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RESEARCH PAPER

Anti-atherogenic effect of statins: role of nitric oxide, peroxynitrite and haem oxygenase-1

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Background and purpose: The pleiotropic effects of HMG-CoA inhibitors (statins), which include anti-inflammation, antioxidation and immunomodulation, are not yet fully understood. The present study was designed to elucidate the role of nitric oxide (NO), peroxynitrite ($ONOO^-$) and haem oxygenase-1 (HO-1) in the anti-atherogenic effect of statins.
Experimental approach: Normal and atherosclerotic New Zealand rabbits were treated with atorvastatin or simvastatin in the presence or absence of inhibitors and promoters of endothelial nitric oxide synthase (eNOS) and HO-1. NO and $ONOO^-$ released from isolated aortae by calcium ionophore were measured with nanosensors placed 6 ± 2 nm from aortic endothelium. Expression of eNOS and HO-1 protein, HO activity, plasma malondialdehyde (MDA) and vessel wall thickness were also measured.
Key results: Hypercholesterolaemia decreased eNOS expression by 31 ± 3%, decreased NO (230 ± 16 vs. 433 ± 17 nmol·L^{-1} control) and increased cytotoxic $ONOO^-$ (299 ± 15 vs. 187 ± 11 nmol·L^{-1} control). The concentration ratio of [NO]/[$ONOO^-$] decreased from 2.3 ± 0.1 (normal) to 0.7 ± 0.1 indicating an increase of nitroxidative stress in atherosclerotic endothelium. Expression of HO-1 protein increased by 20 ± 8% in atherosclerosis and further increased (about 30%) after treatment with statins. Statins partially restored the [NO]/[$ONOO^-$] balance (1.5 ± 0.1 for atorvastatin and 1.4 ± 0.1 simvastatin), decreased MDA and wall thickening. Promoters of eNOS and HO-1 (L-arginine and haemin) ameliorated the [NO]/[$ONOO^-$] ratio while their inhibitors (L-NAME or tin-protoporphyrin) showed no improvement in these ratio.
Conclusions and implications: Atherosclerosis induced an endothelial [NO]/[$ONOO^-$] balance indicative of endothelial dysfunction. Statins showed anti-atherosclerotic effects mediated by HO-1/eNOS, restoring the [NO]/[$ONOO^-$] imbalance and reducing lipid peroxidation.
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Keywords: nitric oxide; peroxynitrite; endothelium; lipid peroxidation; atherosclerosis and haem oxygenase

Abbreviations: eNOS, endothelial nitric oxide synthase; HO, haem oxygenase; iNOS, inducible nitric oxide synthase; LDL, low density lipoprotein; L-NAME, N^G-nitro-L-arginine methyl ester; MDA, malondialdehyde; NAD(P)H, nicotinamide adenine dinucleotide phosphate; SnPP, tin-protoporphyrin; SOD, superoxide dismutase; VW, vessel wall

Introduction

The three haem oxygenases (HO) are the rate-limiting enzymes in the oxidative degradation of haem into carbon monoxide, bilirubin and iron. HO-1 is the inducible form, while HO-2 and HO-3 are constitutively expressed (Maines, 1988). HO-1 is postulated to be a component of cellular defence mechanisms against oxidative stress-mediated injury with anti-atherogenic effects in the vasculature (Elbirt and Bonkovsky, 1999; Yet *et al.*, 2003). The antioxidant activity of HO-1 derives not only from the elimination of pro-oxidant haem but also from the biological activities of its reaction products. Bilirubin, at physiological plasma concentrations, exerts strong antioxidant effects by inhibiting oxidation of low density lipoprotein (LDL) and scavenging a variety of active oxygen species. High normal plasma levels of bilirubin were reported to be inversely related to atherogenic risk and to provide protection against endothelial damage (Baranano *et al.*, 2002; Novotny and Vitek, 2003; Kawamura *et al.*, 2005). Another product of HO activivty, CO, has been recognized as an endogenous mediator that regulates vascular functions. It has mild vasodilatory and antiplatelet aggregating properties through its action on guanylyl cyclase (Ishikawa, 2003). Furthermore, CO has potent anti-inflammatory effects and plays

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a major anti-proliferative role by inhibiting the synthesis of growth factors from vascular cells (Togane *et al.*, 2000; Peyton *et al.*, 2002). While several previous studies demonstrated that nitric oxide (NO) is a determinant in the modulation of the activity of HO-1 leading to resistance of the endothelium to oxidative stress (Motterlini *et al.*, 1996; Durante *et al.*, 1997; Foresti and Motterlini, 1999; Polte *et al.*, 2000; Foresti *et al.*, 2003), others have reported that NO inhibits HO-1 activity (Wang *et al.*, 2003).

Statins (HMG-CoA reductase inhibitors) are now widely prescribed to patients with ischaemic heart disease (Werner *et al.*, 2002). Besides the therapeutic use in hyperlipidaemia, the antioxidant, anti-inflammatory and immunomodulatory benefits of statins have been reported, although the mechanisms underlying these benefits are not yet completely understood (Wierzbicki *et al.*, 2003; Liao, 2005). The present study was designed to elucidate the role of NO, peroxynitrite ($ONOO^-$) and HO-1 in the anti-atherogenic effect of statins. We postulated that a vasoprotective effect of statins might be based on increased NO concentration, induction of HO-1 and decreased $ONOO^-$. This study indicated that HO-1 was a molecular target for statins through the NO pathway and that HO-1 favourably affected the NO/$ONOO^-$ balance and the functional activity of endothelial nitric oxide synthase (eNOS).

Methods

Animal protocol

All procedures used in this study were approved by the Ohio University Animal Care Committee. The study was carried out on adult male New Zealand white rabbits (Harlan Industries, Indianapolis, IN). The animals (1.5–2 kg) were randomized into 10 groups of six animals each: one control group and other groups which were maintained on a 2% cholesterol-containing diet for 2 months. After 1 month on this diet, the animals were treated as follow: non-treated group; atorvastatin group; simvastatin group; atorvastatin + L-NAME (eNOS inhibitor) group; simvastatin + L-NAME group; L-arginine (eNOS substrate) group; atorvastatin + tin-protoporphyrin (SnPP, HO-1 inhibitor) group; simvastatin + SnPP group; and a haemin (HO-1 inducer) group. The doses of statins (3 $mg \cdot kg^{-1} \cdot day^{-1}$, orally), L-NAME (15 $mg \cdot kg^{-1} \cdot day^{-1}$, orally), L-arginine (1.5 $mg \cdot kg^{-1} \cdot day^{-1}$, orally), SnPP (7.5 $mg \cdot kg^{-1} \cdot day^{-1}$, i.v.) and haemin (25 $mg \cdot kg^{-1} \cdot day^{-1}$, i.v.) were chosen from published data (Le Tourneau *et al.*, 1999; Verd *et al.*, 1999; Ishikawa *et al.*, 2001a,b).

Collection of blood samples and isolation of aortas

The animals were anaesthetized with a mixture of ketamine/xylazine (35/2 $mg \cdot kg^{-1}$, i.p.) and then given an overdose of pentothal (120 $mg \cdot kg^{-1}$). Blood was taken through cardiac puncture and plasma was used for biochemical assay of lipid peroxides. The thoracic aorta was excised, placed into cold (4°C) phosphate buffered saline (PBS) and cleaned of adherent connective tissue under a dissection microscope (Leica DME, Miller Precision Optical Instruments, Philadelphia, PA). Each isolated aorta was then cut into two segments along the long vessel axis. One of these segments of aorta was used for electrochemical measurements of NO and $ONOO^-$ and the other segment of the aorta for measurements of eNOS, HO-1 protein expression (by Western blotting analysis), HO-1 activity and total protein concentrations.

Determination of lipid peroxides

The total amount of lipid peroxides in plasma was assayed by the thiobarbituric acid method described previously (Ohkawa *et al.*, 1979).

Nitric oxide and peroxynitrite measurements

Measurements of NO and $ONOO^-$ were performed with two electrochemical nanosensors, their design was based on previously developed and well-characterized chemically modified carbon-fibre technology (Malinski and Taha, 1992). The sensors operated in a three-electrode system, consisting of the sensor working electrode, a platinum wire counter electrode, and a standard reference electrode. The current proportional to NO or $ONOO^-$ concentration was measured simultaneously by sensors (300 ± 30 nm diameter) at constant potential of 0.63 and –0.40 V respectively. An open segment of the aorta was cut into six samples (about 3 × 5 mm each). Each sample was placed in an organ bath filled with 2 mL PBS and gassed continuously with 95% O_2 : 5% CO_2 (flow rate, 2 $mL \cdot min^{-1}$) at 37°C. The sample was immobilized with polyethylene glycol.

A module of NO and $ONOO^-$ sensors was placed in close proximity (6 ± 2 μm) of the surface of the endothelium with the help of a computer-controlled micromanipulator. Then, gas flow (O_2 : CO_2) was terminated and NO and $ONOO^-$ concentrations were measured after injection of a receptor independent stimulus for eNOS agonist, the calcium ionophore A23187 (1 $\mu mol \cdot L^{-1}$). Linear calibration curves were constructed for each sensor from 10 $nmol \cdot L^{-1}$ to 1 $\mu mol \cdot L^{-1}$ before and after measurements with aliquots of NO (1.76 $mmol \cdot L^{-1}$) and $ONOO^-$ (180 $mmol \cdot L^{-1}$) standard solutions, prepared according to previously described methods (Beckman and Crow, 1996).

Determination of eNOS and HO-1 protein expression

eNOS and HO-1 expressions were analysed by using Western blotting methods. Immunoblotting was performed with the use of a monoclonal antibody to eNOS (Sigma, USA) at a dilution of 1:3000 or monoclonal antibody to HO-1 (Calbiochem) at a dilution of 1:2000 in a non-fat milk/PBS. The membrane was subsequently probed with a secondary anti-mouse antibody conjugated to horseradish peroxidase (Amersham Life Sciences) at a dilution of 1:2000 and developed with chemiluminescence. The membrane was then exposed to X-ray film (Kodak), which was subsequently developed. Densitometry was performed by use of the NIH Image software.

Determination of total HO enzyme activity

Haem oxygenase activity was determined in microsomal fractions from tissue homogenates of aortas by monitoring the

conversion of haem into bilirubin. Bilirubin was measured spectrophotometrically according to the method described by Yet *et al*. (1997). Aortas were isolated from the animals, immediately washed, stripped and homogenized in 2× volumes of ice-cold homogenization buffer containing complete protease inhibitors. The homogenate was centrifuged at 10 000× *g* for 15 min at 4°C. The supernatant was further centrifuged at 100 000× *g* for 1 h at 4°C and the aortic microsomal pellets were resuspended in 1 mL of 100 mmol·L^{-1} PBS (pH 7.4) containing complete protease inhibitors. The liver cytosol supernatant fraction from the control animal served as the source of biliverdin reductase. Briefly, the liver from normal control rabbit was homogenized in 100 mmol·L^{-1} PBS (pH 7.4) containing protease inhibitors and centrifugation at 18 000× *g* for 15 min followed by centrifugation at 100 000× *g* for 1 h. The resulting supernatant containing biliverdin reductase was used in the experiment.

A reaction mixture (1 mL) containing the aortic microsomal fraction of the samples (1 mg protein), 33 µmol·L^{-1} haemin (as a substrate for the enzyme), 2 mmol·L^{-1} glucose-6-phosphate, 0.25 U glucose-6-phosphate dehydrogenase, 0.8 mmol·L^{-1} NADPH and 2 mg protein of the prepared liver cytosol (as a source of biliverdin reductase) was incubated at 37°C for 1 h in the dark. The reaction mixture without NADPH generating system served as a blank. At the end of the incubation period, the reaction was stopped by chilling on ice and the bilirubin was extracted by addition of 1 mL chloroform then shaken and centrifuged at 10 000× *g* for 10 min. The absorbance of the chloroform layer was spectrophotometrically measured at 464 and 530 nmol·L^{-1} (Beckmann DU-640 spectrophotometer) and the extracted bilirubin was calculated by the difference in absorbance between 464 and 530 nm (extinction coefficient, ε = 40 mmol·L^{-1}·cm^{-1} for bilirubin in chloroform). HO enzyme activity was expressed as nanomoles of bilirubin formed per milligram of protein per hour (nmol bilirubin mg protein^{-1} h^{-1}).

Statistical analysis

Data are expressed as mean ± SEM, with a value of $P < 0.05$ considered statistically significant. Statistical evaluation was performed by ANOVA, followed by Student's *t*-test. All analysis was made with the statistical software Microcal Origin (Microcal Software, Inc., Northampton, MA).

Results

Effect on endothelial haem oxygenase-1 expression and activity

The expression of HO-1 increased (Figure 1A) with a parallel increase in total HO activity (Figure 2A) in the 'non-treated' atherosclerotic group when compared with the control group. However, treatment of the atherosclerotic groups with statins significantly increased HO-1 expression and the total HO activity when compared with the 'non-treated' atherosclerotic group (Figures 1A and 2A). Interestingly, treatment with L-NAME added to the statin decreased the expression of HO-1 with parallel decreases in the activity of the enzyme, compared with the 'non-treated' atherosclerotic group. In contrast, treatment with L-arginine showed an appreciable

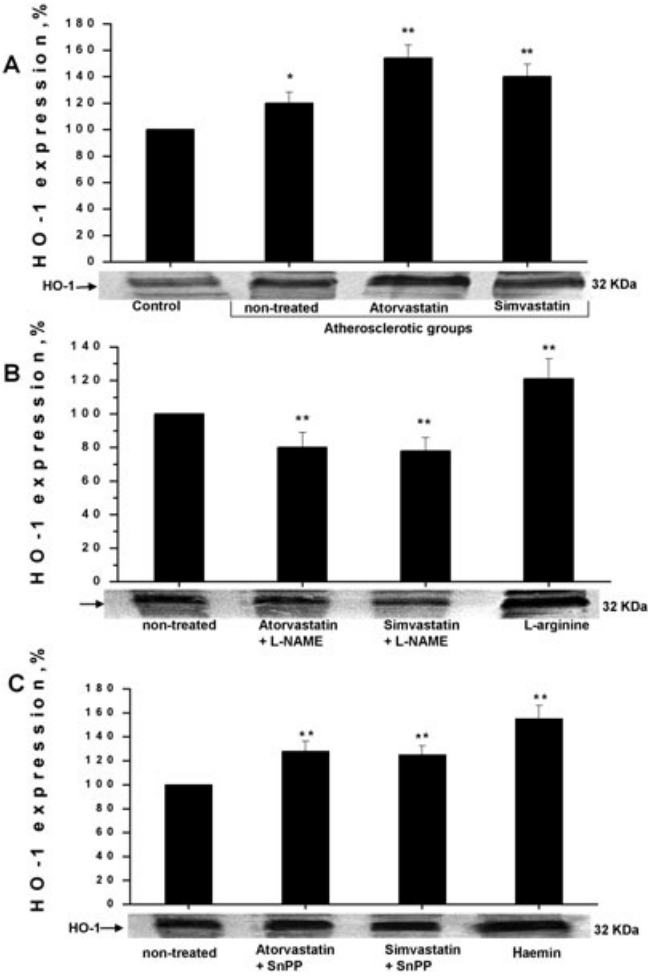

Figure 1 Immunoblots showing the expression of HO-1 protein from isolated aortas of: (A) normal and atherosclerotic rabbits treated with atorvastatin or simvastatin for 1 month; (B) atherosclerotic groups either co-treated with L-NAME and atorvastatin/simvastatin or treated with L-arginine; (C) atherosclerotic group either co-treated with SnPP and atorvastatin/simvastatin or treated with haemin. Representative Western blots and the bar graph show analysis of four separate experiments; *$P < 0.05$ versus control and **$P < 0.05$ versus atherosclerotic group.

increase in HO-1 expression (Figure 1B) and total HO activity (Figure 2B) when compared with the 'non-treated' atherosclerotic group. Adding SnPP to the treatment with statins increased the expression of HO-1 (Figure 1C) and significantly inhibited its activity (Figure 2B), compared with the 'non-treated' atherosclerotic group. The highest increase in HO-1 expression (Figure 1C) and total HO activity (Figure 2B) were observed after treatment with haemin, relative to the 'non-treated' atherosclerotic group.

Endothelial release of nitric oxide and peroxynitrite

Amperometric curves (current proportional to NO or $ONOO^-$ concentration vs. time) are presented in Figure 3A and B respectively; and mean NO and $ONOO^-$ peak concentrations are shown in Figure 3C. Compared with the results of stimulation with the calcium ionophore in the control animals, the peak NO concentration was dramatically decreased while the

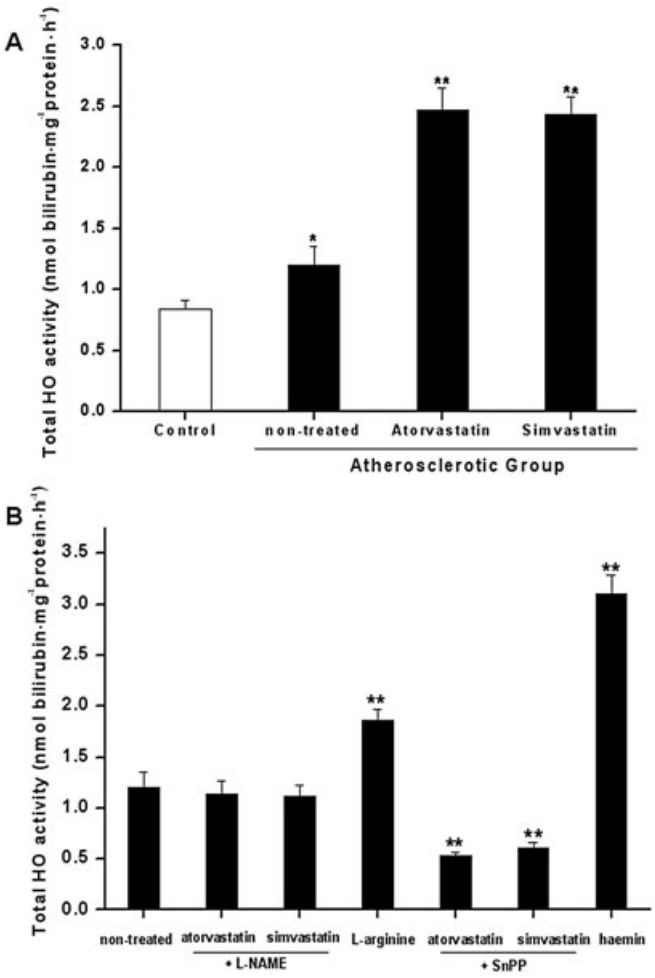

Figure 2 Total activity of HO, measured in the aortas of the different animal groups: (A) normal animals (control) and atherosclerotic animals treated with atorvastin or simvastatin; (B) atherosclerotic groups either treated with statins (atorvastatin or simvastatin) and L-NAME or SnPP or treated with L-arginine or haemin. $n = 6$ for each bar; $^{*}P < 0.05$ versus control group and $^{**}P < 0.05$ versus atherosclerotic group.

peak $ONOO^-$ concentration was increased in the 'non-treated' atherosclerotic rabbits. Treatment of atherosclerotic rabbits with statins partially restored the NO output and significantly reduced $ONOO^-$ release. The ratio of NO/$ONOO^-$ concentrations ($k = [NO]/[ONOO^-]$) was used in this study as an indicator of endothelial dysfunction. At high levels of bioavailable NO and/or low levels of cytotoxic $ONOO^-$, the k value is high indicating normal endothelial function. In normal functioning aortic endothelial cells, the k value was 2.3 (Figure 3D) and in the 'non-treated' atherosclerotic group, there was a threefold reduction in k value, indicating a highly dysfunctional endothelium. However, treatment with statins significantly restored this ratio. Adding L-NAME to the statins prevented the changes in peak NO and $ONOO^-$ concentrations induced by the statins alone and these values (Figure 4A) were now not different from those in the 'non-treated' atherosclerotic group. In contrast, treatment with L-arginine appreciably increased NO release with a concomitant decrease in $ONOO^-$ concentration (Figure 4A). The ratio of $[NO]/[ONOO^-]$ from the groups of animals in Figure 4A are shown in Figure 4C and emphasize the marked reduction in this ratio for statins + L-NAME groups with no significant difference from the ratio in the 'non-treated' atherosclerotic group. Treatment with L-arginine noticeably increased this ratio and appreciably restored the endothelial function. In Figure 4B the effects of adding SnPP to the statins is shown. SnPP did not affect the increase in peak NO induced by the statins but it did repress the decrease in $ONOO^-$ release by statins (compare Figures 3C and 4B). In contrast and a more interesting finding is that, in the atherosclerotic group treated with haemin, the NO peak concentration was partly but significantly increased with a parallel decrease in $ONOO^-$ release, all relative to the 'non-treated' atherosclerotic group. In the assessment of the corresponding $[NO]/[ONOO^-]$ ratios (Figure 4D), treatment with statins + SnPP did not significantly improve this ratio, while there was a marked increase in this ratio between the haemin-treated group and the 'non-treated' atherosclerotic group.

Endothelial nitric oxide synthase expression

There was a marked decreases in eNOS expression in the 'non-treated' atherosclerotic group compared with the control group (Figure 5A) and treatment of the atherosclerotic groups with statins partly reversed this decrease. This reversal of effects on eNOS expression by statins was partly dependent on NO and HO-1. Combined treatment of atherosclerotic groups with statins + L-NAME significantly increased the expression of eNOS enzyme, compared with the 'non-treated' atherosclerotic group (Figure 5B). Moreover, L-arginine produced a significant increase of eNOS expression, compared with the 'non-treated' atherosclerotic group and treatment with statins + SnPP (Figure 5C) also increased the expression of eNOS, to about the same extent as treatment with L-arginine. Similarly, treatment of the atherosclerotic animals with haemin (Figure 5C) produced significant increase in eNOS expression, relative to that in the 'non-treated' atherosclerotic group. A slight ($25 \pm 5\%$) decrease in inducible nitric oxide synthase (iNOS) expression was observed after treatment of atherosclerotic groups with statins (data not shown).

Plasma malondialdehyde (MDA)

Figure 6A shows that MDA concentration increased significantly in the 'non-treated' atherosclerotic group, compared with the basal level in the control group. Treatment of the atherosclerotic group with statins resulted in a remarkable decrease in the MDA level. The per cent change in $ONOO^-$ concentration showed a linear correlation with the increase in MDA concentration (Figure 6B). Addition of either L-NAME or SnPP to the treatment with statins produced no significant changes of plasma levels of MDA, compared with 'non-treated' atherosclerotic group, abolishing the effects of the statins. Interestingly, treatment with either L-arginine or haemin produced partial, but significant, decreases in MDA levels, compared with the 'non-treated' atherosclerotic group.

Histopathological examination of the aortic arch

The histopathological assessments of the aortic arch from the control group revealed a normal structure. The endot-

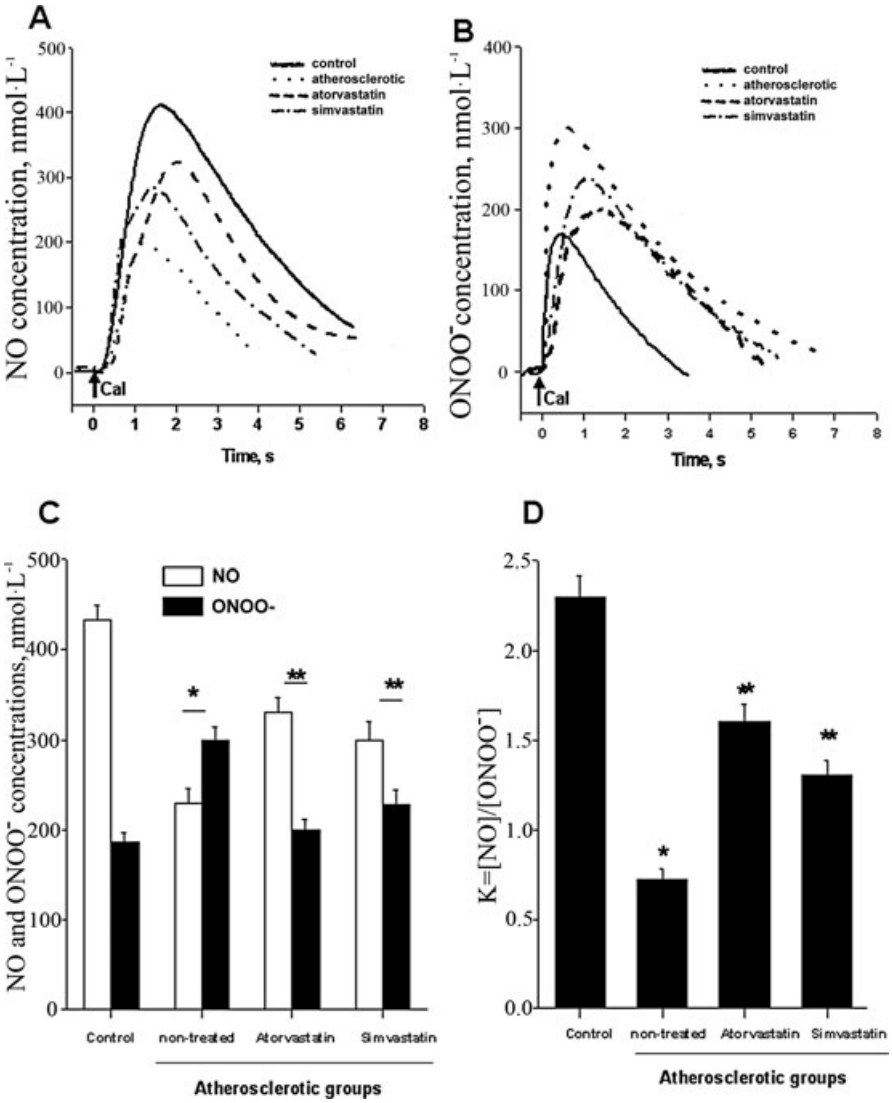

Figure 3 Typical amperograms of the changes in NO (A) and $ONOO^-$ (B) concentrations, recorded by nanosensors from isolated aortas of normal and atherosclerotic rabbits treated with atorvastatin or simvastatin for 1 month. The release of NO and $ONOO^-$ were stimulated by 1 $\mu mol \cdot L^{-1}$ calcium ionophore A23187 (Cal at arrow) in control group and atherosclerotic groups, non-treated or treated with atorvastatin or simvastatin. (C) Calcium ionophore-stimulated maximal NO and $ONOO^-$ release, and (D) ratio of NO concentration to $ONOO^-$ concentration of the above corresponding groups. $n = 6$ for each bar; $^*P < 0.05$ versus control group and $^{**}P < 0.05$ versus atherosclerotic group.

helium was found to form a continuous layer with no particular changes in the intima and there was no accumulation of lipids in the aortic wall (Figure 7A). In contrast, aortas of all rabbits fed on a high-cholesterol diet showed significant fatty streaks and pathological lesions of different intensities between groups. The aortae from the atherosclerotic group presented a thickening of the intima that occurred throughout the whole surface of the examined specimens. Numerous foam cells, lipid droplets and elastic fibres were also found. However, treatment with statins showed a less pronounced deposition of fatty streaks than the non-treated group and the intima was thickened but the athermanous plaques were never as high and wide as the plaques in the aorta of the non-treated animals. The significant increase in vessel wall (VW) thickness in the 'non-treated' atherosclerotic group (Figure 7B) was partly prevented by atorvastatin treatment, although still higher than in the control group. The combination treatment of statin + L-NAME produced aortae with fatty streaks and atherosclerotic lesions larger than those observed with animals treated with statins alone. Qualitatively similar histopathological changes were observed in the aortic arch from animals co-treated with statins + SnPP. However, treatment with either L-arginine or haemin reduced the thickening of the intima with less foam cells and lipid droplets between the first elastic membranes lying under the intima. The mean values for VW (Figure 8B) confirmed that co-treatment with statins and either L-NAME or SnPP did not improve VW, compared with the 'non-treated' atherosclerotic group. In contrast, the VW in either L-arginine or haemin-treated groups was appreciably lowered relative to the 'non-treated' atherosclerotic group.

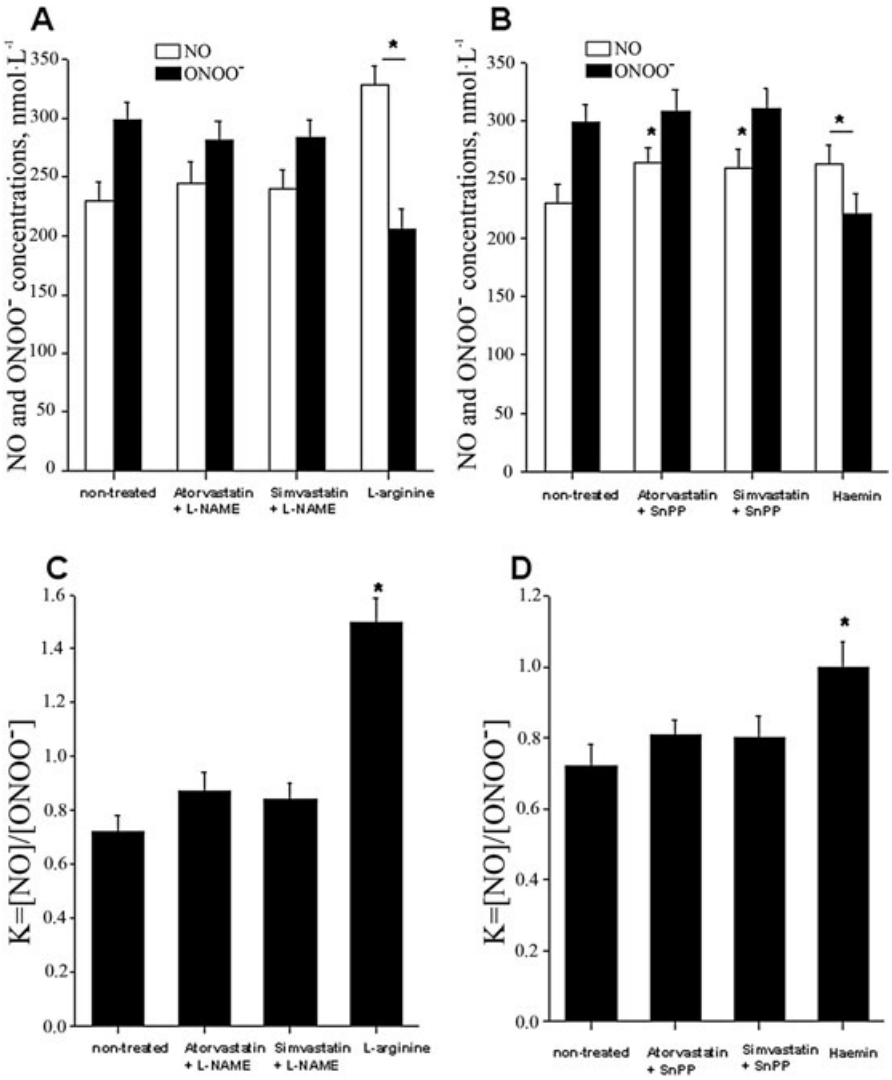

Figure 4 Calcium ionophore-stimulated maximal NO and $ONOO^-$ release from isolated aortas of atherosclerotic groups either co-treated with L-NAME and atorvastatin/simvastatin or treated with L-arginine (A), and from isolated aortas of atherosclerotic group either co-treated with SnPP and atorvastatin/simvastatin or treated with haemin (B). In C & D are shown the ratios of NO concentration to $ONOO^-$ concentration, from the treatment groups shown in A and B. $n = 6$ for each bar; $^*P < 0.05$ versus atherosclerotic group.

Discussion and conclusion

This study sheds new light on the interaction of statins with the eNOS/HO-1 pathways and emphasizes a regulatory interaction between NO and HO-1 to mediate the vasculoprotective actions of statins during atherosclerosis. Restoration of eNOS functional activity with higher [NO]/[$ONOO^-$] ratios and decreased lipid oxidation were probably the most common mechanisms by which HO-1 was able to produce its anti-atherosclerotic effects.

Effect of statins on HO-1 expression and activity through NO, as a signalling molecule

The results from this work indicate that HO-1 was clearly expressed, albeit at a low level, in the arteries of atherosclerotic rabbits, which could be a process of adaptation to hypercholesterolaemia. We postulated that under pathophysiological conditions affecting vascular functions, such as atherosclerosis, increased HO-1 would play a beneficial role by affecting anti-inflammatory and vasodilatory mechanisms. It has already been suggested that induction of HO-1 during atherosclerosis might ameliorate oxidative stress and represent an adaptive response to hypercholesterolaemia (Nakayama *et al.*, 2001).

We characterized the effects of statins on HO-1 expression and activity in aortic tissues of atherosclerotic rabbits and the results demonstrated that the antioxidant enzyme, HO-1, is an intracellular site of action for statins. It was reported that statins induce *in vivo* expression of HO-1, which appears to be a class effect of statins and occurs not only in the vasculature but also extravascularly (Hsu *et al.*, 2006). Moreover, a study by Lee *et al.* (2004) stated that simvastatin up-regulated HO-1 expression in vascular smooth muscle cells, while Uchiyama *et al.* (2006) reported that simvastatin enhanced HO-1 expression in aortic endothelial

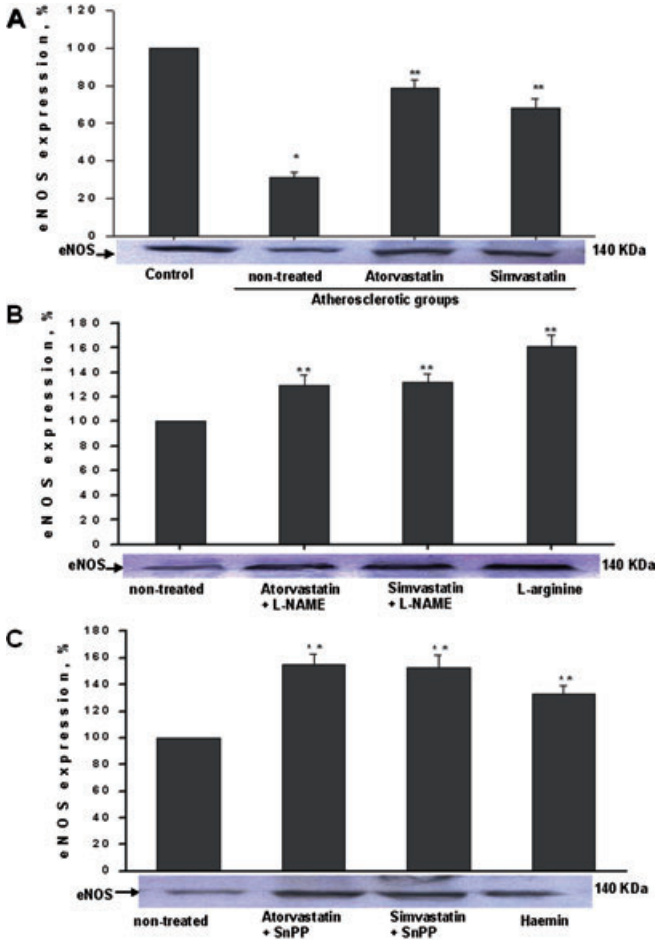

Figure 5 Immunoblots showing the expression of eNOS protein from isolated aortas of: (A) normal and atherosclerotic rabbits treated with atorvastatin or simvastatin for 1 month; (B) atherosclerotic groups either co-treated with L-NAME and atorvastatin/simvastatin or treated with L-arginine; (C) atherosclerotic group either co-treated with SnPP and atorvastatin/simvastatin or treated with haemin. Representative Western blot and bar graph show analysis of four separate experiments; $*P < 0.05$ versus control group and $**P < 0.05$ versus atherosclerotic group.

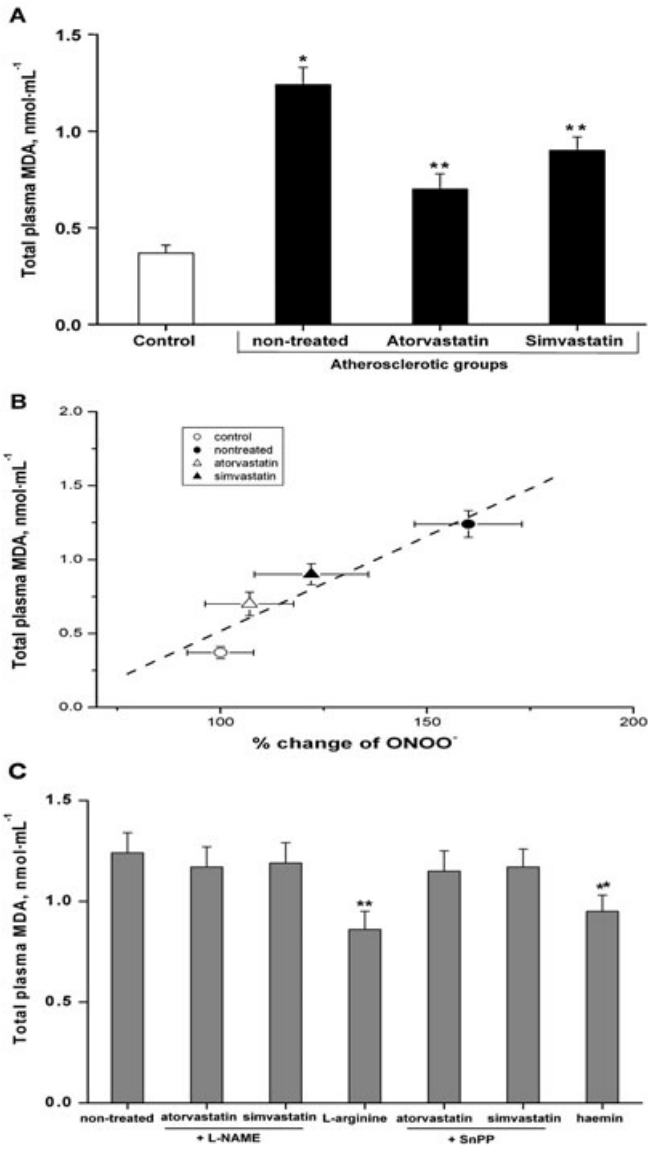

Figure 6 Plasma malondialdehyde (MDA) levels on the different treatment groups: (A) control group and atherosclerotic animals treated with atorvastin or simvastatin; (B) MDA levels plotted against % changes (vs. control) in $ONOO^-$ production in control, non-treated atherosclerotic group and atherosclerotic group treated with atorvastatin and simvastatin; (C) Atherosclerotic groups either co-treated with statins (atorvastatin or simvastatin) and L-NAME or SnPP or treated with L-arginine or haemin. $n = 6$ for each bar; $*P < 0.05$ versus control group and $**P < 0.05$ versus atherosclerotic group.

cells. In contrast, others showed that, the protective effect of statins at their pharmacological concentrations is not mediated by enhancement of HO-1 expression or activity but may involve eNOS expression (Loboda *et al.*, 2006). We believe that the results from our study resolve some of the discrepancies regarding the effects of statins on HO-1 expression, especially when we explored the role of NO in this induction. Interestingly, L-NAME, an inhibitor of eNOS, abolished the HO-1 expression and activity stimulated by statins. In support for this, we found that treatment of atherosclerotic group with L-arginine augmented the increase in HO-1 expression and activity. Therefore, a parallel conclusion can be drawn regarding the involvement of endogenous NO in statin-stimulated HO-1 expression. This has been confirmed by others and has led to speculation that HO-1 is in fact responsible for cytoprotective effects attributed to NO (Motterlini *et al.*, 1996; Foresti and Motterlini, 1999; Polte *et al.*, 2000; Foresti *et al.*, 2003). NO causes the release of free haem from haem proteins and because free haem is known to transcriptionally up-regulate its own degradation by HO, the release of haem by NO may induce HO-1 expression (Durante *et al.*, 1997; Foresti *et al.*, 2006). Moreover, it has been demonstrated that cGMP is a crucial mediator in the endothelial regulation of HO-1 expression and that endothelial protection afforded by the NO/cGMP system is causally related to the induction of HO-1 (Polte *et al.*, 2000). Based on these previous studies and considering the outcome of our experiments, it is plausible that the increased expression of HO-1 by statins could be mediated through NO and might explain some pleiotropic effects of these drugs.

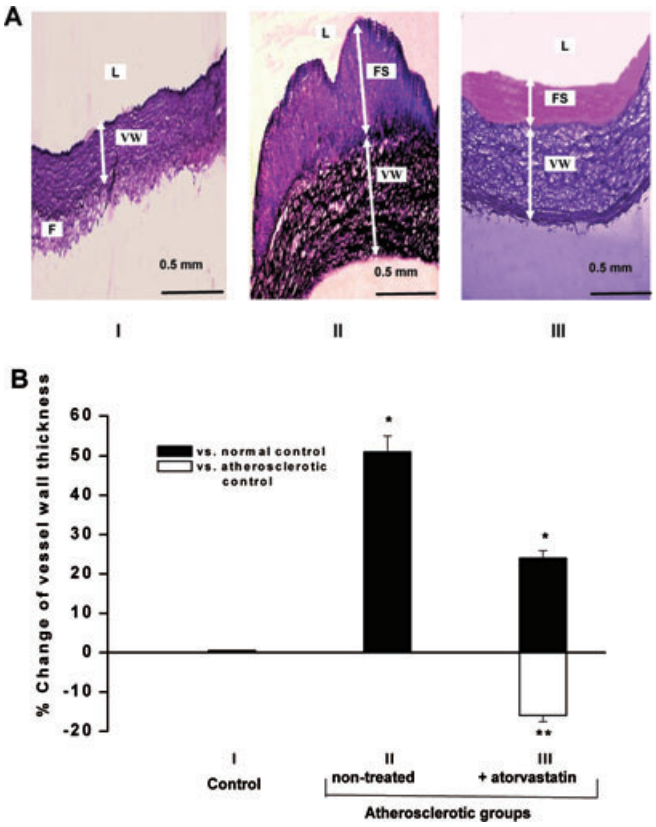

Figure 7 Representative microscopic analysis of lesion area of the aortic arch sections from control and atherosclerotic rabbits treated with atorvastatin for 1 month and stained with haematoxylin and eosin (×100); L, lumen; FS, fatty streak; VW, vessel wall; F, adventitial fatty tissue. (B) % change of the vessel wall thickness of the aortic arch from normal control or from atherosclerotic control was quantitated using an image analysis system. $n = 6$ for each bar; $^{*}P < 0.05$ versus control group and $^{**}P < 0.05$ versus atherosclerotic group.

Role of HO-1 on statin-restored [NO]/[ONOO⁻] ratio

We postulated that induction of HO-1 may play a significant role in the antioxidant and anti-inflammatory effects of statins. Accordingly, we investigated the effect of HO-1 in statin-induced restoration of [NO]/[ONOO⁻] ratios and eNOS functional activity. Atherosclerotic animals were co-treated with statins and SnPP to study how much blocking of HO-1 could affect the vasculoprotective actions of statins. In addition, we treated atherosclerotic animals with haemin (HO-1 inducer) and compared the effects from HO-1 induction with that obtained from statins. This study indicated that, in animals treated with statins, inhibition of HO-1 by SnPP did not affect the increase in NO release and eNOS expression. However, it clearly abolished the decrease in $ONOO^-$ level. As one of the antioxidative roles of statins is shifting the [NO]/[ONOO⁻] balance towards NO with a direct decrease of $ONOO^-$ levels; the increase in $ONOO^-$ level by SnPP during statins therapy, confirmed that HO-1 plays an important role in mediating the antioxidative effect of statins. This HO-1 inhibitor, SnPP, increased the oxidathve stress as reflected by the increase in $ONOO^-$ and overall decrease in [NO]/[ONOO⁻] ratio. In contrast, haemin reduced $ONOO^-$ and oxidative stress and increased [NO]/[ONOO⁻] ratio in atherosclerotic animals. NO and $ONOO^-$ profiles after haemin treatment were similar to those after statin treatment. Thus, the HO-1 enzyme seems to act as an antioxidant mediator during atherosclerosis. These findings revealed a direct effect of HO-1 on increasing the [NO]/[ONOO⁻] ratio, as a result of improvement in the functional activity of eNOS enzyme. In support of this, Ahmed *et al.* (2005) and Turkseven *et al.* (2005) confirmed the vascular cytoprotective mechanism of HO-1 in diabetic rat models. They demonstrated that, while inhibition of HO-1 activity magnified O_2^- production, induction of HO-1 increased eNOS expression level with a concomitant increase in endothelial relaxation and a decrease in iNOS expression and O_2^- generation. Up-regulation of HO-1 expression renders endothelial cells resistant to oxidative stress by increasing other antioxidant genes, including superoxide dismutase (SOD) and catalase which in turn, decreases O_2^- production (Turkseven *et al.*, 2005). Moreover, up-regulation of HO-1 gene expression was shown to decrease the availability of the haem-mediated activation of nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase and O_2^- generation (Taille *et al.*, 2004). This could explain the antioxidant properties of HO-1 and the beneficial effects observed in several cardiovascular diseases in which involvement of oxidases is well described. Our findings suggest that the concentration of $ONOO^-$ (one of the main components of oxidative stress) decreased with an increase of HO-1 expression. Based on these findings, it is plausible to assume that HO-1 and its enzymic products are indeed of functional relevance and responsible, at least in part, for the observed antioxidant and anti-inflammatory effects of statins. Both membrane-bound eNOS and NAD(P)H, as well as mitochondria may contribute to the overall generation of $ONOO^-$ in endothelial cells (Wolin *et al.*, 2005). An increase in $ONOO^-$ formation in endothelium may also be partially attributed to the decrease of activity/expression of xanthine oxidase and/or SOD. The nanotechnological set-up used in this study is not suitable for measuring $ONOO^-$ directly in mitochondria without disturbing cell membranes and stimulating NO and O_2^- release. Therefore, these studies elucidate mainly $ONOO^-$ generation by membrane-bound eNOS and NAD(P)H.

Role of eNOS and HO-1 on lipid peroxidation

Increased lipid peroxidation is a characteristic feature of inflammatory vascular diseases (Cyrus *et al.*, 2001). From this study, a marked increase in MDA (index for lipid peroxidation) was observed in atherosclerotic animals. It appears that $ONOO^-$ must be involved in the oxidation of LDL and generation of oxidized lipids (Radi *et al.*, 1991) and it is more than two orders of magnitude more potent than H_2O_2 in catalysing lipid oxidation (Patel *et al.*, 1996). We demonstrated here that the decrease in the endothelial $ONOO^-$ production by statins correlated with a decrease in lipid peroxidation. Therefore, based on this study, we postulate that the beneficial effect of statins in lowering lipid peroxidation in atherosclerosis involves an increase in eNOS expression, enhanced production of NO, NO-dependent augmentation of HO-1 expression, activity, and increase of the [NO]/[ONOO⁻] ratio, similar to that observed in non-sclerotic animals. Activities of both enzymes, eNOS and HO-1, are necessary for the restoration of the [NO]/[ONOO⁻] balance. As we demonstrated here, either

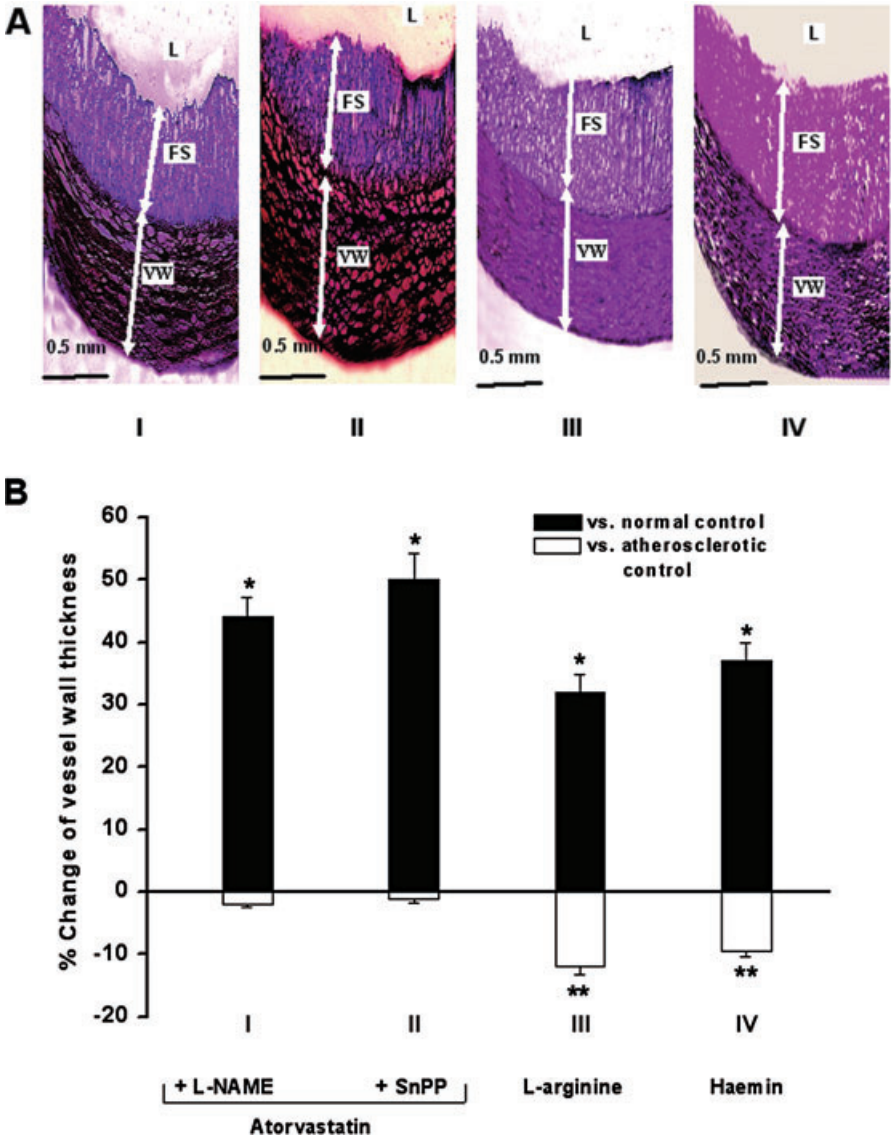

Figure 8 Representative microscopic analysis of lesion area of the aortic arch sections from atherosclerotic rabbits co-treated with atorvastatin and L-NAME or SnPP and treated with either L-arginine or haemin for one month; stained with haematoxylin and eosin (×100); L, lumen; FS, fatty streak; VW, vessel wall; F, adventitial fatty tissue. (B) % change of the vessel wall thickness of the aortic arch from normal control or atherosclerotic control was quantitated using an image analysis system. $n = 6$ for each bar; *$P < 0.05$ versus control group and **$P < 0.05$ versus atherosclerotic group.

inhibition of eNOS or HO-1 abolished the inhibitory effect of statins on lipid peroxidation while L-arginine or haemin decreased lipid peroxidation. Compatible with these findings, plasma lipid peroxides were elevated in the aortas of SnPP-treated atherosclerotic rabbits (Ishikawa *et al.*, 2001a). These authors postulated that the anti-atherogenic properties of HO-1 were mediated through the prevention of lipid peroxidation. The effect of L-arginine in decreasing MDA is most probably due to improved eNOS functional activity, increase in NO, increase in HO-1 expression and decrease of $ONOO^-$ generation. This corroborates with previous observations that L-arginine improved NO generation and increased HO-1 expression in hypercholesterolaemic rabbits (Le Tourneau *et al.*, 1999; Lanteri *et al.*, 2006). Similarly, the potential effect of haemin in decreasing plasma MDA is due to its induction of HO-1, which has strong antioxidative activity that was proven by others (Ishikawa *et al.*, 2001b).

Role of statins, NO and HO-1 on atherosclerotic lesion formation

High cholesterol diet for 2 months produced marked atherosclerotic changes in rabbits, which were evaluated by histopathological examination in the form of marked fatty streaks with multiple plaque lesions and increasing in the thickness of the VW. Statin treatment had led to reduction of the intima of the aorta with atheromatous plaques. The anti-atherogenic effects of statins could be attributed not only to its hypolipidaemic effects but also to its antioxidant activity through increasing the [NO]/[$ONOO^-$] ratio and lowering lipid peroxide formation. In support for this finding, while co-treatment

with statins and L-NAME produced hypolipidaemic effects (data not shown), it failed to produce any improvement in the VW thickness. Also, treatment with L-arginine decreased the formation of atherosclerotic lesions with some improvement in VW thickness.

The study presented here suggests that the increase in HO-1 expression and activity by statins may have an anti-atherosclerotic effect. Several lines of evidence supported this idea. First, inhibition of HO-1 activity, in statin + SnPP groups, prevented the regression of atherosclerotic plaques produced by statins treatment alone. This coincides with the results reported by others, that treatment of atherosclerotic rabbits with SnPP resulted in a significant progression of atherosclerotic lesion formation (Ishikawa *et al*., 2001a). Also, the haemin-treated group had smaller lesions with minor but significant improvements in VW thickness, when compared with the atherosclerotic group. The anti-atherogenic effect of haemin was ascribed to its induction of HO-1 that has anti-oxidant activity. The increase in eNOS functional activity with subsequent increase in bioavailable NO, an improvement of [NO]/[$ONOO^-$] balance and decrease of lipid oxidation are indeed responsible for such anti-atherogenic effect of HO-1 during the atherosclerotic process.

HO-1 plays an important role in many aspects of human physiology, as well as in pathological events such as atherosclerosis. The induction of HO-1 maybe considered a therapeutic strategy for treating inflammatory diseases. In this work, we have identified a novel mechanism related to induction of HO-1 by statins, which is mediated through the NO pathway and could explain some of pleiotropic effects of these drugs. Activation of the HO-1 pathway may be one of the important mechanisms by which statins inhibit inflammatory disorders and protect the endothelium from oxidative damage by increasing the [NO]/[$ONOO^-$] ratio with improvement of eNOS functional activity and decreasing lipid peroxide formation.

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Conflicts of interest

None.

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