Nitric oxide enhances the anti-inflammatory and anti-atherogenic activity of atorvastatin in a mouse model of accelerated atherosclerosis

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Aims
The aim of the present study was to assess whether the addition of a nitric oxide (NO)-donating moiety to atorvastatin enhances anti-inflammatory and anti-atherogenic effects in an animal model of endothelial dysfunction, systemic peroxidation and inflammation, and accelerated atherosclerosis.

Methods and results
Low-density lipoprotein receptor (LDLR)⁻/⁻ mice kept on a high-fat diet (HFD) for 16 weeks underwent photochemical injury to the femoral artery with the local production of oxygen radicals. HFD markedly enhanced cholesterol, inflammatory biomarkers in plasma and in the femoral arterial wall, and atherosclerotic lesions in the aortic arch; inflammation and atherosclerosis were further increased by photochemically generated oxygen radicals. Treatment with the NO-donating atorvastatin NCX 6560 (11.7 mg/kg) was significantly more effective than atorvastatin (10 mg/kg) in reducing the following parameters: lipid-rich lesions in the aortic arch (surface covered: atorvastatin = 24 ± 5%; NCX 6560 = 14.7 ± 3.9%; P < 0.05); the production of radical oxygen species in the aorta (dichlorofluorescein fluorescence intensity per milligram of protein: atorvastatin = 2419 ± 136.7; NCX 6560 = 1766 ± 161.2; P < 0.05); femoral artery intima/media thickness (atorvastatin = 1.2 ± 0.11; NCX 6560 = 0.3 ± 0.14; P < 0.05); circulating interleukin-6 (atorvastatin = 34.3 ± 6.8 pg/mL; NCX 6560 = 17.7 ± 14.4 pg/mL; P < 0.05); and matrix metalloproteinase 2 in the arterial wall (atorvastatin = 55.2 ± 1.9 ng/µg of proteins; NCX 6560 = 45.8 ± 2.6 ng/µg of proteins; P < 0.05).

Conclusion
In conditions of severe endothelial dysfunction, systemic peroxidation and inflammation, and accelerated atherosclerosis, atorvastatin, even at high doses, displays suboptimal anti-atherogenic and anti-inflammatory effects, while the addition of a NO-donating property confers enhanced anti-atherogenic and anti-inflammatory effects.

Keywords
Atherosclerosis • Atorvastatin • Hypercholesterolaemia • Inflammation • Peroxidation • Nitric oxide • NCX 6560

1. Introduction
Dyslipidaemia, inflammation, endothelial activation, and endothelial dysfunction co-operate in determining an individual’s propensity to develop atherosclerosis. Statins not only reduce plasma cholesterol but also decrease systemic inflammation, as documented by a reduction of interleukin-1β (IL-1β),¹ CD40L,² and high-sensitivity C-reactive protein,³ blunt endothelial activation, as shown by the reduction of E-selectin or soluble intercellular adhesion molecule-1 (sICAM-1),⁴ and ameliorate impaired endothelial function, as shown by the restoration of the production of nitric oxide (NO).⁵ Statins exert these activities by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase which is central not only to cholesterol metabolism (via the liver) but also to intracellular signalling in vascular cells.⁶

However, in conditions of severe functional endothelial injury, such as those occurring in strong hypercholesterolaemia associated with enhanced oxidative stress, statins may not be able to re-establish a normal endothelial function, and in particular to restore normal NO synthesis.⁷,⁸

Systemic inflammation, exacerbated by the formation of reactive oxygen species (ROS) from oxidized lipoproteins, contributes to endothelial dysfunction and accelerates atherosclerosis.⁹ For instance,
in familial hypercholesterolaemia, statins, although reducing cholesterol, do not completely reverse endothelial dysfunction and require higher doses and longer lasting administration to display beneficial effects on the vasculature.10–13 Similar to human familial hypercholesterolaemia, low-density lipoprotein receptor (LDLR)−/− mice present dyslipidaemia, impaired endothelial function, and an altered redox balance associated with reduced NO bioavailability and accelerated atherosclerosis.14,15 The administration of a high-fat diet (HFD) to these mice further increases total and low-density lipoprotein (LDL) plasma cholesterol, boosts systemic inflammation and peroxidation, and leads to the rapid development of atherosclerosis.16,17 In these animals, impaired endothelial function and accelerated atherosclerosis can be prevented by S-nitrosothiol, a NO-donating agent, but not by l-arginine, a NO precursor, probably due to peroxynitrite formation and nitric oxide synthase (NOS) uncoupling.18–20 This observation suggests that in conditions of strong hyperlipidaemia and systemic peroxidation, exogenously supplied NO may take the place of endogenous NO and block the phenomena leading to atherosclerosis.

Recently, a new class of compounds, the NO-donating statins, have been reported to associate the ability to donate bioactive NO to the capacity to inhibit HMG-CoA reductase, and thus to display a variety of biological effects in addition to those of statins, including anti-thrombotic and anti-inflammatory properties.21–23 The aim of the present study was to compare the anti-inflammatory and anti-atherothrombotic properties of the NO-donating atorvastatin, NCX 6560, with those of atorvastatin in an animal model of severe endothelial dysfunction, oxidative stress, and accelerated atherosclerosis, i.e. LDLR−/− mice fed a cholesterol-rich diet and undergoing intravascular photochemically induced generation of oxygen free radicals.

2. Methods

2.1 Animals and drug treatments

C57BL6/j mice were obtained from Charles River (Calco, Italy); LDLR−/− mice, on a C57BL6/j background, were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Male LDLR−/− mice were maintained on a standard diet [SD; Altromin–R. Rieper S.p.A., Bolzano, Italy; 20% (wt/wt) proteins, and 4.6% fat] until the fourth week of age and then randomly divided into two groups, one continuing on a SD and the other shifted to a HFD [Tekland Custom Diet, WI, USA; diet #TD 95286; 20.5% (wt/wt) fat, 1% cholesterol, 19.5% casein, and no sodium cholate] for another 19 weeks. Starting from week 16, mice were randomly assigned to the following treatments: vehicle (controls); atorvastatin (5, 10, 20 mg/kg); or NCX 6560 (5.85, 11.7, or 23.4 mg/kg), dissolved in dimethyl sulfoxide (DMSO)−1−1 mice fed a cholesterol-rich diet and undergoing intravascular photochemically induced generation of oxygen free radicals.

2.2 Photochemical generation of oxygen radicals

Photochemical generation of oxygen radicals in the femoral artery was induced as previously described.24,25 Mice were anaesthetized with xylazine (5 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.) and placed in the supine position. The adequacy of anaesthesia was monitored by standard methods (observation of respiration and colour of mucous membranes, loss of pedal reflex, and loss of reflective response to tactile stimuli). Their body temperature was maintained at 36°C by a heating pad during surgery. The femoral artery was surgically exposed and transilluminated with a polarized green light (wavelength 540 nm), using a xenon lamp with a heat-absorbing filter, through an optic fibre positioned 5 mm away from the arterial segment (Hamamatsu Photonics, Shizuoka, Japan). Green light irradiation was maintained for 25 min. After the first 5 min, Rose Bengal (Sigma), diluted in phosphate-buffered saline (PBS), was infused into the tail vein for 5 min in a volume of 0.2 mL, at a dose of 20 mg/kg. In control animals, instead of Rose Bengal, PBS was infused for 5 min. The photochemical activation of intravascular Rose Bengal induces the formation of ROS, resulting in endothelial damage.24–27 The cutaneous wound was then closed, and animals were returned to their cages. After 21 days, mice were killed by an overdose of pentobarbital (100 mg/kg, i.v.), the chest and abdominal cavities were opened, and a catheter was inserted into the left cardiac ventricle. The vascular system was washed with saline and then perfused with a solution of 2% glutaraldehyde and 1% paraformaldehyde in 0.1 mM PBS, pH 7.4, at physiological pressure (90–100 mmHg) for 10 min. The femoral artery was then removed and fixed overnight in the same fixative. For the aorta en face preparations, mice were killed 21 days after photochemical injury to the femoral artery by an overdose of pentobarbital (100 mg/kg, i.v.), a cannula was inserted into the left ventricle, and the aortic tree was fixed by perfusion with ice-cold PBS containing 4% paraformaldehyde, 5% sucrose, and 2 μM EDTA.

2.3 Measurement of atherosclerotic lesions

Atherosclerotic lesions were evaluated in paraffin sections of femoral arteries collected 21 days after photochemically induced generation of oxygen radicals, stained with Haematoxylin and Eosin, and analysed using a specific software package, as previously reported24,28 (see Supplementary material online for detailed methods).

2.4 Immunohistochemistry

Paraffin sections of the ascending segment of the aortic arch were stained for macrophages. Briefly, sections (3 μm thick) were incubated with a rat anti-mouse Mac-3 monoclonal primary antibody (dilution 1:20; BD Pharmingen, San Diego, CA, USA) to identify macrophages. After rinsing with PBS, the slides were developed with biotinylated rabbit anti-rat IgG (dilution 1:200; Vector Laboratories, Burlingame, CA, USA). Antibody reactivity was detected using horseradish peroxidase-conjugated biotin–avidin complexes (Vector Laboratories) and developed with diaminobenzidine tetrahydrochloride substrate (Dako, Denmark, Glostrup). The sections were photographed with a Leica microscope, and the presence of macrophages (brown stain) was evaluated qualitatively.

2.5 Atherosclerosis-related inflammatory biomarkers

Matrix metalloproteinase-2 (MMP-2), cyclo-oxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) were measured in extracts of femoral arteries collected 21 days after the photochemical generation of oxygen radicals. Femoral arteries were homogenized in lysis buffer (40 mM Tris–HCl, 0.3 M NaCl, 1 mM EDTA, 0.05% NaNO3, and 1% NP-40) at 4°C for 1 h and then centrifuged at 1000 × g for 15 min. Supernatants were stored at −80°C until assay. Total MMP-2 was measured by zymography, as previously described,25,29 and COX-2 and iNOS were measured by western blotting (see Supplementary material online). Intra- and interassay coefficients of variation of MMP-2 measurements were 2.3 and 3.9%, respectively (n =
8); intra-assay coefficients of variation of COX-2 was 3.85% (n = 8). Each sample was tested in triplicate for MMP-2, iNOS, and COX-2.

Tissue protein content was measured by the Bio-Rad (Milan, Italy) protein assay.

Tumour necrosis factor-α (TNFα), interleukin-6 (IL-6), sICAM-1, and serum amyloid A (SAA) were determined by ELISA (R&D Systems Inc., Minneapolis, MN, USA for TNFα, IL-6, and sICAM-1; and Life Technologies, Grand Island, NY, USA for SAA) in serum samples taken immediately before femoral artery removal. Each sample was tested in triplicate. All the ELISAs were highly specific; sensitivity and coefficients of variation (CVs) of the assays were as follows: for IL-6, sensitivity 1.6 pg/mL, intra-assay CV = 3.9%, and interassay CV = 6.1%; for TNFα sensitivity 5.1 pg/mL, intra-assay CV = 6.5%, and interassay CV = 7.3%; for sICAM-1, sensitivity 0.029 ng/mL, intra-assay CV = 9.2%, and interassay CV = 7.3%; and for SAA, sensitivity 0.3 μg/mL, intra-assay CV = 4.4%, and interassay CV = 7.0%.

2.6 Serum lipids
Non-anticoagulated blood was drawn from the tail vein in the morning after an overnight fast, at baseline (before starting HFD) and after 19 weeks of HFD, and serum was prepared. Total cholesterol, high-density lipoprotein cholesterol and triglycerides were determined by a commercial colorimetric assay (Menarini Pharmaceuticals, Firenze, Italy). Low-density lipoprotein (LDL) cholesterol was derived from total and high-density lipoprotein cholesterol.21

2.7 Nitric oxide-related parameters
Plasma nitrite and nitrate (NO₂/NO₃), the NO degradation end-products,21 were measured by a colorimetric, non-enzymatic method (Oxford Biomedical Research, Rochester Hills, MI, USA), and plasma cyclic guanosine monophosphate (cGMP), a marker of the in vivo biological effects of NO,21 was measured by ELISA (GE Healthcare, Milan, Italy), as reported elsewhere (see Supplementary material online). Each sample was tested in triplicate. The sensitivity of the ELISA was 1.0 pmol/mL, intra-assay CV = 5.7%, and interassay CV = 11.3%.

Endothelial nitric oxide synthase (eNOS) phosphorylated at Ser(1177) (p-eNOS; BD Pharmingen, Milan, Italy) was assessed by western blotting in extracts of femoral arteries prepared as reported above and tested in triplicate.

2.8 Endothelial function
Endothelium-dependent relaxation, a sensitive marker of endothelial function,30 was assessed in spontaneously hypertensive rats, which represent an established and standardized model of endothelial dysfunction.31 Detailed methods are reported in the Supplementary material online.

2.9 Blood pressure measurement
Systolic blood pressure was measured in conscious LDLR⁻/⁻ mice on HFD and in eNOS⁻/⁻ mice (Jackson Laboratory, Maine Harbour, Bar Harbor, Maine, USA), after a period of training and acclimation to the procedure, at baseline and after 3 weeks of treatment with atorvastatin (10 mg/kg) or NCX 6560 (11.7 mg/kg) using a non-invasive computerized tail-cuff system (BP-2000, Visitech Systems, Apex, NC, USA), as described elsewhere.21

2.10 Peroxidation indexes
2.10.1 Reactive oxygen species assay
The formation of ROS in aorta was measured by the use of the OxiSelect ROS assay kit (Cell Biolabs, San Diego, CA, USA; sensitivity limit of 10 pM for dichlorofluorescein) according to the manufacturer’s instructions (see Supplementary material online for detailed methods).32 Each sample was tested in triplicate.

2.10.2 Urinary 8-iso-PGF₂α
Twenty-four hour urine samples were collected from mice placed in metabolic cages before and 1, 7, 14, and 21 days after photochemical generation of oxygen radicals, supplemented with the antioxidant 4-hydroxy-tempo (1 mmol/L; Sigma Chemical Co., St Louis, MO, USA) and stored at −20 °C until extraction. Urinary 8-iso-PGF₂α was measured by a previously described, gas chromatography-mass spectrometry validated, radio-immunoassay method,23 and results are expressed as nanograms per milligram of creatinine.

2.11 Platelet-activation markers
Platelet surface P-selectin was measured in whole blood by flow cytometry (EPICS XL-MCL, Beckman Coulter, Miami, FL, USA), as previously described.25 One hour after last drug administration, a mixture of collagen (150 μg/mL) and adrenaline (0.25 μM) was injected intravenously to induce in-vivo platelet activation, and blood was collected after 2 min in 4% sodium citrate for platelet flow cytometry. Blood samples, diluted 1:10 with PBS, were stained with a phycoerythrin-labelled anti-CD41 anti-body (Leo,D2, Emfret Analytics, Wurzburg, Germany) as a platelet identifier and with a fluorescein isothiocyanate-labelled anti-P-selectin antibody (CD62P, Emfret Analytics) for 30 min in the dark. The reaction was stopped with 1% paraformaldehyde (1 mL). Platelet P-selectin is reported as the percentage of positive cells.25

2.12 Plasma drug levels and serum creatine kinase
Blood was collected by cardiac puncture in sodium citrate (4%), centrifuged at 10 000 × g for 4 min at 4 °C, and plasma stored at −80 °C for later analysis. Atorvastatin and its active metabolites were determined by liquid chromatography quadruple mass spectrometer (Applied Biosystem, SCIEX API 5000 mass spectrometer) using an Agilent technology 1000 high performance chromatography pump and a CTC prep and load platform autosampler (CTC Analytics AG, Zwingen, Switzerland) with a target lower limit of quantitation of 0.0897 nM and quantified using Analyst software (Applied Biosystem/MDS SCIEX, Toronto, ON, Canada), as described.21

Serum creatine kinase (CK) was measured in LDLR⁻/⁻ mice on HFD treated with NCX 6560 (11.7 mg/kg) or atorvastatin (10 mg/kg) for 3 weeks,34 using a commercially available kit (creatinine kinase, NAC, Assay; Diagnostic Chemicals Ltd, Charlottetown, PEI, Canada), within 7 days from serum preparation. CK activity was expressed as units/litre (U/L).

2.13 Statistical analysis
Data were analysed by one-way ANOVA, followed by the Newman–Keuls multiple comparison test between all groups. The correlation between different parameters was assessed by Spearman’s test. All analyses were performed using GraphPad Prism 4.00 for Windows software (GraphPad Software, San Diego, CA, USA; www.graphpad.com). Data are expressed as arithmetic means ± SEM. A P value of <0.05 was considered statistically significant.

3. Results
3.1 Effects of a high-fat diet and of photochemically generated oxygen radicals on atherosclerosis-related parameters in LDLR⁻/⁻ mice
Three weeks of HFD strikingly enhanced total and LDL cholesterol of LDLR⁻/⁻ mice, while triglyceride levels were not significantly modified (Figure 1A, Table 1).
Intimal hyperplasia of the femoral artery 3 weeks after photochemical injury was markedly enhanced in HFD-fed LDLR\textsuperscript{−/−} mice compared with SD-fed LDLR\textsuperscript{−/−} or with wild-type mice (Figure 1B).

LDLR\textsuperscript{−/−} mice on a HFD developed much more extensive aortic atherosclerotic lesions than LDLR\textsuperscript{−/−} mice on a SD (Figure 1C). This effect was particularly evident at the level of the aortic arch (Figure 1D), where the surface covered by plaques was enhanced several-fold by HFD. Photochemically induced generation of oxygen radicals further increased aortic plaque formation (Figure 1C–E). The formation of ROS in aortas was also markedly enhanced in LDLR\textsuperscript{−/−} mice on HFD compared with LDLR\textsuperscript{−/−} mice kept on a SD, and photochemical injury to the femoral artery further increased ROS in the aorta (Figure 1F).

LDLR\textsuperscript{−/−} on a SD, and to a greater extent LDLR\textsuperscript{−/−} on a HFD, had enhanced amounts of MMP-2, COX-2, and iNOS in the femoral artery vascular wall compared with wild-type mice, which is compatible with inflammatory cell infiltration, a phenomenon further enhanced by photochemical injury (Figure 2A–C).

Administration of a HFD to LDLR\textsuperscript{−/−} mice also enhanced systemic inflammation, as documented by a rise of serum IL-6, TNF-α, and sICAM-1 (Figure 2D–F); IL-6 and TNF-α, and not significantly sICAM, were further increased after the photochemical generation of oxygen radicals (Figure 2D–F).

Moreover, serum levels of amyloid A were significantly increased in LDLR\textsuperscript{−/−} mice on HFD, and even more so after the photochemical generation of ROS in the femoral artery, in comparison with wild-type mice and LDLR\textsuperscript{−/−} on SD. Taken together, these data show that the term administration of a HFD to LDLR\textsuperscript{−/−} mice, in association with a burst of oxygen radicals generated photochemically, represents a model of systemic inflammation, in vivo peroxidation, endothelial dysfunction, and accelerated atherosclerosis.
3.2 Different effects of a nitric oxide-donating atorvastatin and of atorvastatin in a model of accelerated atherosclerosis

Although both atorvastatin and NCX 6560 reduced lipidic plaques in the aorta, NCX 6560 was significantly more effective (Figure 3A–C), in particular at the level of the aortic arch (Figure 3B and D).

Macrophage infiltration in aortic plaques, evaluated by immunohistochemistry, was reduced in NCX 6560-treated LDLR^−/− mice on HFD more than by atorvastatin (Figure 3E).

NCX 6560 was also more effective than atorvastatin in reducing the proliferation of neointima of injured femoral arteries (Figure 4A), except at the highest dose tested, at which both treatments almost abolished neointimal proliferation (Figure 4A). Moreover, the inhibitory effect of NCX 6560 on intimal proliferation appeared earlier than that of atorvastatin, with a statistically significant reduction of neointima being already evident 2 weeks after the beginning of treatment (Figure 4B).

NCX 6560 reduced the expression of MMP-2 in the injured femoral arterial wall significantly more than atorvastatin (Figure 5A), while femoral artery COX-2 and iNOS were reduced by NCX 6560 and atorvastatin to a similar extent (Figure 5B and C).

### Table 1: Effect of treatment on total serum cholesterol, low-density lipoprotein (LDL)-cholesterol and triglycerides

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Atorvastatin, 5 mg/kg</th>
<th>NCX 6560, 5.85 mg/kg</th>
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<tr>
<td>Total cholesterol (mmol/L)</td>
<td>25.8 ± 3</td>
<td>24.0 ± 0.98</td>
<td>21.6 ± 1</td>
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<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>18.9 ± 1</td>
<td>19.9 ± 1.1</td>
<td>17.0 ± 0.7</td>
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<tr>
<td>Triglycerides (mmol/L)</td>
<td>6.3 ± 0.7</td>
<td>4.2 ± 0.4</td>
<td>5.2 ± 0.4</td>
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<tr>
<td>Atorvastatin, 10 mg/kg</td>
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<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>25.8 ± 3</td>
<td>21.9 ± 2.6</td>
<td>16.2 ± 2.7*</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>18.9 ± 1</td>
<td>13.9 ± 0.5**</td>
<td>15.2 ± 1*</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>6.3 ± 0.7</td>
<td>5.5 ± 0.4</td>
<td>4.0 ± 0.3</td>
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<tr>
<td>Atorvastatin, 20 mg/kg</td>
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<tr>
<td>Total cholesterol (mmol/L)</td>
<td>25.8 ± 3</td>
<td>20.9 ± 2.1</td>
<td>10.9 ± 2.1**</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/L)</td>
<td>18.9 ± 1</td>
<td>14.2 ± 0.7*</td>
<td>9.1 ± 0.7***</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>6.3 ± 0.7</td>
<td>3.7 ± 0.2</td>
<td>4.6 ± 0.4</td>
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</table>

Low-density lipoprotein receptor (LDLR)^−/− mice on a high-fat diet (HFD) were treated with atorvastatin or NCX 6560 for 3 weeks. Data are expressed as means ± SEM; n = 8 mice per group. Values in LDLR^−/− mice on a standard diet were as follows: total cholesterol = 6.3 ± 0.8 mmol/L; LDL cholesterol = 2.4 ± 0.2 mmol/L; and triglycerides = 2.9 ± 0.3 mmol/L.

*P < 0.05, **P < 0.01 vs. vehicle treated.

#P < 0.01 vs. equivalent atorvastatin.

Figure 2: Effect of a HDF and of photochemically generated oxygen radicals on vascular and systemic inflammation. Expression of inflammatory proteins in the femoral arterial wall and in serum of wild-type mice kept on a standard diet (SD; white columns), LDLR−/− on a SD (grey columns), LDLR−/− mice on a HFD (black columns) and LDLR−/− mice on HFD (hatched columns) 3 weeks after photochemical generation of oxygen radicals. (A) MMP-2 levels were measured by zymography. COX-2 levels (B) and iNOS levels (C) were measured by western blotting. Serum IL-6 (D), TNFα (E), and sICAM (F) were assessed by ELISA. *P < 0.05, **P < 0.01 by ANOVA followed by the Newman–Keuls multiple comparisons test (n = 6–8 per group).
Figure 3  Effect of treatment on atherosclerotic lesions. Atorvastatin (black columns) and NCX 6560 (grey columns) reduced the surface of total aorta (A), aortic arch (B) and thoracic aorta (C) covered by lipid-rich plaques of LDLR^{-/-} mice on HFD following photochemical generation of oxygen radicals. (D) Representative examples of mouse entire aortas prepared using the en face method with Sudan IV. (E) Sections of mouse ascending aorta stained with Haematoxylin and Eosin (1A, 2A, and 3A) and analysed by immunohistochemistry for the presence of macrophages (1B, 2B, and 3B) after 16 weeks on a HFD and treated for 3 weeks with vehicle, atorvastatin (10 mg/kg), or NCX 6560 (11.7 mg/kg). *P<0.05, **P<0.01 vs. vehicle; and #P<0.05, ###P<0.01 vs. equivalent atorvastatin by ANOVA followed by the Newman–Keuls multiple comparisons test (n=6–8 per group).

Figure 4  Effect of treatment on vascular inflammation in vehicle- (white columns), atorvastatin- (black columns) and NCX 6560 (grey columns)-treated mice. (A) Dose-dependent effect of treatment on intimal thickening after photochemical injury to the femoral artery in LDLR^{-/-} mice on HFD. (B) NCX 6560 (11.7 mg/kg) already produced a reduction in femoral artery intima/media thickness after 2 weeks of treatment. Intima/media thickness in LDLR^{-/-} sham-operated mice was 0.03 ± 0.007 (n = 4). *P<0.05, **P<0.01 vs. vehicle; and #P<0.05, ###P<0.01 vs. equivalent atorvastatin by ANOVA followed by the Newman–Keuls multiple comparisons test (n=6–8 per group).
NCX 6560, but not atorvastatin, reduced IL-6 serum levels in a dose-dependent manner in LDLR\(^{-/-}\) mice kept on a HFD and in which oxygen radicals had been generated photochemically (Figure 5D). The IL-6-lowering effect of NCX 6560 was rapid, with a statistically significant reduction being already evident 1 week after the beginning of treatment (Figure 5E). NCX 6560 also reduced sICAM-1 significantly more than atorvastatin at the highest dose tested; plasma TNF\(\alpha\) was equally reduced by both treatments (Table 2). Finally, SAA was reduced in a dose-dependent manner by NCX 6560 and more effectively than by the equivalent dose of oral atorvastatin (see Supplementary material online, Table S1).

### 3.3 Effects of treatments on serum cholesterol

HFD induced a significant increase of body weight (from 30.2 ± 1.2 to 40 ± 2.5 g; \(P < 0.05\)), with no differences in weight gain between the two treatment groups (data not shown). Total and LDL-cholesterol were reduced in a dose-dependent manner by both atorvastatin and NCX 6560 (Table 1), but the latter was significantly more active at the highest dose tested (23.4 mg/kg; Table 1), confirming previous observations.\(^{21}\) Moreover, neither NCX 6560 nor atorvastatin significantly modified triglyceride levels.

Treatment of LDLR\(^{-/-}\) mice on HFD for 3 weeks with the combination of an NO donor, isosorbide-5-mononitrate (20 mg/kg), and atorvastatin (11.7 mg/kg) reduced total cholesterol more than atorvastatin or isosorbide-5-mononitrate alone (see Supplementary material online, Table S2).

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**Table 2 Effect of treatment on serum levels of tumour necrosis factor \(\alpha\) (TNF\(\alpha\)) and soluble intercellular adhesion molecule-1 (sICAM-1)**

<table>
<thead>
<tr>
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<th>Vehicle</th>
<th>Atorvastatin, 10 mg/kg</th>
<th>NCX 6560, 11.7 mg/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF(\alpha) (pg/mL)</td>
<td>43.4 ± 4.7</td>
<td>13.1 ± 3.2*</td>
<td>10.5 ± 3.6*</td>
</tr>
<tr>
<td>sICAM-1 (ng/mL)</td>
<td>525.4 ± 19.7</td>
<td>435.7 ± 12.7</td>
<td>412.3 ± 24.8**</td>
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</table>

LDLR\(^{-/-}\) mice on a HFD were treated for 3 weeks with atorvastatin or NCX 6560 by oral administration. Data are expressed as means ± SEM; \(n = 8\) mice per group. 
*\(P < 0.05\), **\(P < 0.01\) vs. vehicle-treated group.
#\(P < 0.01\) vs. equivalent atorvastatin.
3.4 Nitric oxide-related biomarkers

NCX 6560 significantly enhanced plasma concentrations of nitrites and nitrates, the NO degradation end-products, while atorvastatin was almost inactive (Figure 6A).

Moreover, NCX 6560 induced a marked, and dose-dependent, increase in plasma cGMP, in contrast to atorvastatin, which enhanced it slightly and only at the highest dose tested (Figure 6B).

The expression of eNOS in femoral arteries was enhanced by NCX 6560, more than by equivalent atorvastatin (Figure 6C).

Finally, the effect of NCX 6560 or of atorvastatin on NO-related endothelial dysfunction was assessed in spontaneously hypertensive rats, which typically present impaired acetylcholine-induced relaxation of aortic rings.31 Five days of treatment with NCX 6560 (46.8 mg/kg) improved acetylcholine-induced relaxation significantly more than the equimolar dose of atorvastatin (Figure 6D).

3.5 Peroxidation indexes

NCX 6560 (11.7 mg/kg for 3 weeks) significantly reduced formation of ROS in extracts of aorta, while atorvastatin was ineffective (see Supplementary material online, Figure S2).

Urinary excretion of 8-iso-PGF2α, a marker of systemic lipid peroxidation, was strongly increased in LDLR−/− mice on HFD in the 24 h following photochemically induced ROS formation, and subsequently tended to return to baseline in the following 3 weeks. Treatment with NCX 6560 (11.7 mg/kg, p.o. for 3 weeks) reduced the rise of 8-iso-PGF2α significantly more than atorvastatin (Table 3).
3.6 Atorvastatin plasma levels

Plasma levels of atorvastatin and of its active metabolites, 2-hydroxy-atorvastatin and 4-hydroxy-atorvastatin, increased in a dose-related manner and similarly in NCX 6560 and atorvastatin treated mice. Atorvastatin levels ranged between 35 and 45 ng/mL for atorvastatin-treated mice and between 30 and 65 ng/mL for NCX 6560-treated mice; 2-hydroxyatorvastatin levels were between 80 and 120 ng/mL and between 50 and 110 ng/mL for atorvastatin- or NCX 6560-treated groups, respectively; 4-hydroxy-atorvastatin levels were between 5 and 20 ng/mL for both compounds.

Serum CK levels, a marker of possible statin-induced myopathy, were strongly elevated by 3 weeks treatment with atorvastatin (10 mg/kg; from 57.7 ± 6.5 to 198.7 ± 6.8 U/L; P < 0.0001), but not by NCX 6560 (11.7 mg/kg; 117 ± 12.1 U/L) in LDLR−/− mice on HFD.

4. Discussion

We show here that the prolonged administration of a HFD to LDLR−/− mice in association with the in vivo generation of oxygen radicals represents a model of severe endothelial dysfunction, in vivo peroxidation, inflammation, and accelerated atherosclerosis.

In fact, transillumination with polarized light of an exposed femoral artery of mice infused with Rose Bengal, a procedure that produces superoxide anions, strongly increased lipidic plaque deposition in the aorta and enhanced femoral artery intima hyperplasia in hypercholesterolaemic mice and was associated with an increased urinary excretion of 8-iso-PGF2α, with vascular ROS production, and with systemic and vascular inflammation.

In this model, atorvastatin, despite its lipid-lowering and anti-inflammatory activity, only partly prevented atherosclerosis and inflammation, while a NO-releasing atorvastatin showed superior activity. This suggests that in conditions of severe endothelial injury associated with the generation of oxygen radicals, the direct supply of NO allows the attainment of a significant anti-atherosclerotic effect by the enhancement of the vascular protective effects of atorvastatin unrelated to lipid lowering. The conclusion that NO donation confers superior anti-atherosclerotic activity to atorvastatin is supported by several observations. First, plasma IL-6 was reduced as early as 1 week after the beginning of treatment with NCX 6560, while atorvastatin required at least 3 weeks. Indeed, clinical observations show that atorvastatin, even at high doses, requires at least 3 weeks to show anti-inflammatory effects. Second, the anti-atherosclerotic activity of NCX 6560 was superior to that of atorvastatin despite a similar reduction of serum cholesterol and similar drug plasma levels. Third, while there was a statistically significant correlation between the reduction of total serum cholesterol and that of femoral artery intimal hyperplasia after 3 weeks of treatment with both atorvastatin and NCX 6560 (r = 0.5316, P = 0.0109 and r = 0.5113, P = 0.0212, respectively), this correlation was no longer evident for NCX 6560 after a shorter treatment period (2 weeks, r = 0.065, P = 0.7328), suggesting that the early anti-atherosclerotic effect of NCX 6560 is largely independent from its cholesterol-lowering activity. The prevention of neo-intimal proliferation by NCX 6560 can thus be attributed to the ability of NO to inhibit smooth muscle cell proliferation and leucocyte adhesion to the arterial wall. Fourth, the administration of NCX 6560, but not atorvastatin, strongly enhanced the levels in plasma of NO-degradation products and of cGMP, confirming the in vivo release of biologically relevant amounts of NO. Fifth, NCX 6560, but not atorvastatin, reduced the expression in the injured arterial wall of some inflammatory proteins, e.g. MMP-2, which participate in atherosclerotic vascular remodelling. A direct effect of NCX 6560-released NO on the vascular wall is documented by the decrease of inflammatory markers (MMP-2, COX-2, and iNOS) in the vessel wall, by enhanced endothelium-dependent vasorelaxation, by the lowering of blood pressure in eNOS−/− mice, and by the increase of phosphorylated eNOS in the vascular tissue.

In our model, the photochemical generation of oxygen radicals in the femoral artery produced strong systemic peroxidation, as documented by the increase of the urinary excretion of 8-iso-PGF2α, a marker of peroxidation typically increased in atherosclerosis, and by the enhanced generation of ROS in the aortic wall. NCX 6560 reduced vascular and systemic peroxidation more thanatorvastatin, suggesting that NO donation potentiates the in vivo antioxidant action of atorvastatin.

In our model, the photochemical generation of oxygen radicals in the femoral artery also produced systemic inflammation, as shown by the rise of circulating IL-6 and TNFα. Raised plasma IL-6 predicts recurrent events in patients with acute coronary syndromes. In patients with severe dyslipidaemia, IL-6 levels are lowered by atorvastatin only after more than 6 weeks of treatment, while TNFα and sICAM-1 are reduced earlier. In our model, while the two treatments reduced serum TNFα and sICAM-1 to the same extent, only NCX 6560 reduced IL-6 strongly and precociously, further suggesting that the systemic anti-inflammatory effect is probably due to the NO released by NCX 6560. IL-6 stimulates the expression of genes for acute-phase reactants, such as SAA, which is in fact increased by dietary cholesterol and is associated with the development of
atherosclerosis in LDLR<sup>−/−</sup> mice. In our model, SAA was further increased after photochemically induced peroxidation, and it was reduced by treatment with NCX 6560 significantly more than by atorvastatin, in accordance with the effects on IL-6 levels.

At the highest dose tested, NCX 6560 reduced cholesterol significantly more than atorvastatin, in agreement with previous data. This is in line with observations showing that repeated administration of sodium nitroprusside, a short-acting NO donor, reduces serum cholesterol in LDLR<sup>−/−</sup> mice and that S-nitrosothioguanine (GSNO), another NO donor, inhibits lipogenesis by hepatocytes in peroxidation and strong atherosclerosis-related inflammation, atorvastatin may enhance its effectiveness in disease conditions of atherosclerotic effects. The addition of a NO-donating property to anti-atherogenic and anti-inflammatory activity, while a NO-donating atherosclerosis, atorvastatin, even at high doses, displays a suboptimal dysfunction, systemic peroxidation and inflammation, and accelerated treatment. The doses of statins we used in our model seem high at a glance, compared with those used in humans. However, animal doses should not be extrapolated to humans by a simple body weight conversion, but should be normalized for the body surface area, an index which correlates several biological parameters across different mammalian species. Based on body surface area, when converted to an average human being, the doses of NCX 6560 preventing atherosclerosis and reducing inflammation in our model ranged between 33 and 131 mg/day. In agreement with this, plasma levels of atorvastatin and its metabolites in our mice were in the range of those found in humans after 6 weeks of treatment with 20 mg/day atorvastatin. Phase I studies in subjects with high LDL cholesterol have shown that, up to 144 mg/day, NCX 6560 is well tolerated and already produces marked lipid lowering 2 weeks after the beginning of treatment.

In conclusion, our study shows that in a model of severe endothelial dysfunction, systemic peroxidation and inflammation, and accelerated atherosclerosis, atorvastatin, even at high doses, displays a suboptimal anti-atherogenic and anti-inflammatory activity, while a NO-donating atorvastatin has strong and prompt anti-inflammatory and anti-atherosclerotic effects. The addition of a NO-donating property to atorvastatin may enhance its effectiveness in disease conditions of in vivo peroxidation and strong atherosclerosis-related inflammation, such as those found in high-risk patients with acute coronary syndromes.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**References**


