Protective effect of atorvastatin on acute systemic inflammation-induced endothelial dysfunction in hypercholesterolaemic subjects

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Aims Recent studies suggest an association between acute inflammation and deterioration of arterial function. The effect of acute inflammation on endothelial function and the role of treatment with statins have not been investigated in subjects with dyslipidaemia.

Methods and results In this randomized, placebo-controlled, double-blind study, we generated a transient systemic inflammation by Salmonella typhi vaccination in 50 volunteers with mild hypercholesterolaemia after 4 days of treatment with atorvastatin 40 mg or placebo once daily. Endothelium-dependent flow-mediated dilation (FMD) of the brachial artery and circulating levels of endothelial and inflammatory markers were measured before and 8 h after the vaccine. Vaccination produced a decline on FMD at 8 h (absolute decrease of 2.55%, \( P = 0.001 \)), indicating an unfavourable effect on endothelial function. In contrast, in atorvastatin-treated subjects, FMD was preserved after vaccination (decrease of 0.15%, \( P = 0.005 \) vs. placebo). The vaccination-induced decline in plasma level of nitric oxide metabolites (by 6.0 \( \mu \text{mol/L}, P = 0.007 \)) and antioxidant capacity (by 20.6 \( \mu \text{mol/L}, P = 0.001 \)) in the placebo group were completely abolished by atorvastatin (\( P = 0.038 \) and \( P = 0.005 \), respectively, vs. placebo). In contrast, atorvastatin had no significant effect on cytokine levels.

Conclusion Acute inflammation is aetiologically associated with the deterioration of vasomotor and systemic endothelial function in hypercholesterolaemic patients. Atorvastatin effectively abrogates these deleterious effects.

Introduction

Besides the relationship of low-grade inflammation with atherosclerosis,1,2 accumulating evidence suggests that even acute inflammatory conditions may transiently increase risk of cardiovascular events.3–5 This effect may be partly mediated by an impairment of arterial performance following acute inflammation. We6 and other investigators7–10 have recently shown that a mild acute inflammation induced by Salmonella typhi vaccination deteriorates arterial function even in healthy subjects. Indices of arterial performance are important markers of cardiovascular disease and independent predictors of the corresponding risk.11,12

Statins decrease cardiovascular outcomes both in secondary and primary prevention settings.13 The major amount of this salutary action of statins is attributed to lipid lowering. However, a part of the statin-related benefit is perhaps independent of changes of blood lipids.14,15 This is further evidenced by studies showing that statins may improve cardiovascular performance even in subjects without overt hyperlipidaemia.15,16 These ‘pleiotropic’ effects are largely accounted for by a favourable action profile of statins on endothelial function, inflammation, or thrombosis.17 Low-grade inflammation is activated and arterial performance is impaired in hypercholesterolaemic patients.18,19 However, it has not been investigated whether a superimposed acute inflammatory stimulus may accentuate this inflammatory milieu or further aggravate vascular function in such patients. Moreover, data regarding any potential role of statins in preventing the impairment of arterial function induced by acute inflammation are limited.20 Accordingly, in the present randomized, double-blind, placebo-controlled study, we investigated through a cause-and-effect testing (with the S. typhi vaccine model) and a thorough humoral investigation (involving several markers/mediators of inflammation, systemic endothelial function, and oxidant stress) whether acute inflammation further deteriorates conduit artery endothelial function in hypercholesterolaemic patients and whether short-term (4 day) pre-treatment with atorvastatin protects from the arterial endothelial response to inflammation.

KEYWORDS

Statin; Endothelium; Hypercholesterolaemia; Inflammation; Nitric oxide

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Methods

Study population

We studied 50 untreated individuals with mild hypercholesterolemia (mean age 41.5 years, 30 men) who were referred to our department. All patients received nutritional-hygienic therapy and were studied within 1 month from initial presentation. All subjects were free of cardiovascular disease, they were clinically well, and taking no cardiovascular medications or antioxidant vitamins. They had not had any infection, they did not report use of anti-inflammatory or steroid substances during the past 2 months, and they had not received any typhoid vaccination in the previous 6 months. No female participant was on oral contraceptives or oestro-gen replacement therapy. The study complies with the Declaration of Helsinki. The study protocol was approved by our Institutional Research Ethics Committee and all subjects gave written informed consent.

Study design

The study was carried out using a randomized, double-blind, placebo-controlled design and consisted of two treatment arms, one with atorvastatin and one with placebo. All vascular studies were performed in a quiet, temperature-controlled room at 23 °C. Subjects had fasted for at least 6 h and had abstained from caffeine, ethanol, and flavonoid-containing beverages intake for at least 12 h before each session. Baseline measurements for the evaluation of endothelial function of the brachial artery were performed with ultrasonography in the morning after a 20 min rest period in the supine position. Venous blood was drawn into vacutainer tubes. The study participants were subsequently randomized to taking atorvastatin 40 mg or placebo once daily, between 7 and 9 p.m., for a 4 day period. After repeating all vascular and biochemical measurements in the morning of the 5th day (end of 4 day treatment period: pre-vaccine time point), subjects were vaccinated with the S. typhi capsular polysaccharide vaccine (0.025 mg, Typhim Vi, Pasteur Merieux MSD, France),6–10 which was injected into the deltoid muscle of the subjects’ dominant arm. All measurements were repeated at 8 h after injection (post-vaccine time point).

Evaluation of endothelial function

Flow-mediated dilatation (FMD) is predominantly dependent on endothelial nitric oxide (NO) release and can be used as an estimate of endothelial function. Resting and hyperaemic arterial diameters and flows and FMD of the right brachial artery were determined with ultrasonography (EnVisor C, Philips) by using a high-resolution, extended frequency range transducer of 3–12 MHz (Broadband Linear Array Transducer L12-3), as previously described.19,21–23 FMD was calculated as the percent increase in brachial artery diameter during hyperaemia compared with the resting value. Endothelium-independent nitrate-induced dilatation (NID) was measured after delivering a single 0.4 mg dose of nitroglycerin spray sublingually. Repeatability of FMD in our unit (mean variability between two measurements performed by the same operator 3 h apart) is 1.36% (absolute value).

Measurement of biochemical markers/mediators

Immediately after the acquisition of venous blood, plasma or serum was separated by centrifugation (3000 g at 4 °C for 15 min), placed in aliquots, and then stored at −70 °C. High-sensitivity C-reactive protein (hs-C-reactive protein) was measured by immunophelometric assay (Dade Behring, Marburg, Germany). Interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumour necrosis factor-α (TNF-α) were measured using ELISA (R&D Systems, Minneapolis, MN, USA). ELISA was also used to determine endothelin-1 and endothelin-1 (1–21), Biomedica, Vienna, Austria) and oxidized LDL (Oxidized LDL ELISA, Merckodia, Uppsala, Sweden). Circulating level of plasma total NO metabolites (nitrate and nitrite, NOx) was measured colorimetrically (R&D Systems, Minneapolis, MN, USA). Plasma total antioxidant capacity (TAC) was also measured colorimetrically (ImAnOx, Immunodiagnostik AG, Bensheim, Germany).

Total cholesterol, HDL-cholesterol, triglycerides, and glucose levels were measured with standard methods. LDL-cholesterol level was calculated using the Friedewald formula.

Statistical analysis

Sample size calculation was based on the hypothesis that acute inflammation would be associated with an absolute decrease in FMD of at least 2%. Therefore, on the basis of data from our unit showing that the standard deviation of FMD for subjects with characteristics similar to those of our study population was 2.4%, we estimated that 24 subjects per treatment group (48 in total) would provide 80% power at the 5% level of significance to detect such a difference between groups treated with atorvastatin or placebo. However, to provide better confidence, we finally recruited 50 subjects.

Continuous variables are expressed as mean value (standard deviation), whereas categorical variables as absolute and/or relative frequencies. All continuous variables were tested for normal distribution by using the Kolmogorov–Smirnov criterion. For skewed variables (hs-C-reactive protein, IL-6, IL-1β, and endothelin), data are expressed as median value (25th–75th percentile), and logarithmic transformation was performed prior to analysis. At baseline, numerical parameters among the two study subgroups were compared using t-test for unpaired measures. Contingency tables and the χ² test were applied for categorical variables.

Between atorvastatin and placebo group, variables of brachial artery function and biochemical variables 8 h after vaccination were compared with analysis of covariance (ANCOVA) to adjust for pre-vaccine values. Similarly, the effect of atorvastatin during the 4 day pre-treatment period was evaluated with ANCOVA, which compared pre-vaccine variables between groups with simultaneous adjustment for baseline values. The vaccination effect within each group was evaluated by Student’s t-test for paired measures comparing post-vaccine values vs. pre-vaccine values. Exact P-values < 0.05 were considered statistically significant. Data analysis was performed with SPSS software, version 10.1 (SPSS Inc., Chicago, IL, USA).

Results

There were no significant differences in baseline characteristics between the study groups (Table 1). The vaccination effect within each group is reported as the 8 h post-vaccine mean value minus pre-vaccine mean value. The net effect of atorvastatin vs. placebo in the 4 day pre-treatment substudy is defined as atorvastatin effect (pre-vaccine minus baseline mean value) minus placebo effect (pre-vaccine minus baseline mean value). In the case of skewed variables, geometric means (anti-logs of mean log values) were used for calculating effects.

Systemic haemodynamic effects

Pre-vaccine treatment period

The 4 day atorvastatin regimen did not cause any significant change in blood pressure or heart rate (all P = NS).

Vaccination

Vaccination significantly increased heart rate (by 5.0 b.p.m., P = 0.01 within the placebo group) but did not influence blood pressure. Atorvastatin did not abrogate the vaccination-related increase in heart rate (increase of...
3.8 b.p.m. within the atorvastatin subgroup, \( P = 0.013 \) (between groups \( P = 0.75 \)).

**Effects on lipid profile**

**Pre-vaccine treatment period**

Compared with placebo, atorvastatin significantly decreased total cholesterol (by 18.9 mg/dL, \( P = 0.004 \)) and LDL-cholesterol (by 23.3 mg/dL, \( P = 0.003 \), [Figure 1]) after the 4 day treatment period. We observed no changes regarding the levels of HDL-cholesterol or triglycerides ([Figure 1]).

**Vaccination**

Vaccination had no significant effect on the lipid profile within or between the study subgroups ([Figure 1]).

**Effects on systemic inflammation, endothelial function, and oxidant stress**

**Pre-vaccine treatment period**

Compared with placebo, treatment with atorvastatin did not affect the levels of inflammatory markers/mediators at 4 days ([Figure 2]). Oxidized LDL was significantly decreased with atorvastatin (by 7.6 U/L, \( P = 0.032 \), [Figure 3]). NO\(_x\) level was non-significantly increased (by 4.5 m\(\text{mol/L} \), [Figure 3]), whereas endothelin-1 and TAC did not change significantly with atorvastatin ([Figure 3]).

**Vaccination**

In the placebo group, vaccination gave rise to a prominent increase in IL-6 (by 3.36 pg/mL, \( P < 0.001 \), [Figure 2]) level and also resulted in a significant reduction in NO\(_x\) level (by 6.0 \(\text{mol/L} \), \( P = 0.007 \)) and TAC (by 20.6 \(\text{mol/L} \), \( P = 0.001 \), [Figure 3]). Pre-treatment with atorvastatin did not prevent the effect of the vaccination on IL-6 (an increase of 2.61 pg/mL within the atorvastatin subgroup, \( P = 0.001 \); between groups \( P = 0.37 \), [Figure 2]). On the other hand, atorvastatin abrogated the unfavourable effect of vaccination on NO\(_x\) level (a decrease of 0.6 m\(\text{mol/L} \) within the atorvastatin subgroup, \( P = 0.89 \); between groups \( P = 0.038 \), [Figure 3]) and TAC (an increase of 6.0 \(\text{mol/L} \) within the atorvastatin subgroup, \( P = 0.42 \); between groups \( P = 0.005 \), [Figure 3]).

**Effects on brachial artery endothelial function**

**Pre-vaccine treatment period**

Atorvastatin pre-treatment did not induce any significant effect on brachial artery resting or hyperaemic diameter and flow, reactive hyperaemia, FMD ([Figure 4]), or NID.

**Vaccination**

Vaccination was associated with an unfavourable effect on FMD in the placebo-treated group (absolute decrease by 2.55%, \( P = 0.001 \), [Figure 4]). This effect occurred because

| Table 1 Baseline characteristics of the study subgroups |
|------------------------------------------|---------|----------|
the vaccine induced a decline of hyperaemic brachial artery diameter in the placebo group (a reduction of 0.08 mm, \( P = 0.03 \), Figure 4). In contrast, pre-treatment with atorvastatin fully abrogated the vaccination-induced reduction, both of the hyperaemic diameter (an increase of 0.04 mm within the atorvastatin subgroup, \( P = 0.29 \); between groups \( P = 0.024 \), Figure 4) and of FMD (absolute decrease of 0.15% within the atorvastatin subgroup, \( P = 0.74 \); between groups \( P = 0.005 \), Figure 4). No significant within- or between-group effect of vaccination was observed regarding resting brachial artery diameter (Figure 4) and flow, the percentage degree of reactive hyperaemia or NID (all \( P = \text{NS} \)).

Discussion

This is the first study, to the best of our knowledge, to demonstrate through a cause-and-effect relationship that acute systemic inflammation aggravates endothelial dysfunction in conduit arteries of patients with uncomplicated, untreated, mild hypercholesterolaemia. Furthermore, a short-term atorvastatin regimen decreases total and LDL-cholesterol level and abrogates the deterioration of endothelium-dependent vasomotion induced by an acute inflammatory stimulus in such subjects. Interestingly, these vascular effects are associated with changes in the circulating level of NO metabolites and indices of oxidant state, but not with
changes in the level of certain inflammatory markers/mediators.

**Clinical implications**

Our study may have important clinical implications. LDL and its oxidized forms are implicated in the pathogenesis of atherosclerosis through the modulation of the inflammatory cascade, and these mechanisms are intensified in subjects with high cholesterol levels.1,24,25 There is no doubt that the type of acute inflammation induced by the vaccine in this study is not related to chronic, low-grade, subclinical inflammation that promotes atherogenesis.1,2 However, accumulating data suggest that acute systemic inflammatory responses (such as those in acute infections or after surgery) are clinically significant, since they are associated with a short-term increased risk of a cardiovascular event.2–5 In part, this risk may be accounted for by a deterioration of arterial function following acute inflammation.6–10 Interest-ingly, after a real-life acute infection, the degree of FMD impairment is almost similar to that observed in this study.26 The present study establishes an aetiological relationship between acute inflammation and endothelial dysfunction in hypercholesterolaemic patients and indicates that an acute inflammatory insult can further compromise endothelial function even in subjects with presumed abnormal baseline endothelial performance.

An additional important implication is that our study aetiologically establishes a protective effect of atorvastatin against inflammation-induced impairment of endothelium-dependent vasomotion. This is pathophysiologically relevant, given that preservation of endothelial function of peripheral arteries is associated with normal coronary vaso-reactivity27 and fewer cardiovascular outcomes.12 However, since there are, perhaps, differences between the inflammatory response following an acute infection or other real-life inflammatory stimuli and the response that is generated by the S. typhi vaccine model, the exact clinical relevance of our observations remains to be established. Finally, our results provide mechanistic links regarding the beneficial effect of early administration of statins in patients with acute coronary syndromes28 and the reduction in perioperative mortality in patients undergoing major surgery.29

**Mechanisms**

**Effect of acute inflammation on conduit artery endothelial function**

Considering that vasodilatory response to reactive hyperaemia is dependent on endothelial NO,21 the decrease of FMD following vaccination might be attributed to an adverse effect on NO bioavailability. This notion is further supported by recent evidence showing a decrease in NO activity after vaccination with the S. typhi vaccine.9 In the placebo-treated patients, endothelial deterioration was accompanied by diminished level of NO metabolites. This implies that a decreased NO production probably accounts for the observed vascular effects. Most likely, this effect is not mediated via an acute reduction in the expression of endothelial NO synthase.30 but rather post-translational downregulation may have played a role. The finding of reduced TAC after vaccination may signal a state...

**Figure 3** Changes in circulating levels of endothelial markers/mediators and indices of oxidant stress in the atorvastatin and placebo groups before and 4 days after treatment and 8 h after vaccination. Probability values on top of graphs as in Figure 1. Triangles indicate mean changes from baseline; bars, their 95% CIs. *P = 0.007, †P = 0.001 (paired t-test between the pre-vaccine and post-vaccine values within the placebo group).
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The protective effect of atorvastatin

Our study is the first to demonstrate that, in hypercholesterolaemic patients, atorvastatin manifests a protective endothelial effect against acute inflammation. This is in line with a recent study showing that simvastatin may prevent endothelial dysfunction induced by intravenous endotoxin in healthy subjects. Atorvastatin did not influence the changes of cytokine levels after vaccination. Level of IL-6, a principal mediator of the acute-phase response, increased after vaccination in a similar manner, both in the atorvastatin and the placebo subgroups. Therefore, it seems that the anti-inflammatory effect of atorvastatin does not involve the initial phase of the inflammatory cascade, but rather the other components of the pathway are modified. Interestingly, there is evidence that atorvastatin may beneficially regulate the expression of genes controlling the synthesis of proteins that participate in the inflammatory cascade as early as 12 h after administration. Whatever the exact anti-inflammatory action of atorvastatin, this drug preserved NO-mediated actions during acute inflammation, despite the initiation of acute-phase response. This beneficial effect occurred in parallel with a prevention of the vaccination-induced reduction of circulating NO metabolites and plasma TAC. S. typhi vaccine increases oxidant stress, and atorvastatin may have favourably modulated the production of reactive oxygen species from endothelial cells and monocytes. The possible anti-oxidant effect of atorvastatin that is implied by our results is also in line with a previous study showing that antioxidants counterbalance the deleterious endothelial effects induced by this vaccine. Furthermore, other effects of atorvastatin, such as the reduction in the levels of LDL-cholesterol and oxidized LDL, may have contributed to our results. Indeed, there is evidence that even an acute decrease in LDL-cholesterol level with apheresis may in its own benefit endothelial function. The protective effect of atorvastatin on the observed arterial effects. The protective effect of atorvastatin on the observed arterial effects.

Specific comments

Our study did not demonstrate a beneficial effect on FMD after 4 days of atorvastatin treatment, although other studies have shown that this drug may improve resistance

of low NO bioavailability (more rapid inactivation) due to increased oxidant stress. This is in line with the evidence that the vaccine model increases oxidant stress. In line with all relevant previous studies, we observed no significant change of circulating hs-C-reactive protein, IL-1β, and TNF-α levels 8 h after vaccination. However, IL-6 level exhibited a prominent rise with inflammation. Furthermore, there is unambiguous evidence that the vaccine model generates an acute phase response. In particular, body temperature6 and white blood cell count6,7 increase after 8 h, whereas hs-C-reactive protein level rises prominently at a later stage.6,10 Interestingly, tissue IL-1β production may increase after the S. typhi vaccine, despite a lack of change in circulating level of IL-1β.7,10 Nevertheless, our study cannot reach a definitive conclusion regarding which cytokine is responsible for the endothelial effects of inflammation. Despite the prominent rise of IL-6 level after the vaccine, it is rather unlikely that IL-6 is implicated with our results. Indeed, IL-6 also increased in the atorvastatin-treated subgroup, in which the endothelial dysfunction was abrogated, and furthermore, existing evidence refutes any direct adverse endothelial effect of IL-6 in humans. On the other hand, after considering that tissue production (but not blood level) of IL-1β increases 8 h after vaccination7–10 and that IL-1β may attenuate endothelium-dependent vasomotor responses,10 we speculate that IL-1β may perhaps account, at least partly, for the arterial effects of the vaccine observed in this study.

Specific comments

Our study did not demonstrate a beneficial effect on FMD after 4 days of atorvastatin treatment, although other studies have shown that this drug may improve resistance
artery endothelial function after a few days. Our study was not designed to detect a change in FMD in unstressed (non-vaccine-induced) conditions, and moreover, arterial bed-specific factors perhaps play a role. Further studies are needed to elucidate whether the beneficial effects of atorvastatin we observed are dose-dependent, whether they represent a statin class effect, and whether they apply to other populations as well.

In our study, we measured total nitrites and nitrates (NOx) in an attempt to quantify NO metabolism, given the evidence showing that plasma NO metabolites are associated with FMD, both in chronic and acute conditions. Since plasma NOx level is affected by changing patterns of nitrate intake, we instructed our subjects to adhere to the diet that was prescribed at initial presentation. Nevertheless, the relationship between FMD response and changes of NOx observed in this study is associative and not necessarily aetiologial.

Finally, taking into consideration the remarkable similarity of the acute-phase response (as expressed by cytokine, mainly IL-6 increase) in the present and previous studies, we further confirm the reproducibility of this model of vascular inflammation introduced by Hingorani et al.7

In conclusion, this is the first study to demonstrate through a causative link that, in individuals with mild hypercholesterolaemia, acute systemic inflammation impairs endothelial function. Furthermore, atorvastatin pretreatment abrogates the effect of acute inflammation on endothelial performance, with a simultaneous blunting of the inflammation-induced reduction of NO metabolites and plasma antioxidant reserve. These findings may have important implications, given the importance of endothelial performance for cardiovascular function and the potential of therapeutic interventions with statins.

Conflict of interest: none declared.

References

Clinical vignette

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Coronary artery perforation during primary PCI: an easily resolved case for a dramatic complication

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A 55-year-old male diabetic and hypercholesterolemic patient presented with acute lateral wall myocardial infarction. Coronary angiography showed a significant stenosis of distal right coronary artery (RCA), a total chronic occlusion of a small left circumflex, and the left anterior descending (LAD) and acute coronary occlusion of the marginal branch (MB) (Panel A). The patient underwent MB primary angioplasty (Panel A). The patient was treated with 300 mg of clopidogrel and 8000 I.U. of heparin. The MB occlusion was crossed with guide wire, and pre-dilatation was performed using 2.0 x 15 mm balloon (Maverick, Boston Scientific). Just after a Janus stent (2.75 x 23 mm, Sorin) deployment, there was angiographic evidence of coronary perforation (type III of Hells-Classification) (Panel B). At that moment, we inflated a balloon (3.0 x 12 mm, Aria-Sorin) at the perforation site, giving neutralization therapy with protamin (55 mg) towards the heparin effect (activated clotting times 165 s). We inflated the balloon five times, 5 min each inflation. The control angiogram showed a closure of the site of perforation and a final TIMI-III flow and MBG-II (Panel C). The patient, asymptomatic and hemodynamically stable, was admitted to the intensive care unit with aspirin (100 mg), clopidogrel (75 mg), atorvastatin (20 mg) and insulin. After 2 days, another coronary angiography was performed to complete the procedure for LAD disobstruction and RCA angioplasty. During this procedure, we performed an intravascular ultrasound for the control of the MB (Panel D) that showed the site of perforation well repaired and haematoma behind the proximal part of the stent deployed in the MB. The patient was discharged after 5 days as asymptomatic and haemodynamically stable.


Panel B. Coronary perforation (arrow). (B1) Balloon inflation and extravasation of the contrast to the pericardium (arrows).

Panel C. Closure of the site of perforation.

Panel D. Intravascular ultrasound. (D1) Axial image: site of perforation (arrow), haematoma (arrows). (D2) Longitudinal image: haematoma (arrows).