Simvastatin Reduces Endothelial NOS: Caveolin-1 Ratio but not the Phosphorylation Status of eNOS In Vivo

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In vivo evidence for the pleiotropic effects of simvastatin on the nitric oxide synthase system is limited. Aims: To determine if simvastatin can affect the endothelial nitric oxide synthase cascade. Methods: New Zealand white rabbits (n=15) were divided: Group 1 (control) was fed a normal rabbit diet; Group 2 (MC) received a normal rabbit diet with 1% methionine (M) plus 0.5% cholesterol (C) and 5% peanut oil (atherogenic diet); Group 3 received the same diet as the MC group plus 5 mg/kg/day simvastatin (S) orally (MCS). After 4 weeks, the abdominal aorta was collected and analyzed. Results: Total cholesterol (TC) and total homocysteine (tHcy) were not significantly different between MCS and MC. Endothelial function was only reduced in MC (p<0.05). Although eNOS significantly increased in MC and MCS (p<0.01), simvastatin treatment significantly reduced endothelial caveolin-1 by 35% (p=0.038), causing a 2.5-fold (p=0.026) increase in the eNOS: caveolin-1 ratio. The phosphorylation of eNOS at the threonine 495 site or serine 1177 site was not affected by diet or treatment; however, a positive correlation between the two phosphorylation sites was observed (r²=0.5, p=0.01). Conclusion: In vivo pleiotropic effects of statin therapy include decreasing endothelial caveolin-1. Other therapies designed to affect eNOS phosphorylation in vivo might be useful in further preventing CVD.


Key words; Simvastatin, eNOS, Thre-eNOS, Ser-eNOS, Caveolin-1

Introduction

One of the most successful treatments for cardiovascular disease has been the inhibition of the HMG CoA reductase enzyme using statin therapy. This results in decreased plasma low density lipoprotein cholesterol (LDL), modestly higher plasma high density lipoprotein cholesterol (HDL), and also additional pleiotropic effects, of which the clinical importance remains uncertain. Among these potentially beneficial, non-lipid, pleiotropic effects, interactions with the nitric oxide system have been reported. For example, endothelial cell culture studies clearly show the positive effects of statin therapy on decreasing caveolin-1 abundance1 and increasing eNOS2. Feron and colleagues showed that cultured bovine aortic endothelial cells incubated with atorvastatin reduced caveolin-1 abundance by up to 75%, which resulted in the over-activation of eNOS activity by over 40% in the basal state and over 100% after agonist stimulation but did not change total eNOS expression1. In addition, work published by Hernandez-Perera and colleagues showed that neither atorvastatin or simvastatin increased eNOS protein expression in the same cell type but impaired the downregulation of eNOS caused by oxidised LDL2. Moreover, studies have also focused on the role of statins in the phosphorylation status of eNOS in cell culture. For example, incubation of cultured bovine aortic endothelial cells with lovastatin or pravastatin phosphorylated eNOS at the serine 1179
site but not at the threonine 495 site\textsuperscript{3, 4}).

In vivo studies in ApoE-deficient mice revealed that caveolin-1 is increased in this model of atherosclerosis in whole aortic and heart isolates, and that rosuvastatin treatment maintains caveolin-1 at control levels with no changes in any group regarding eNOS abundance\textsuperscript{5}; however, considering that caveolin-1 is expressed in smooth muscle cells\textsuperscript{6} and macrophages\textsuperscript{7}, it remains unclear if the change in caveolin-1 was at the aortic endothelial layer, which is the site of eNOS regulation.

Thus, as studies elucidating the role of statins in the endothelial cell NOS system have been performed on endothelial cell cultures or whole organ lysates, it remains uncertain whether statins can regulate the eNOS system at the endothelial cell layer in vivo. Thus, to better understand whether statin therapy has pleiotropic effects on the NOS system in endothelial cells in vivo, this study was designed to determine if the beneficial effects of simvastatin therapy on vascular function involved effects on the eNOS system, specifically at the aortic endothelial cell layer.

**Methods**

Male New Zealand White rabbits at three months of age were randomized into 3 groups and fed the following diet for 4 weeks. Group (a) control, (\(n=5\)), Con); Group (b) received a normal rabbit chow diet supplemented with 0.5% cholesterol + 1% methionine + 5% peanut oil (\(n=9\), MC); and group (c) received a normal rabbit chow diet supplemented with 0.5% cholesterol + 1% methionine + 5 mg/kg/day simvastatin orally + 5% peanut oil (\(n=5\), MCS). Blood was collected weekly from the main ear artery and immediately spun at 5000 rpm for 5 minutes, and the serum was collected and snap frozen in liquid nitrogen. The animals were housed in individual cages and maintained at a constant temperature of approximately 21\(^\circ\)C. Food and water were supplied ad libitum. The experiments were carried out according to the National Health and Medical Research Council “Australian Code of Practice for the Care and Use of Animals for Scientific Purposes” (6th Edition, 1997). The animals were then sacrificed by an overdose of an intravenous injection of ketamine and xylazine via the main ear vein as previously described in our laboratory\textsuperscript{8, 9}. The aorta was then excised and cleaned of connective tissue and fat and used for isometric tension studies, and another section was immersed in freshly prepared 4% paraformaldehyde solution in 1x PBS, pH 7.4 and fixed overnight.

**Isometric Tension in the Abdominal Aorta**

Abdominal aortae closest to the bifurcation to the femoral arteries from each rabbit were dissected and mounted in organ baths. The rings were mounted between two metal hooks in organ baths attached to force displacement transducers (Grass FT03) coupled to a data acquisition system (MacLab).

The baths were filled with Krebs solution, kept at a constant temperature of 37\(^\circ\)C, and continuously bubbled with 95% O\(_2\)/5% CO\(_2\). After 1 hour, vessels were gently stretched to a resting tension of 2.5 g. After 15 minutes, the vessels were gently re-stretched to a resting tension of 2.5 g. After the vessels reached the plateau tension, maximum constriction was determined by a high potassium Krebs solution (KPS5, 124 mM K\(^+\)). After the plateau (6 minutes), vessels were rinsed with Krebs solution. After 45 minutes, the vessel rings were subjected to a phenylephrine concentration curve (\(10^{-8}\)-\(10^{-5}\) M, half log units). After the contraction reached 30% of maximum and reached plateau, the aortic rings were subjected to an acetylcholine dose response curve (\(10^{-8}\)-\(10^{-6}\) M, half log units)\textsuperscript{10}.

**Semi-Quantitative Immunohistochemistry**

The aortic rings were processed using paraffin in one batch and mounted on a single chuck to maintain equal cutting thickness\textsuperscript{11}. Sections were cut at 5 microns, dewaxed through xylene, rehydrated through alcohol and water, and then placed in 10 mM TrisHCl pH7.4 buffer to equilibrate tissue pH. Then sections were incubated with anti-eNOS antibody (1:100 dilution in 1% goat serum in 10 mM Tris HCl, pH7.4; Transduction Laboratories, Cat# 610296), or anti caveolin-1 antibody (1:50 dilution in 1% goat serum in 10 mM Tris HCl, pH7.4; ZYMED Laboratories, Cat# 03-6000), or anti 495-Thr-eNOS antibody (1:100 dilution in 1% goat serum in 10 mM Tris HCl, pH7.4; Transduction Laboratories, Cat# 612706), or anti 1177-Ser-eNOS antibody (1:100 dilution in 1% goat serum in 10 mM Tris HCl, pH7.4; Transduction Laboratories, Cat# 612096), or anti 1177-Ser-eNOS antibody (1:100 dilution in 1% goat serum in 10 mM Tris HCl, pH7.4; Transduction Laboratories, Cat# 612392) or CD31 (1:100 dilution in 1% goat serum in 10 mM Tris HCl, pH7.4; Chemicon, Cat# CBL468), or a monoclonal antibody to Aspergillus niger glucose oxidase (as negative IgG control; Dakocytomation, Cat#X0931) overnight at room temperature. This was followed by a 5-minute wash in 10mM TrisHCl pH 7.4 and then incubation with the 'envision' molecule (Dako Corporation). Sections were then washed in 10 mM TrisHCl pH 7.4, reacted with DAB chromagen, counterstained with hematoxylin, dehydrated and mounted.
All stained images of the top, bottom, left and right of each aorta were captured on a digital camera (40x magnification). The endothelial layer was traced using the ‘ribbon’ tool in MCID analysis software (MCID Core; InterFocus Imaging, Linton, UK). The average was calculated of all 4 images per artery. The endothelial layer was quantified using MCID by setting the hue, saturation, and intensity to detect the brown DAB reaction. This was repeated three times, and each time a new hue, saturation, and intensity were set. The intensity and proportional area were recorded for all proteins and all three tracings were averaged and used for data analysis. All data points are arbitrary units and normalized to control as ‘1’. This was previously published by our laboratory [10-12].

**Data Analysis**

All data points were analyzed by ANOVA followed by the Newman-Keuls multiple comparison post-hoc test. A p<0.05 was accepted as significant in all cases. All data are expressed as the mean ± SEM. Data points were excluded if the point was >2SD from the mean.

**Results**

Blood total cholesterol (TC, Fig. 1A) and total homocysteine (tHcy, Fig. 1B) were both increased by the atherogenic diet from weeks 1 to 3, but only cholesterol was increased in week 4. Simvastatin did not significantly affect the plasma concentration of either.

**Immunoquantification and Endothelial Function**

There was a significant increase in endothelial NOS in both the MC and MCS groups; however, endothelial caveolin-1 significantly decreased by 35% (p=0.038) by simvastatin treatment (Fig. 2, bottom left graph). This led to a significant increase in the eNOS: caveolin-1 ratio by 2.5-fold compared to the control (2.52 ± 0.7 vs. 1.0 ± 0.05) and improvement in vascular reactivity. Endothelial function was impaired compared to the control in MC (p<0.05, Fig. 2 middle bottom). Simvastatin virtually inhibited the development of endothelial dysfunction. There was no difference in endothelial layer integrity, as shown by CD31. There was no detectable difference in serine (1177) or threonine (495) phosphorylation sites; however, a positive correlation between the two sites in the MC and MCS groups was shown (r²=0.5, p=0.01, Fig. 2, bottom right).

**Discussion**

The major finding of this investigation was that simvastatin treatment maintains endothelial cell function during atherogenesis via decreasing endothelial cell caveolin-1, but not changes in eNOS or the phosphorylation of eNOS at the 1177 serine or 495 threonine site.

The lack of a lipid-lowering effect in the simvastatin-treated group has also been observed by Thakur and colleagues, who showed no effect of 5 mg/kg/day simvastatin treatment for 4 weeks [13], and Chen showed similar effects in mice [14]; however, others show lipid-lowering effects of simvastatin. For example,
Further studies aimed at determining whether homocysteine can impair the hypolipemic effects of simvastatin are warranted. In addition, statin therapy did not restore endothelial function to normal. The reason for this effect remains unknown; however, plasma homocysteine remained elevated throughout the statin treatment and hyperhomocysteinemia is well documented to cause endothelial dysfunction via separate mechanisms, including increased oxidative stress and reduction in eNOS co-factors.

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Alfon and colleagues showed reduced TC by simvastatin treatment in rabbits fed 0.5% cholesterol +15% coconut oil for 10 weeks. In addition, Nachtigal showed that simvastatin treatment reduced TC in rabbits fed 0.4% cholesterol for 2 weeks. The reasons for these discrepancies remain unclear; however, it is clear from clinical studies that statin therapy can reduce TC in most cases. In our study, we hypothesize that the elevated level of plasma homocysteine could be impairing the effect of simvastatin on lowering TC, as previous studies suggested possible interactions between statin and homocysteine.

Further studies aimed at determining whether homocysteine can impair the hypolipemic effects of simvastatin are warranted. In addition, statin therapy did not restore endothelial function to normal. The reason for this effect remains unknown; however, plasma homocysteine remained elevated throughout the statin treatment and hyperhomocysteinemia is well documented to cause endothelial dysfunction via separate mechanisms, including increased oxidative stress and reduction in eNOS co-factors.

It remains unclear whether the roles of statins in the prevention of CVD are solely related to its lipid-
lowering effects or whether other pleiotropic effects remain important\textsuperscript{23}. In this study, we suggest that simvastatin does have important non-lipid pleiotropic effects. Simvastatin treatment did not decrease total plasma cholesterol or LDL-C over this short treatment period, yet had important, potentially beneficial, vascular effects that reduced endothelial caveolin-1 protein. This suggests that the effect of statin therapy on the inhibition of HMGCoA reductase is independent of total plasma cholesterol levels and could possibly reduce endothelial intracellular cholesterol content, which has reduced endothelial caveolin-1.

Recent studies have suggested that eNOS must reside in plasmalemmal invaginations (caveolae) for maximal acetylcholine-evoked stimulation\textsuperscript{24}. The mechanism whereby acetylcholine induces NO release is complex\textsuperscript{25}. It has been suggested that, at rest, eNOS is anchored to the caveolae by caveolin-1. The activity of eNOS is inhibited by caveolin-1. In this state, eNOS produces a low level of basal NO. Upon agonist stimulation, the influx of Ca\textsuperscript{2+} activates calmodulin, which then displaces caveolin-1, allowing phosphorylation\textsuperscript{26} and activation of eNOS and increased production of NO; therefore, we suggest that the decrease in caveolin-1 described in this study could allow calmodulin to further activate eNOS and thus improve agonist stimulated endothelial function.

The eNOS: caveolin-1 ratio could be regarded as a marker of eNOS function. This is supported by evidence of complete artery relaxation in caveolin-1 knockout mice (which lack caveolin-1 and caveolae) by low concentrations of bradykinin\textsuperscript{27, 28}. In addition, the incubation of mouse aortic rings with a peptide mimicking the eNOS-caveolin-1 binding site inhibits artery relaxation by acetylcholine\textsuperscript{29}. We have previously shown that the eNOS: caveolin-1 ratio is increased in the endothelial layer overlying plaques in rabbits\textsuperscript{11}, suggestive of eNOS inhibition; however, taken together, the current results allude to the possibility that statin therapy could reduce the eNOS: caveolin-1 ratio in endothelial overlying plaques, which might enhance plaque remodeling, leading to a more stable plaque phenotype.

Several published studies have shown the inverse correlation between the phosphorylation of eNOS at the serine 1177 site and threonine 495 sites after agonist stimulation\textsuperscript{30-31}. In our study, we did not determine the correlation of both sites after acetylcholine stimulation, but used virgin sections of aorta. In this case we showed a positive correlation ($r^2 = 0.5$, $p = 0.01$) between these two sites in non-agonist-stimulated aorta. We also report that simvastatin did not alter the phosphorylation of eNOS at either site in the aortic endothelial layer in vivo. This is in stark contrast with in vitro studies, which clearly show that statins can phosphorylate eNOS at these sites\textsuperscript{32-34}. Further studies aimed at quantifying the levels of phosphorylated eNOS after agonist stimulation in the aortic endothelial layer will determine whether statin treatment can enhance the phosphorylation of eNOS in vivo.

Despite no change in the eNOS: caveolin-1 ratio in the MC group after 4 weeks of the atherogenic diet, the endothelium remained dysfunctional, indicating that other factors such as eNOS uncoupling, reduction in substrate or reduced NO bio-availability could be the cause at this time point. Other in vitro studies clearly show that cholesterol can increase caveolin-1 concentration. For example, Feron and colleagues demonstrated that bovine aortic endothelial cells cultured in the presence of human serum from hypercholesterolemic volunteers upregulated caveolin abundance without any effect on eNOS\textsuperscript{35}. In addition, a landmark study by Li and colleagues showed that human umbilical vein and renal microvascular endothelial cells can upregulate caveolin-1 if exposed to homocysteine, which is suggested by the authors, to occur by increasing cellular cholesterol content\textsuperscript{36}. We show that aortic endothelial caveolin-1 did not change in the group fed the atherogenic diet, which raised both plasma cholesterol and homocysteine over a period of four weeks. This discrepancy could be explained by the duration of experiments, in that cell culture studies were performed within 48 hours whereas the in vivo study presented here was performed after 4 weeks. Further studies aimed at shorter time end points could shed light on the regulation of aortic endothelial caveolin-1 during the atherogenic diet.

In conclusion, we show that simvastatin does not directly affect eNOS or the phosphorylation of eNOS, but decreases caveolin-1 in vivo. These results suggest that therapies directed to the phosphorylation of eNOS in vivo in conjunction with statin therapy might help reduce the burden of CVD.

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**Disclosures**

The authors have no disclosures.
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