Prolonged statin-associated reduction in neutrophil reactive oxygen species and angiotensin II type 1 receptor expression: 1-year follow-up

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Aims

Our study investigated reactive oxygen species (ROS) generation and angiotensin II type 1 receptor (AT₁-R) expression in primed polymorphonuclear leukocytes (PMNs) of dyslipidaemic subjects over prolonged statin treatment.

Methods and results

Sixteen untreated dyslipidaemic subjects with moderately increased cardiovascular risk (National Cholesterol Education Program, Adult Treatment Panel III) were studied before and during long-term (1 year) simvastatin treatment. Neutrophils from dyslipidaemic subjects generated more ROS in comparison with cells from healthy control subjects. After 1 year of simvastatin treatment, ROS production (delta N-formyl-Met-Leu-Phe-induced generation and area under the curve) was significantly reduced. At baseline, AT₁-R mRNA expression was also higher in dyslipidaemic subjects than in healthy controls and it was reduced after clinical treatment with simvastatin. In a subgroup of patients, a reduced angiotensin II-induced ROS generation was also observed upon clinical simvastatin treatment. Moreover, a direct effect of statin on the upregulated AT₁-R expression was demonstrated in vitro in neutrophils of untreated dyslipidaemic subjects.

Conclusion

A consistent reversion of pro-inflammatory oxidative functional response and reduction of AT₁-R expression in primed PMNs was observed in patients during long-term statin treatment. The AT₁-R reduction over treatment may contribute to the normalization of dysregulated neutrophil activation which occurs in the pre-clinical phase of atherosclerosis.

Keywords

Neutrophils • Reactive oxygen species • Angiotensin II AT1 receptors • Statins • Dyslipidaemic subjects • Cell function

Introduction

Although the role of polymorphonuclear leukocytes (PMNs) as an inflammatory cell type in the defence against bacterial components have been long recognized in sepsis and local infections, a direct involvement of neutrophils in the first phases of the atherosclerotic processes such as endothelial dysfunction and initial plaque formation has been suggested only recently.1

The fully activated PMNs release reactive oxygen species (ROS), proteases, and granule contents to the surrounding milieu, resulting in oxidative stress and lipid peroxidation.2,3 Moreover, compelling evidence in neutrophils supports a role for ROS as signalling molecules in diverse intracellular pathways including phagocytosis, secretion, gene expression, and apoptosis that further modulate the inflammatory response, particularly during the early phases of inflammation.4 As regards the functional alterations which
occur at the cellular level in atherosclerosis, a primed state of PMNs, associated with increased oxidative stress and pro-inflammatory cytokine release, has been described both in animals and in humans with high cardiovascular risk, hypercholesterolaemia, hypertension, diabetes, and renal failure.\textsuperscript{5–10} However, a reduced superoxide anion generation was also described in dyslipidaemia.\textsuperscript{11} Recently Jacobi et al.\textsuperscript{12} reported pro-inflammatory changes in umbilical endothelial cells induced by primed PMNs from patients treated with haemodialysis, demonstrating that functionally altered PMNs can be direct mediators of endothelial cell injury and therefore involved in the initial stage of vascular atherosclerosis.

Angiotensin II is one of the most potent pro-atherogenic factors, being inflammatory and hypertrophic stimuli mediated through the angiotensin II type 1 receptor (AT\textsubscript{1}-R).\textsuperscript{13} Few studies in humans demonstrated an upregulation of the expression of platelet AT\textsubscript{1}-R in hypercholesterolaemia and showed that AT\textsubscript{1}-R antagonism may improve hypercholesterolaemia-associated endothelial dysfunction.\textsuperscript{14,15} Moreover, we have recently demonstrated that PMNs of healthy subjects at high cardiovascular risk show increased AT\textsubscript{1}-R expression when compared with subjects at low risk.\textsuperscript{16} Angiotensin II is a major mediator of oxidative metabolism, and the renin–angiotensin system could be involved in the increased oxidative stress associated with hyperlipidaemia.\textsuperscript{10,17–19}

Statins may interfere at various levels, including a modulation of oxidation pathways, with the processes leading to atherosclerosis.\textsuperscript{20} Previous studies from our group showed that short-term simvastatin administration could affect PMN cellular properties.\textsuperscript{9,16} However, although atherosclerosis may be viewed as a ‘continuum’ from cellular changes to clinical events, no study has previously determined the effect of prolonged statin treatment, as usually indicated in the clinical setting, on the intracellular ROS levels in PMNs. Moreover, no data are available on AT\textsubscript{1}-R expression following a long-term statin treatment. Thus, the present observational study was designed to investigate whether a change in the pro-inflammatory pattern (ROS generation and AT\textsubscript{1}-R expression) of primed PMNs of hypercholesterolaemic subjects bearing a moderately increased risk for vascular events (and therefore affected by early and pre-clinical cellular manifestations of atherosclerosis) could be observed during a prolonged simvastatin treatment. Moreover, as secondary endpoints, we sought to investigate the potential modulation of angiotensin II-induced ROS generation by simvastatin and to evaluate the direct in vitro effects of simvastatin on AT\textsubscript{1}-R expression in these cells.

Methods

Subjects

In 16 healthy dyslipidaemic subjects, whites, PMNs were isolated from venous blood (see what follows) (i) before any pharmacological treatment and (ii) during short-term (1 month) and (iii) long-term (1 year) simvastatin treatment (20 mg assumed at 10 PM) to investigate ROS production and to assess the mRNA AT\textsubscript{1}-R expression. The subjects were enrolled consecutively at our Lipid Clinic (Clinical Medicine, University of Insubria, Varese, Italy), where they came for evaluation of dyslipidaemia. As for inclusion criteria to be enrolled in this study aimed at evaluating potential differences occurring after 1 year of treatment, the dyslipidaemic subjects were at increased cardiovascular risk showing a ‘moderate risk’ for vascular events according to the National Cholesterol Education Program—Adult Treatment Panel III (ATPIII) guidelines,\textsuperscript{21} and no disease was found after a clinical examination and routine laboratory tests. Moreover, the subjects were included in the study if a lipid-lowering pharmacological treatment with statins was clinically indicated and they did not assume any pharmacological treatment. We considered ongoing clinical infection and/or the presence of infections in the previous 3 months as well as a smoking habitus and competitive sporting activities as exclusion criteria. Of the initially evaluated 20 patients who fulfilled the inclusion criteria, three smokers were excluded, one patient dropped out from the study at the 1-year evaluation. No patient had to be excluded because of infections and none was involved in competitive sporting activities. The patients were studied after 6 weeks of life-style modification including dietary treatment (qualitative counselling) and recommendations for mild physical activity. The patients were then asked to maintain the same level of physical activity and a similar diet; besides the evaluations scheduled for the study on neutrophils, they were followed for clinical evaluations and standard laboratory exams at the third and the sixth month after starting the statin.

Since only a few studies have been performed in humans on the altered cellular properties of neutrophils in subjects prone to develop atherosclerosis, a group of healthy subjects was included to determine whether the increased-risk patient population actually differed at baseline from subjects showing a low cardiovascular risk. Healthy subjects were enrolled while evaluated for a general clinical check-up. To be included, the subjects had to be in the same age range as our patient population, to show a low cardiovascular risk according to ATPIII guidelines, to be free of diseases in personal history, and, as for the increased-risk patient studied, they had to be free of any pharmacological treatment. The exclusion criteria were the same as those for the increased-risk patient group. All the healthy subjects asked to be enrolled as potential controls agreed to be evaluated.

All the patients were evaluated during a clinical visit on the basis of familiar and personal history. Blood samples were obtained to perform routine laboratory examination and to isolate circulating PMNs for subsequent studies by using heparinized tubes between 8.00 and 9.00 AM, after a fasting night. All the subjects were previously asked not to take coffee, tea, chocolate, or cola-containing substances for the 24 h preceding the evaluations. Table 1 shows the descriptive statistics of clinical and laboratory findings of the dyslipidaemic subjects at baseline and healthy subjects. Our study complies with the Declaration of Helsinki; the local Ethics Committee has approved the research protocol, and informed consent has been obtained from the subjects.

Cell isolation

Whole blood was allowed to sediment on dextran at 37°C for 30 min. Supernatant was recovered and PMNs were isolated by standard density-gradient centrifugation as described previously.\textsuperscript{1} Contaminating erythrocytes were eliminated by 10-min hypotonic lysis in distilled water with added NH\textsubscript{4}Cl 8.2 g/L, KHCO\textsubscript{3} 1.0 g/L, and EDTA 37.0 mg/L. Cells were then washed three times in NaCl 0.15 M. Purity and viability of PMNs preparations were always >95%, and no platelets or erythrocytes could be detected either by light microscopic examination or by flow cytometric analysis.

Determination of reactive oxygen species generation

Intracellular ROS levels were assessed by using the redox-sensitive dye dichlorodihydrofluorescein-diacetate (C-DCDHF-DA; Molecular
Table 1 Descriptive statistics of the clinical and laboratory parameters of increased-risk patients at baseline and healthy subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients, n = 16 (8 M, 8 F)</th>
<th>Healthy subjects, n = 16 (8 M, 8 F)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>57 (49–64)</td>
<td>54 (46–62)</td>
<td>0.56</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27 (25–29)</td>
<td>25 (22–28)</td>
<td>0.18</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>134 (126–150)</td>
<td>125 (116–125)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>85 (80–90)</td>
<td>83 (75–85)</td>
<td>0.17</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>287 (258–338)</td>
<td>193 (172–202)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL-c (mg/dL)</td>
<td>50 (47–57)</td>
<td>60 (44–63)</td>
<td>0.17</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>141 (115–190)</td>
<td>93 (81–168)</td>
<td>0.03</td>
</tr>
<tr>
<td>LDL-c (mg/dL)</td>
<td>203 (181–240)</td>
<td>112 (101–127)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Apolipoprotein A (mg/dL)</td>
<td>141 (127–159)</td>
<td>144 (120–156)</td>
<td>0.83</td>
</tr>
<tr>
<td>Apolipoprotein B (mg/dL)</td>
<td>163 (137–224)</td>
<td>95 (88–104)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>High-sensitive C-reactive protein (mg/L)</td>
<td>2.98 (1.35–4.86)</td>
<td>0.75 (0.6–1.07)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

M, males; F, females; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol.

Protein Eukaryotic Mini Kit (Eppendorf, Hamburg, Germany) and the human AT1-Rs (Applied Biosystems). Cycles included one 2-min hold (50°C); one 10-min and 45 15-s cycles of denaturation (95°C). Raw data were analysed by the ABI prism SDS software (Applied Biosystems). Levels of mRNA were quantitated by reference to a standard curve generated by performing serial dilutions (1:10; 1:50; 1:100; 1:1000; 1:10 000) of the total mRNA obtained from PMNs. The values were then normalized for ct values of 18S ribosomal RNA.

Angiotensin II-induced reactive oxygen species generation in polymorphonuclear leukocytes of dyslipidaemic subjects before and during statin treatment

In PMNs of a subgroup of six dyslipidaemic subjects (the last six enrolled), the direct effect of (i) angiotensin II (50 μM) alone, (ii) simvastatin (10 μM) alone, and (iii) angiotensin II after simvastatin incubation (1 min) was studied before and during short-term clinical statin treatment. Results were expressed as delta percentage (ΔFI) variations between resting production and 30-min responses induced by in vitro stimulation with the different compounds.

**In vitro effect of simvastatin on angiotensin II type 1 receptor mRNA in polymorphonuclear leukocytes obtained by untreated dyslipidaemic subjects**

In the subgroup of six dyslipidaemic subjects, PMNs were also evaluated once (when the subjects did not assume any pharmacological treatment) to investigate the mRNA AT1-R expression (i) at baseline, (ii) after incubation with angiotensin II (50 μM), (iii) after incubation with simvastatin (10 μM), and (iv) after co-incubation of both angiotensin II and simvastatin.

**Effect of angiotensin II on reactive oxygen species generation in polymorphonuclear leukocytes of healthy subjects**

In PMNs of a subgroup of six healthy subjects (the last six enrolled), the direct effect of angiotensin II (0.1–50 μM) on ROS generation was also tested. Moreover, in order to assess the ability of simvastatin to counteract the angiotensin II-induced ROS generation, cells were pre-treated (1 min) with simvastatin (10 μM) before the incubation with angiotensin II. Results are expressed as delta percentage (ΔFI) variations between resting production and 30-min responses induced by in vitro stimulation with the different compounds.

**Statistical analysis**

Data are presented as median and 25–75th percentile range (IQR). Comparisons between dependent measures were performed with the Wilcoxon signed-rank test. The Bonferroni correction was applied for post hoc comparisons. Comparisons between independent measures were performed with the Mann–Whitney U test. The mean difference and its 95% confidence interval (95% CI) were computed to quantify both changes in time and differences between cases and healthy subjects. The relationships between the functional parameter changes (ROS) and AT1-R variations occurring during the 1-year follow-up, and changes in lipid parameters (LDL cholesterol,
apoprotein B) were investigated by linear regression analysis. For the in vitro experiments comparing the effects of different stimuli, a multivariable regression model was used, with calculation of robust standard errors to account for intra-experiment correlation. A term for interaction of angiotensin II and simvastatin was included in the model.

Calculations were performed using commercial software (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com and Stata 10, Stata Corp, College Station, TX, USA). A two-sided \( P < 0.05 \) was retained for statistical significance.

## Results

### Reactive oxygen species generation from polymorphonuclear leukocytes of dyslipidaemic individuals and healthy subjects

PMNs of dyslipidaemic subjects showed an increased ROS generation following stimulation with fMLP when compared with healthy subjects both as regards the \( \Delta \) fMLP-induced ROS production and AUC (Table 2); the mean difference and 95% CI between controls and untreated dyslipidaemic individuals was \(-86.3 \text{ FI} (\text{-151.6 to } -21.1) \) and \(-1152 \text{ FI} \times \text{ min} (\text{-2421 to } 118.4) \), respectively.

The \( \Delta \) fMLP-induced ROS production and AUC were significantly reduced after 1-year simvastatin treatment (Table 2) (mean differences (95% CI) between untreated dyslipidaemic individuals and 1-year values: \(72.9 \text{ FI} (-3.8 to 149.7)\) and \(1145 \text{ FI} \times \text{ min} (-110.6 to 2400)\)); the values obtained at the 1-year evaluation were reduced to values similar to those observed in healthy subjects \((P = 0.12 \text{ and } P = 0.45 \text{ for the } \Delta \text{ and AUC, respectively})\).

The PMA-induced ROS generation did not significantly differ between untreated dyslipidaemic subjects and controls, nor was significant difference observed during treatment either as regards the \( \Delta \) PMA-induced ROS production and AUC (Table 2).

Figure 1 shows a typical pattern of ROS generation in one dyslipidaemic individual at baseline and during treatment and its matched control.

### Effect of statin in vitro and during clinical administration of treatment on angiotensin II-induced reactive oxygen species generation in polymorphonuclear leukocytes of dyslipidaemic individuals

When the direct effects of angiotensin II alone, simvastatin alone, and angiotensin II after simvastatin incubation were studied in vitro before statin treatment (in PMNs of dyslipidaemic individuals at baseline), both angiotensin II and simvastatin alone or co-incubated were found to increase ROS generation \((P = 0.0002, P = 0.031, \text{ and } P = 0.0086, \text{ respectively})\). Moreover, no difference was observed between the \( \Delta \) variations obtained after 30-min stimulation with angiotensin II alone or co-incubated with simvastatin \([n = 6; \text{ angiotensin II: } 21.06 \text{ (IQR: 8.75–29.54); simvastatin + angiotensin II: 11.58 \text{ (IQR: 5.77–21.88), } P = 0.75])\). On the contrary, after 1 month of clinical simvastatin treatment, the angiotensin II-induced ROS generation was significantly reduced \([\text{mean difference (95% CI): 11.6 FI (0.9–22.4)}]\) (Figure 2).

### In vitro effect of simvastatin on angiotensin II type 1 receptor mRNA in polymorphonuclear leukocytes in patients at baseline

When the PMNs of dyslipidaemic untreated subjects were treated in vitro with angiotensin II, simvastatin alone, and co-incubated with

## Table 2 Reactive oxygen species generation in isolated polymorphonuclear leukocytes obtained from controls and dyslipidaemic patients at baseline and during statin treatment

<table>
<thead>
<tr>
<th></th>
<th>Healthy subjects</th>
<th>Patients</th>
<th>Patient follow-up</th>
<th>Patient follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td></td>
<td>1 month</td>
<td>1 year</td>
</tr>
<tr>
<td><strong>Resting</strong></td>
<td>31.9 (23.5–40.2)</td>
<td>43.3 (22.4–69.5)</td>
<td>33.3 (22.5–49.3)</td>
<td>36.1 (26.1–45.6)</td>
</tr>
<tr>
<td><strong>PMLP-Δ</strong></td>
<td>59.4 (45.3–71.3)</td>
<td>130.2 (107.6–181.3)</td>
<td>128.4 (58.2–230.5)</td>
<td>78.5 (63.5–113.8)</td>
</tr>
<tr>
<td><strong>PMLP-AUC</strong></td>
<td>932.7 (679.0–1600.0)</td>
<td>2176.0 (1244.0–3443.0)</td>
<td>1777.0 (969.3–3431.0)</td>
<td>1151.0 (906.9–1649.0)</td>
</tr>
<tr>
<td><strong>PMA-Δ</strong></td>
<td>216.1 (129.6–267.4)</td>
<td>270.8 (134.4–513.1)</td>
<td>152.3 (92.0–532.4)</td>
<td>323.5 (249.0–415.5)</td>
</tr>
<tr>
<td><strong>PMA-AUC</strong></td>
<td>3049.0 (1914.0–3721.0)</td>
<td>4581.0 (2166.0–6085.0)</td>
<td>4545.0 (2095.0–7534.1)</td>
<td>5136.0 (2207.0–6188.0)</td>
</tr>
</tbody>
</table>

* \( P \)-values (Mann–Whitney U test): patients at baseline vs. controls; † \( P \)-values (Wilcoxon signed-rank test): patients at baseline vs. 1 year.

Reactive oxygen species levels were expressed as values of fluorescence intensity measured at resting time (1 min), as \( \Delta \) values after 30-min stimulation (31-min stimulation—resting values) with both N-formyl-Met-Leu-Phe (fMLP) or phorbol myristate acetate (PMA) and AUC (measured as total values of reactive oxygen species generated during the 31 min of evaluation). Values were expressed as median with 25th and 75th percentile. For further details, see Methods section.
angiotensin II and simvastatin, AT₁-R expression was significantly affected. In particular, the incubation with simvastatin reduced significantly and by a similar amount AT₁-R both in the presence and in the absence of angiotensin II [simvastatin alone—mean difference (95% CI) 4.77ΔCt (2.77–6.77); simvastatin + angiotensin II—mean difference (95% CI) 5.17ΔCt (3.17–7.17); interaction: P = 0.67]. Cells treated with angiotensin II alone did not differ from untreated cells (P = 0.23) (Figure 4).

In vitro effect of angiotensin II on reactive oxygen species generation in polymorphonuclear leukocytes of healthy donors

As shown in Figure 5, incubation of PMNs with angiotensin II (0.1–50 μM) induced an increase of ROS generation; this increase was concentration-dependent and reached the statistical significance at the concentration of 50 μM (P < 0.05 vs. resting value). Pre-treatment with simvastatin (10 μM) significantly reduced the angiotensin II-induced effects (P < 0.05).
Relationship between the clinical parameters and polymorphonuclear leukocyte functional response changes during statin treatment

The body mass index and the blood pressure values did not significantly change during follow-up. As expected, total cholesterol, LDL cholesterol, and apolipoprotein B were significantly reduced when comparing baseline (baseline values: see Table 1) with 1-year evaluation [1-year values, P vs. baseline values: 205 (IQR: 173–232), P < 0.001; 120 (IQR: 104–149), P < 0.001; 101 (IQR: 88–119), P < 0.001, respectively], whereas HDL cholesterol was borderline-increased [1-year values, P vs. baseline values: 52 (IQR: 49–65), P = 0.049]. Triglycerides and apolipoprotein A did not change significantly during follow-up. Moreover, C-reactive protein was significantly reduced during treatment [1-year values, P vs. baseline values 1.6 (IQR: 1.0–2.7), P = 0.01]. No significant relationship was observed between both the functional parameter changes (ROS) and AT1-R variations occurring during the 1-year follow-up, and the variations observed in LDL cholesterol, apolipoprotein B, and C-reactive protein (data not shown).

Discussion

The main finding of this study is the observation that patients on clinical simvastatin treatment over a long-term follow-up (1-year treatment) elicit changes of the altered PMN properties of dyslipidaemic subjects, including total intracellular ROS generation and AT1-R expression, and that the reduction observed during statin treatment in AT1-R expression contributes to the normalization of key cellular functions in human neutrophils.

Increasing experimental data identify peripheral immune cells and particularly PMNs as culprit cells in the processes associated with endothelial cells modifications linked with initial atherosclerotic vascular damage. Oxidative stress plays a key role in the pathophysiology of several major cardiovascular diseases, and antioxidant effects of statins have been demonstrated in animal studies. We describe here at a cellular level that a complete normalization in ROS generation after a membrane stimulation by fMLP occurs during prolonged simvastatin treatment in primed human PMNs of subjects at moderately increased vascular risk. The prolonged follow-up has important clinical implications as regards the findings reported here on PMN functional responses. The population studied is both healthy and at ‘intermediate’ cardiovascular risk, thus with early and pre-clinical atherosclerosis: the demonstrated prolonged action on cellular pro-inflammatory response pattern using a statin treatment which is clinically indicated for lifelong therapy is therefore particularly interesting in the cell type studied (neutrophils), known to be involved in the early phases of the atherosclerotic process. Previously, in hypercholesterolaemic patients, statin administration for 1 and 2 months was reported to reduce the urinary excretion of the isoprostane 8-iso-prostaglandin F₂α, an index of systemic oxidative stress. Moreover, in hypercholesterolaemic subjects followed for 12 weeks, atorvastatin-induced reductions in systemic ‘oxidation markers’ (oxidation products produced by myeloperoxidase and NO-derived oxidants) were independent of statin-induced...
alterations in total cholesterol, LDL cholesterol, and apolipoprotein B. In line with those results, in our study, the lipid profile changes were independent of the variations observed in ROS generation by PMNs.

An important role for peripheral leukocytes in angiotensin II-dependent inflammation was recently demonstrated in hypercholesterolaemic mice in which the adhesion of leukocytes to venular endothelium (and subsequent tissue migration) was directly related to AT1-R expression on the white blood cells themselves and it has been suggested that an inflammatory state may induce feedforward mechanisms in which increasing AT1-R expression in leukocytes maintains inflammation mediated through these receptors. In this study, though performed in a limited number of subjects, the treatment with simvastatin was associated with a marked and prolonged downregulation of the increased mRNA AT1-R expression observed in PMNs of dyslipidaemic subjects at moderately increased cardiovascular risk. The AT1-R mediates pro-inflammatory effects of angiotensin II by activating transcription factors such as NF-kB, and activation by angiotensin II of p38MAPK, ERK1/2, and JNK1/2 was observed in human neutrophils. We previously reported that a short-term simvastatin treatment is able to reduce mRNA AT1-R expression in PMNs of high-risk subjects, therefore this study rules out potential tolerance phenomena which could have occurred during longer clinical treatment. Moreover, the mRNA expression after 1-year treatment was even lower than the value observed in the healthy subjects.

Besides the statin effects observed in our study on ROS generation induced after non-specific stimulation (such as the membrane stimulation by fMLP and thus possibly resulting in vivo from diverse pro-inflammatory pathways, the angiotensin II-induced ROS generation by PMNs of these individuals was blunted during clinically administered statin treatment, showing a reduced functional response to angiotensin II in terms of oxidative stress of PMNs obtained from treated patients. PMNs activation is carried out as a series of successive events that are initiated by interaction of external signal through G-protein-coupled receptors that activated phospholipase C. This interaction splits membrane phospholipids that finally result in intracellular Ca2+ efflux from non-mitochondrial pools, and intracellular Ca2+ increases drastically. A typical stimulus acting via G-protein-coupled receptor is fMLP, whereas PMA, due to its ability to penetrate easily into cells and its structural similarity to diacylglycerol, is a strong activator of protein kinase C. In our patients, the lack of statin-treatment-induced changes in ROS generation after stimulation with PMA, associated with a significant reduction of fMLP-stimulated generation, could suggest a modulation of ROS generation by statin at the membrane site, either mediated by reduced LDL or through cellular changes. The latter may include the reduced AT1-R mRNA expression observed in our patients. This interpretation of data is supported by the finding of a direct action of statin on AT1-R expression in our in vitro experiments showing for the first time that simvastatin (alone or co-incubated with angiotensin II) is able to reduce the upregulated mRNA AT1-R expression of PMNs of untreated dyslipidaemic individuals. The inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase by statins decreases isoprenoid intermediates such as farnesyl-PP and geranylgeranyl-PP, which leads to an inhibition of isoprenylation of small GTPases. Rac1 and Rac2 are present in human neutrophils and are relevant for the cellular function, being Rac2 the predominant GTPase isoform and a necessary integral component of NADPH oxidase, whereas Rac1 is the predominant expressed isoform in human monocytes; the activation of Rac2 by angiotensin II is exerted through multiple signalling pathways, involving Ca2+/calceinurin and protein kinases. Moreover, previous observations in PMNs of healthy donors have pointed to a direct effect of simvastatin on the membrane-associated Rac1 after angiotensin II stimulation.

Previously, only the selective angiotensin II-stimulated oxide anion production was shown to be reduced in neutrophils by fluvasatin and it has been reported in monocytes that the in vitro pre-incubation with lovastatin was able to interfere with superoxide anion production. Moreover, the decreased ROS generation observed during simvastatin treatment in our clinical longitudinal study seems to be associated with the clinical administration and/ or related to the interference with angiotensin II-mediated stimulation, whereas the crude effect of in vitro simvastatin alone resulted in a slight increase of ROS generation in PMNs of untreated subjects. The in vitro effect of statins on enhanced pro-inflammatory molecules generation has already been described, and for pre-treatment of monocytes with similar doses of simvastatin used in our in vitro study (10 μM), an increase in reactive oxygen intermediates has been observed. The direct involvement of angiotensin II in ROS generation in PMNs is also sustained by our in vitro experiments in healthy donors: incubation of cells with angiotensin II significantly increased ROS generation in a dose-dependent manner, and this effect was reduced in the presence of simvastatin. These data show that the intracellular oxidative stress of PMNs may be modulated by the effects of simvastatin treatment on the AT1-R.

In conclusion, our longitudinal study in dyslipidaemic individuals with moderately increased risk for cardiovascular events suggests that the 1-year follow-up that functional modulation of PMN oxidative response and AT1-R expression (both intimately involved in the initial atherosclerotic process) may be related to statin treatment. Therefore, statin is consistently associated with the reversion of the dysregulated PMN function occurring during the pre-clinical development of atherosclerosis. This hypothesis could deserve further validation within randomized placebo-controlled clinical trials.

Conflict of interest: none declared.

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