Statins normalize vascular lysyl oxidase down-regulation induced by proatherogenic risk factors

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Aims Statins are lipid-lowering drugs widely used in the management of vascular diseases. Clinical and experimental evidence suggest that statins improve endothelial function by both cholesterol-lowering-dependent and -independent mechanisms. We have previously shown that endothelial dysfunction induced by risk factors and proinflammatory cytokines is associated with down-regulation of lysyl oxidase (LOX), a key enzyme modulating extracellular matrix maturation and vascular integrity. Our aim was to analyse whether statins could normalize LOX expression impaired by proatherogenic risk factors.

Methods and results We observed that pharmacological concentrations of statins (atorvastatin and simvastatin) modulated LOX transcriptional activity, counteracting the down-regulation of LOX (at the mRNA, protein, and activity level) caused by tumour necrosis factor-α (TNFα) in porcine, bovine, and human aortic endothelial cells. Geranylgeraniol but not farnesol reversed this effect, suggesting the involvement of geranylgeranylated proteins. In accordance, inhibitors of RhoA/Rho kinase also counteracted LOX down-regulation caused by TNFα, and over-expression of a RhoA dominant-negative mutant mimicked statin effects. Statins were also able to counteract the decrease in LOX expression produced by atherogenic concentrations of LDL by a similar mechanism and to partially prevent the increase in endothelial permeability elicited by these lipoproteins. Finally, in the in vivo porcine model of hypercholesterolaemia, we observed that statins abrogated the reduction of vascular LOX expression triggered by high plasma levels of LDL.

Conclusion These data indicate that statins normalize vascular LOX expression altered by atherogenic risk factors through a RhoA/Rho kinase-dependent mechanism. Thus, modulation of LOX by statins could contribute to vascular protection and to the cardiovascular risk reduction achieved by this therapy.

KEYWORDS Atherosclerosis; Endothelial function; Statins; Lysyl oxidase; Porcine model

1. Introduction Statins are drugs widely used in the management of vascular diseases. These drugs are competitive inhibitors of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. Statins have demonstrated clinical benefits in primary and secondary prevention of coronary heart disease; however, some of their vascular protective actions cannot be solely explained by their plasma cholesterol-lowering effects.1–5 There is a growing evidence that some effects of statins, including the improvement of endothelial function and their anti-inflammatory effects, are achieved before a significant reduction in plasma cholesterol levels occurs.6–10 Although the underlying molecular mechanisms are not fully understood, depletion of isoprenoid bioavailability and the consequent inactivation of small GTPases have been associated with these non-lipid-related effects.11 Lysyl oxidase (LOX) is an extracellular copper enzyme that catalyses the formation of lysine and hydroxylysine-derived cross-links in collagen and elastin chains. This activity is essential to ensure normal extracellular matrix assembly and determines its mechanical properties.12,13 LOX inhibition leads to connective tissue abnormalities and has been related with pathological processes including cardiovascular diseases.14,15 In this regard, we have previously reported that proatherogenic risk factors and proinflammatory cytokines that impair endothelial function decrease LOX expression/activity, effect that seems to be associated with a disturbance of endothelial barrier integrity.16–20

In this paper, we show that statins abolish the down-regulation of LOX produced by tumour necrosis factor-α (TNFα) and atherogenic concentrations of LDL (in vitro and
in vivo). Thus, LOX could be regarded as a new target gene of statins in the vascular wall.

2. Methods

A more detailed description of this section could be found in Supplemental material online.

2.1 Cell culture

Cell culture studies were performed in porcine, bovine, and human aortic endothelial cells (PAECs, BAECs, and HAECs, respectively). All the procedures were approved by the Reviewer Institutional Committee on Human Research of the Hospital of Santa Creu i Sant Pau that conforms to the Declaration of Helsinki.

2.2 LDL isolation

Human LDL were obtained from fresh plasma by sequential ultracentrifugation ($d = 1.019-1.063$ g/mL) as described previously. The study was approved by the Reviewer Institutional Committee on Human Research of the Hospital of Santa Creu i Sant Pau that conforms to the Declaration of Helsinki.

2.3 Real-time PCR

Total RNA was isolated using RNeasy™ (Qiagen). Quantification of LOX mRNA levels was performed by real-time PCR as described.

2.4 LOX activity

LOX activity was measured in the supernatant of endothelial cells by a high-sensitive fluorescent assay as described previously.

Figure 1  (A) Statins increase LOX mRNA levels and counteract the down-regulation of LOX induced by TNFα. PAECs were pre-treated with atorvastatin (ATOR) or simvastatin (SIM) 18 h before TNFα addition (1 ng/mL). After 21 h, LOX mRNA levels were evaluated by real-time PCR and were normalized by 18S RNA. Results come from three different experiments performed in triplicate and are expressed as mean ± SEM. (B) LOX mature protein levels were evaluated in cell supernatants by western blot. Unchanged levels of β-actin from cell lysates are shown as a loading control. Autoradiograms are representative of two independent experiments performed in duplicate. (C) LOX activity was measured in supernatants from PAECs pre-treated with SIM (0.1 μmol/L) and stimulated with TNFα (48 h). Results are mean ± SEM of four independent experiments performed by triplicate ($P < 0.05$: *, vs. control cells; †, vs. cells incubated with TNFα alone; #, vs. control cells and cells incubated with TNFα + SIM or TNFα + ATOR).
2.5 Western blot analysis

Proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters (Bio-Rad) as reported previously.\(^{21,25}\)

2.6 Transient transfection assay

Transient transfections were carried out in BAECs with a pGL3/LOX luciferase construct previously reported,\(^{18}\) a wild-type RhoA expression vector, a RhoA dominant-negative mutant (RhoAT19N), or the corresponding empty vector (pcDNA3) together with the pSVβ-gal.

2.7 Transendothelial exchange

Endothelial permeability was determined by the exchange of FITC-dextran (Mr 40 000; Sigma) through the endothelial monolayer in a Transwell\(^{11}\) system (Cultek) as described previously.\(^{17}\)

2.8 In vivo animal model

Female pigs (Landrace/Largewhite; body weight at initiation: 32 ± 4 kg) were randomized into two groups: normolipaemic animals (\(n = 5\)), fed with a normal chow, and hyperlipaemic animals (\(n = 15\)), fed with a cholesterol-rich diet (2% cholesterol, 1% cholic acid, and 20% beef tallow), for 100 days.\(^{21}\) The hyperlipaemic group was divided into three subgroups, one receiving simvastatin (2.5 mg/kg) (\(n = 5\)), one receiving pravastatin (5 mg/kg) (\(n = 5\)), and one treated with placebo (\(n = 5\)). At the end of the study, the animals were euthanized with a thiopental overdose.\(^{26}\) All procedures were in accordance with institutional guidelines and were approved by the Committee on Animal Research and Ethics of the Cardiovascular Research Center. These procedures conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.9 Immunohistochemistry

Sections of 5 μm were obtained from vessel samples as described previously.\(^{27}\) LOX immunohistochemistry was assessed with a rabbit anti-LOX antibody (kindly provided by Dr Kirschmann, Children’s Memorial Research Center, Chicago, IL, USA).

2.10 Statistical analysis

Data are expressed as mean ± SEM. Means were compared by one-factor ANOVA followed by Fisher PLSD to assess specific group differences. For in vivo studies, statistical differences between groups were analysed by the Mann-Whitney U test. Differences were considered significant at \(P < 0.05\).

3. Results

3.1 Statins increase LOX mRNA levels and counteract LOX inhibition produced by TNFα in endothelial cells

We evaluated whether statins affect TNFα-induced LOX inhibition. PAECs were pre-incubated with statins (atorvastatin or simvastatin) for 18 h followed by stimulation with TNFα (1 ng/mL, 21 h). As previously reported, TNFα decreased LOX expression (54%).\(^{19}\) Interestingly, increasing concentrations of atorvastatin and simvastatin, comparable to those used clinically (0.05–0.1 μmol/L), induced basal LOX mRNA levels and significantly abolished the negative effect of TNFα on LOX mRNA levels (Figure 1A). Similarly, simvastatin counteracted the down-regulation of LOX produced by TNFα in HAECS (see Supplementary material online, Figure S1). The up-regulation of LOX mRNA induced by statin treatment abolished the inhibition of LOX protein levels and activity produced by TNFα (Figure 1B and C). Although in PAEC statins alone slightly increased LOX mRNA levels, this effect was not observed on mature LOX protein or LOX activity levels. Finally, we ruled out that simvastatin effects were related to either TNFR1 or TNFR2 protein level modification (data not shown).

3.2 RhoA/ROCK pathway is involved in TNFα-stimulated LOX down-regulation

We tested the role of isoprenoid intermediates of the cholesterol biosynthetic pathway in the effect of statins. PAECs were incubated with geranylgeraniol and farnesol in the presence of simvastatin and TNFα. Geranylgeraniol but not farnesol reversed the effect of simvastatin on TNFα-induced LOX mRNA down-regulation, suggesting the involvement of geranylgeranylated proteins (Figure 2A). Consistent with these results, toxin B (an inhibitor of Rho proteins) and Y-27632 (a ROCK inhibitor) induced baseline LOX mRNA levels and also counteracted TNFα-induced LOX down-regulation (Figure 2B).
down-regulation of either LOX expression (Figure 2B) or LOX activity (see Supplementary material online, Figure S2), suggesting the involvement of the RhoA/ROCK cascade.

3.3 Statins modulate LOX transcriptional activity counteracting the inhibition caused by TNFα

To further characterize the molecular mechanism involved in the effect of statins, we analysed whether these drugs modulate LOX transcriptional activity. In transfection experiments, simvastatin dose-dependently increase LOX transcriptional activity and counteracted the decrease of promoter activity caused by TNFα (Figure 3A). Furthermore, in concert with mRNA data, geranylgeraniol but not farnesol abolished the effect of simvastatin on the TNFα-dependent response (Figure 3B). Accordingly, RhoA/ROCK pathway inhibitors (toxin B and Y-27632) counteracted the inhibitory effect of TNFα on LOX transcriptional activity (Figure 3C). Finally, over-expression of a RhoA dominant-negative mutant (RhoAΔN) abrogated the inhibition elicited by TNFα mimicking simvastatin effect (Figure 3D).

3.4 PKC is involved in the modulation of LOX expression via RhoA

We have previously reported the involvement of PKC on the down-regulation of LOX produced by TNFα. Moreover, small GTP-binding Rho proteins have been linked to PKC. Thus, we tested the effect of calphostin C (a PKC inhibitor) on RhoA-mediated LOX modulation. As shown in Figure 3D, co-transfection with a wild-type RhoA construct (w-RhoA) produced a decrease in LOX transcriptional activity. Interestingly, calphostin C partially counteracted the decrease in LOX transcriptional activity caused by this over-expression of RhoA. Altogether, these results support the involvement of RhoA/ROCK in the transcriptional regulation of LOX and suggest that PKC acts downstream RhoA in this event.

3.5 Statins and RhoA/ROCK inhibitors restore LOX expression and endothelial barrier function disturbed by LDL

In order to determine whether statins could also counteract LOX down-regulation produced by atherogenic concentrations of LDL, PAECs were pre-incubated with simvastatin

Figure 3 Statins abolish TNFα-mediated decrease in LOX transcriptional activity through a RhoA/ROCK-dependent mechanism. (A) BAECs were transfected with the pGL3/LOX construct and pre-treated with simvastatin (SIM; 0.1 μmol/L) in the presence or in the absence of TNFα (1 ng/mL, 18 h). Luciferase and β-galactosidase activities were determined as described in Section 2 (P < 0.05: *, vs. control cells; †, vs. cells incubated with TNFα alone; #, vs. control cells, and cells treated with TNFα + SIM 0.05). (B) BAECs were pre-treated with simvastatin alone (SIM; 0.1 μmol/L) or simvastatin plus geranylgeraniol (GER, 3 μmol/L) or farnesol (FAR, 3 μmol/L) before TNFα addition and LOX transcriptional activity was evaluated (P < 0.05: *, vs. control cells; †, vs. cells incubated with TNFα alone; ‡, vs. control cells, and cells treated with TNFα + SIM; #, vs. control cells, and cells treated with TNFα + SIM, TNFα + SIM + GER). (C) LOX transcriptional activity from BAECs transfected with the pGL3/LOX plasmid stimulated with TNFα in the presence or in the absence of toxin B (ToxB; 1 ng/mL) or Y-27632 (5 μmol/L) (P < 0.05: *, vs. control cells; †, vs. cells incubated with TNFα alone; #, vs. control cells, and cells treated with TNFα + Y-27632). (D) Luciferase activity from BAECs transfected with the pGL3/LOX construct and either a RhoA dominant-negative (RhoAΔN) or a wild-type RhoA (w-RhoA) treated or not with calphostin C (Calph; 200 nmol/L). Results, expressed as a per cent of controls, come from three independent experiments performed by triplicate (P < 0.05: *, vs. control cells; †, vs. cells incubated with TNFα alone; ‡, vs. w-RhoA alone; $, vs. control cells, and w-RhoA + Calph).
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4. Discussion

LOX dysregulation has been involved in the onset and progression of several cardiovascular diseases.\textsuperscript{14–16} Recently, we have shown that atherogenic risk factors such as hyperhomocysteinaemia and hypercholesterolaemia and pro-inflammatory cytokines down-regulate endothelial LOX and impair endothelial barrier integrity, suggesting a role for this enzyme in the pathogenesis of atherosclerosis.\textsuperscript{17–20} In this context, therefore, pharmacological control of LOX could be a potential therapeutic tool to limit atherosclerosis progression. The present study shows that statins abolish the down-regulation of endothelial LOX produced by pro-atherogenic stimuli and delineate the molecular mechanism underlying such effect.

Clinical and experimental evidence suggest that statins improve endothelial function\textsuperscript{29–31} by both lipid-lowering-dependent and -independent mechanisms.\textsuperscript{3,4,6,10} Indeed, besides their well-known effect on plasma cholesterol, HMG-CoA reductase inhibitors prevent the synthesis of isoprenoid intermediates needed for protein prenylation, a post-translational modification essential for the proper subcellular localization and biological function of small GTP-binding proteins such as RhoA.\textsuperscript{32} In the present study, we show that in endothelial cells, statins (simvastatin and atorvastatin) normalized LOX expression (mRNA and protein levels) down-regulated by TNF\textsubscript{α} preserving LOX levels (\texttextemdash\textasciitilde20\% inhibition) was observed in animals treated with statins; however, this lipid-lowering effect was not statistically significant (Table 1). Furthermore, neither hypercholesterolaemia nor statin treatment significantly modified plasma TNF\textsubscript{α} levels [normolipaemic animals (76.93 ± 15.87 pg/mL), hypercholesterolaemic animals (84.67 ± 24.04 pg/mL), and hypercholesterolaemic animals treated with simvastatin (89.38 ± 48.02 pg/mL) or pravastatin (75.57 ± 22.14 pg/mL)]. However, this atherogenic diet reduced LOX expression in porcine abdominal aorta as we had described previously.\textsuperscript{17} Interestingly, the down-regulation of LOX mRNA levels observed in hypercholesterolaemic animals was partially counteracted by statin treatment (either simvastatin or pravastatin) (Figure 5A). Moreover, immunohistochemical analysis showed that LOX immunostaining, which is clearly observed in the luminal endothelium from healthy vessels, is decreased by hypercholesterolaemia, whereas statin treatment (simvastatin or pravastatin) restores a similar pattern to that observed in normolipaemic animals (Figure 5B).

Table 1 Plasma lipid profile in pigs under normolipaemia or hyperlipaemia with or without statin treatment

<table>
<thead>
<tr>
<th></th>
<th>Total cholesterol (mg/dL)</th>
<th>LDL-cholesterol (mg/dL)</th>
</tr>
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<tbody>
<tr>
<td>Normolipaemia</td>
<td>77.9 ± 9.7</td>
<td>45.6 ± 12.9</td>
</tr>
<tr>
<td>Hyperlipaemia</td>
<td>589.8 ± 141.2*</td>
<td>443.9 ± 38.34*</td>
</tr>
<tr>
<td>Hyperlipaemia + simvastatin</td>
<td>452.5 ± 142.1*</td>
<td>354.6 ± 144*</td>
</tr>
<tr>
<td>Hyperlipaemia + pravastatin</td>
<td>431.9 ± 97.6*</td>
<td>345.2 ± 96.7*</td>
</tr>
</tbody>
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Results are mean ± SD.

*\textit{P} < 0.05 vs. normolipaemic animals.
activity. In fact, statins increased basal LOX mRNA levels and LOX transcriptional activity, although they did not significantly modify LOX activity or protein levels. These data suggest that statins interfere cellular mechanisms that negatively regulate LOX expression leading to the increase in basal LOX expression and counteracting the inhibitory effect of TNFα (or LDL) on LOX expression. The ability of statins to modulate 'per se' gene expression thereby counteracting the deleterious effects exerted by proatherogenic stimuli has been shown previously. The effect of statins was reversed by geranylgeraniol but not by farnesol and was mimicked by specific inhibitors of RhoA/ROCK (toxin B and Y-27632) and a RhoA dominant-negative. Thus, LOX expression is negatively regulated by the RhoA/ROCK pathway and thereby inhibition of this pathway promotes LOX expression. Like statins, both toxin B and Y-27632 up-regulated basal LOX mRNA levels but did not significantly modify LOX activity. Previous studies have shown that LOX activity could not reflect actual changes in LOX mRNA. The complex post-translational processing of LOX in endoplasmic reticulum/Golgi, which includes cleavage of signal peptide, protein glycosylation, addition of copper and lysine tyrosylquinone cofactor formation, and its final extracellular proteolytic processing by bone morphogenetic protein-1 to yield the active form, could explain, at least in a part, that changes in LOX activity do not quantitatively reflect those of LOX mRNA levels. Finally, our results suggest that PKC is a downstream effector of RhoA involved in the regulation of LOX by TNFα, and on the basis of our previous data, either PKCα or β could be involved in this effect. This is in agreement with the well-established activation of both PKC and RhoA by TNFα and with previous reports showing a cross-talk between RhoA and PKC in endothelial cells. In fact, inhibition of Rho abrogates PKC activation in endothelial cells (HUVECs and PAECs) and similarly, the blockade of Rho isoprenylation by atorvastatin interferes the RhoA/PKC cross-talk. The inhibition of RhoA geranylgeranylation is considered key for the improvement of endothelial function by statins. Indeed, in endothelial cells, statins up-regulate eNOS expression and decrease pre-proendothelin-1 transcription through a RhoA-depnding mechanism. Multiple studies have shown that Rho GTPases participate in TNFα signalling and that RhoA is involved in several deleterious effects exerted by LDL on endothelial cells. In this context, we observe that LDL also down-regulates LOX through a RhoA/ROCK-depending mechanism and that simvastatin reverses both LOX down-regulation

Figure 5  Vascular LOX down-regulation caused by hypercholesterolaemia is prevented by statins. (A) LOX mRNA levels in the vascular wall from abdominal aorta samples corresponding to normolipaemic pigs (NORMO) or animals fed with a hyperlipaemic diet (HYPER) treated or not with simvastatin (HYPER + SIM) or pravastatin (HYPER + PRA) (P < 0.05; *, vs. NORMO; †, vs. HYPER). (B) Masson Trichromic staining (upper panel) and LOX expression (lower panel) in abdominal aorta samples from pigs under a normolipaemic (NORMO) or hyperlipaemic (HYPER) diet either untreated or treated with simvastatin (HYPER + SIM) or pravastatin (HYPER + PRA). Arrows highlight endothelial LOX expression.
and the increase in endothelial permeability induced by LDL. Although the improvement of endothelial barrier function by simvastatin has been previously shown,57,58 our present results suggest that this effect could be dependent at least in part of a catalytically active LOX. Taken together, these results provide evidence on the prominent role of RhoA/ROCK in the regulation of LOX and point towards LOX as a new target gene for statins in the vascular wall.

Furthermore, in the porcine model of hypercholesterolaemia, we show that statins restored vascular LOX expression, although in this model statins only slightly decreased LDL-cholesterol (not statistically significant).26,58,59 This effect was observed in immunohistochemical and real-time PCR analyses at statin dosages (simvastatin 2.5 mg/kg/day) that led to circulating levels similar to those reached in clinical practice (52.2 ± 16.7 nmol/L; Badimon et al., unpublished data).60 Although it has been reported that statins can modulate circulating TNFα levels,61 under our experimental conditions, neither hypercholesterolaemia nor statin treatment significantly modified basal TNFα levels. The up-regulation of LOX was similar in animals treated with simvastatin or pravastatin, a hydrophilic statin that exhibits lower lipid-lowering potency62 but that has shown pleiotropic effects in multiple studies in humans and in animal models including non-human primates.9,63 Our results suggest that the normalization of vascular LOX expression could be a novel mechanism associated with the improvement of endothelial function produced by these drugs through mechanisms beyond plasma cholesterol lowering. It should be noted, however, that simvastatin as well as pravastatin produced a similar inhibitory trend on plasma LDL-cholesterol and that although it was not statistically significant, it could have biological consequences and a not negligible impact on LOX. Finally, regarding the potential consequences of statin treatment in humans, since LDL is a powerful inhibitor of LOX expression it is likely that the lipid-lowering-independent effects of statins could be as relevant as the lipid-lowering-independent effects of these drugs on the modulation of vascular LOX expression.

In summary, we show that an increase in LOX gene transcription is the primary mechanism accounting for the normalization of LOX expression caused by statins. These drugs are able to interfere signalling pathways thereby regulating the activity of a set of transcription factors,64,65 that modulate vascular gene expression and vascular function.23,63,66 Our findings provide the first evidence that pharmacological control of LOX by statins could contribute to the vascular beneficial actions of these drugs and to the cardiovascular risk reduction achieved by this therapy. Further studies are necessary to identify the RhoA downstream mechanisms involved in the up-regulation of LOX by statins and gain more insight into the biological implications of such regulation.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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Conflict of interest: none declared.

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