellular trafficking of vitally important cellular proteins. Furthermore, these changes were found in human subjects receiving an orally administered and commonly prescribed preparation in doses that are highly relevant to everyday clinical practice.

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