Hsp90 and Caveolin Are Key Targets for the Proangiogenic Nitric Oxide–Mediated Effects of Statins

Agnès Brouet,* Pierre Sonveaux,* Chantal Dessy, Stéphane Moniotte, Jean-Luc Balligand, Olivier Feron

Abstract—3-Hydroxy-3-methylglutaryl (HMG)–coenzyme A reductase inhibitors or statins exert direct beneficial effects on the endothelium in part through an increase in nitric oxide (NO) production. Here, we examined whether posttranslational modifications of the endothelial NO synthase (eNOS) could account for the proangiogenic effects of statins. We used endothelial cells (ECs) isolated from cardiac microvasculature, aorta, and umbilical veins, as well as dissected microvessels and aortic rings, that were cultured on reconstituted basement membrane matrix (Matrigel). Tube or precapillary formation was evaluated after statin treatment, in parallel with immunoblotting and immunoprecipitation experiments. Atorvastatin stimulated NO-dependent angiogenesis from both isolated and outgrowing (vessel-derived) ECs, independently of changes in eNOS expression. We found that in macro- but not microvascular ECs, atorvastatin stabilized tube formation through a decrease in caveolin abundance and its inhibitory interaction with eNOS. We also identified the chaperone protein hsp90 as a key target for the proangiogenic effects of statins. Using geldanamycin, an inhibitor of hsp90 function, and overexpression of recombinant hsp90, we documented that the statin-induced phosphorylation of eNOS on Ser1177 was directly dependent on the ability of hsp90 to recruit Akt in the eNOS complex. Finally, we showed that statin promoted the tyrosine phosphorylation of hsp90 and the direct interaction of Akt with hsp90, which further potentiated the NO-dependent angiogenic processes. Our study provides new mechanistic insights into the NO-mediated angiogenic effects of statins and underscores the potential of these drugs and other modulators of hsp90 and caveolin abundance to promote neovascularization in disease states associated or not with atherosclerosis. (Circ Res. 2001;89:866-873.)

Key Words: statin ■ angiogenesis ■ nitric oxide ■ hsp90 ■ caveolin

It is now established that statins, in addition to their ability to improve serum lipid profile,1 exert beneficial effects on endothelial function through an increase in nitric oxide (NO) production and/or bioavailability.2 A major area in which such effects of statins on peripheral cells would be valuable is therapeutic angiogenesis, eg, the development of neovascularization in ischemic tissues. NO has, indeed, been identified as a downstream mediator of various growth factors initiating the angiogenic signaling cascade in endothelial cells (ECs).3 Recently, Kureishi et al4 reported that simvastatin could promote angiogenesis in ischemic limbs of normocholesterolemic rabbits. These authors further documented that statins could acutely induce Akt-dependent endothelial NO synthase (eNOS) phosphorylation in cultured ECs and therefore proposed that the Akt activation of eNOS could account for the long-term beneficial effects of simvastatin observed in vivo. Although the reversal of this effect by mevalonate suggested an inhibitory effect of the statin on the isoprenoid synthesis and downstream isoprenylation/activation of small G proteins,5 the mechanism by which statins activate both Akt and eNOS remains enigmatic. Akt can, indeed, be activated by Ras, the activation of which is dependent on isoprenylation.5 However, if statins inhibit Ras isoprenylation, this should produce a decrease in Akt activation. Among the other identified pathways leading to phosphatidylinositol 3-kinase (PI(3)K)/Akt activation, a calcium-dependent6 process has been reported. Interestingly, we showed that statin treatment can promote the Ca2+-dependent activation of eNOS through a decrease in caveolin abundance.7 The Akt activation of eNOS could therefore be conditional on the disruption of the caveolin/eNOS complex. However, according to the EC types, the extent of this interaction may vary dramatically,8 and the effects of statins on eNOS activation are therefore likely to differ according to the endothelial bed. Altogether, these data prompted us to further investigate how the different posttranslational modifications of eNOS identified so far could lead to the NO-mediated angiogenic effects of statins.

We used several models of freshly isolated and outgrowing ECs cultured on extracellular matrices that were either acutely or chronically exposed to atorvastatin. This work provides new mechanistic insights into the NO-mediated angiogenic effects of statins and, we think, opens new
perspectives in the development of therapeutic strategies targeting cardiac or peripheral angiogenesis.

Materials and Methods

Cell Culture

Mouse cardiac microvascular ECs (CMECs) and human umbilical vein ECs (HUVECs) were freshly prepared according to standard isolation procedures. Bovine aortic ECs (AECs) were purchased from Clonetics and used at early passages. Serum-starved ECs were usually exposed to atorvastatin (0.1 to 1 µmol/L) after a 30- to 60-minute preincubation with various pharmacological modulators, as follows (from Sigma except when indicated): 5 mmol/L Nω-nitro-L-arginine methyl ester (L-NAME), 1 mmol/L mevalonate, 20 µmol/L BAPTA-AM, 1 µg/mL geldanamycin (Invitrogen Life Technologies), 10 µmol/L LY294002, 100 nmol/L okadaic acid, and 10 nmol/L calyculin.

Aorta-derived ECs (AoECs) and coronary microvessel–derived ECs (CoMECs) were obtained by incubating small rat aorta rings (1 mm length) and human microvessels (dissected from endocardial biopsies), respectively, for 5 to 7 days in Matrigel (see below) in the presence of 20% FCS/DMEM. In this model, atorvastatin was added from day 0 to day 5, and the medium was replaced every 24 hours with fresh drug solution. Cultures of AoECs and CoMECs organized in a network were then challenged at day 5 by incubation under serum-free conditions for 24 to 48 hours with or without some of the drugs mentioned above.

The identity and purity of the different ECs used in this study were routinely validated by indirect immunofluorescence using von Willebrand factor antibodies. In addition, serum-stimulated cholesterol efflux measurements were carried out to evaluate the functional pool of caveolin in the different ECs (see online Materials and Methods available at http://www.circresaha.org).

Angiogenesis Assay on Matrigel

The formation of capillary-like structures was assessed by plating (CMECs, AECs, and HUVECs) on or culturing (AoECs, CoMECs) in basement membrane matrix preparation (growth factor–reduced Matrigel [Becton Dickinson]) distributed in 24-well multidishes. Tube formation was observed using an inverted phase-contrast microscope (Zeiss Axiovert 25), and images were captured with a videographic system (Pixera Pro). The tube formation index was determined by measuring the length of tubes in random fields from each well using the Image J program. Statistical analyses were made using the Student t test or one-way ANOVA where appropriate.

In some experiments, ECs were recovered from Matrigel after a 5-minute incubation with 50 mg/mL collagenase under constant agitation, followed by two sequences of washing/centrifugation (2000 rpm for 5 minutes at 4°C).

Transfection and Peptide Loading by Reversible Permeabilization

HUVECs were transfected with the hsp90 cDNA (a gift from Dr W.C. Sessa, Yale University, New Haven, Conn) using Lipofectamine (Invitrogen) according to the manufacturer’s protocol; an irrelevant plasmid encoding β-galactosidase was used as a control to obtain identical amounts of transfected DNA in each condition. HUVECs were also reversibly permeabilized, as previously reported, to introduce synthetic peptides derived from the caveolin scaffolding domain (CSD).

Immunoprecipitation (IP) and Immunoblotting (IB)

ECs were processed for IB or IP as described previously; antibodies were from BD Transduction Labs, except phospho-eNOS antibody, which was from New England Biolabs.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Figure 1. Atorvastatin stimulates NO-dependent angiogenesis in CMECs and AoECs. Shown are representative pictures of freshly isolated CMECs plated on Matrigel for 3 hours (A through C) and of AoECs grown in Matrigel for 5 days and placed under serum-free conditions for 2 more days (D through F). The following treatments were performed as detailed in Results: A and D, vehicle; B and E, atorvastatin (1 µmol/L); C and F, atorvastatin+L-NAME (5 mmol/L). Inset in panel D, Typical endothelial tube organization arising from a cultured aortic ring.

Results

Atorvastatin Stimulates NO-Dependent Angiogenesis in Two Models of EC Primary Cultures

We first examined the ability of statins to stimulate and/or stabilize tube formation from CMECs and AoECs cultured on Matrigel. As shown in Figure 1B, exposure of freshly isolated CMECs to atorvastatin rapidly led to the formation of capillary-like structures on the Matrigel surface; this proangiogenic effect was maximal after 3 hours and almost completely blocked by the coincubation with the NO synthase inhibitor L-NAME (Figure 1C). Atorvastatin similarly promoted AoEC network organization but with a very distinct time course because of the nature of this model of tissue culture. Aortic rings had, indeed, to be cultured for 5 to 7 days in Matrigel to exhibit EC proliferation and tube formation (see Figure 1D, inset). The presence of atorvastatin during this period of cell growth did not significantly influence the serum-driven formation of the AoEC network. However, when the vessel culture was deprived of serum for 48 hours, we observed that pretreatment with statins dramatically stabilized the network organization (Figure 1E), whereas the precapillaries formed in the absence of statins completely degenerated (Figure 1D). Importantly, the addition of L-NAME at the time of serum deprivation abrogated the beneficial effects of statin pretreatment (Figure 1F).
Atorvastatin Decreases the Extent of the Caveolin/eNOS Interaction in AoECs but not in CMECs

Because atorvastatin treatment did not alter the expression of eNOS in either CMECs or AoECs (see Figure 1, bottom left), we examined whether the NO-mediated angiogenic process reported in Figure 1 could, instead, originate from the modulation by statins of posttranslational modifications of eNOS. eNOS activity has, indeed, been shown to be either decreased by the interaction of the enzyme with the scaffolding protein caveolin or increased after recruitment of the heat shock protein, hsp90. Accordingly, ECs were recovered from Matrigel after exposure to atorvastatin (St) or vehicle (V), and the extent of caveolin/eNOS and hsp90/eNOS interactions in either condition was examined. As shown in Figure 2A (top left panel), the amount of caveolin immunoprecipitated by eNOS antibodies was not significantly altered by the statin treatment in CMECs ($P > 0.1$, $n = 3$), whereas it appeared dramatically decreased in atorvastatin-treated AoECs ($-73 \pm 7\%$, $P < 0.01$, $n = 3$).

To better appreciate the absolute amounts of eNOS interacting with caveolin in AoECs, we also performed reverse IP, using caveolin antibodies to immunoprecipitate eNOS, and compared the eNOS immunoblot signal with that resulting from the direct IP using eNOS antibodies. Densitometric analysis of immunoblots as presented in Figure 2A (bottom panels) revealed that, whereas in CMECs <5% of the cell eNOS pool was bound to caveolin (and not altered by the statin treatment), 44±8% ($n = 6$) of eNOS was found in the caveolin immunoprecipitate from AoEC extracts; importantly, this amount was reduced to 8±5% ($n = 6$) when AoECs had been exposed to atorvastatin for 5 days (see Figure 2A).

Atorvastatin Decreases the Abundance of Caveolin in Macrovascular but not Microvascular ECs

As depicted in Figure 2A (top right), the extent of caveolin recovered from the caveolin IP was reduced in AoECs exposed to atorvastatin. This prompted us to examine the effects of statin exposure on the absolute amounts of caveolin in AoECs and CMECs. Figure 2B confirmed that the AoEC pool of caveolin was reduced by 26±5% ($n = 3$, $P < 0.01$) in the presence of atorvastatin, whereas, as expected, the 3-hour exposure of CMECs to the statin did not alter the abundance of caveolin. To further assess whether this difference was dependent on the cell type (eg, macro- versus microvascular) or the experimental model (eg, isolated versus outgrowing ECs), we also evaluated the effects of statins on caveolin expression in isolated bovine aortic ECs (AECs) and in outgrowing ECs recovered from human coronary microvessels (CoMECs) cultured in Matrigel. Figure 2B shows that the endothelial bed was critical because both outgrowing and isolated aortic ECs revealed a significant decrease in caveolin abundance after statin treatment, whereas microvascular outgrowing (CoMECs) and isolated cells (CMECs) did not show any alteration in caveolin expression (see also Figure 2C).

Remarkably, when the data presented in Figure 2B were normalized to the number of cells (see Figure 2C), the cellular pool of eNOS (and of hsp90) was not significantly different between the different cell types tested (not shown). Importantly, we also documented that supplementation of the culture medium with mevalonate (the immediate downstream

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**Figure 2.** Atorvastatin decreases the extent of the caveolin/eNOS interaction in AoECs but not in CMECs. ECs were recovered from Matrigel after exposure to atorvastatin (St) (1 μmol/L) or vehicle (V). A, CMEC and AoEC lysates were immunoprecipitated with eNOS (left) or caveolin (right) antibodies and analyzed by IB with caveolin (top) and eNOS (bottom) antibodies. B, Lysates were also directly analyzed by IB with caveolin antibodies. Lysates from outgrowing CoMECs and isolated AECs were also used for comparison. These experiments were repeated 2 to 3 times with similar results. C, Densitometric analysis of caveolin IB presented in panel B; expression levels are normalized to number of cells (open bars, control; hatched bars, statin). D, Effects of atorvastatin treatment on cholesterol efflux (ie, serum-stimulated decrease in [3H]cholesterol cell content) from AECs and CMECs. *$P < 0.01$ ($n = 3$) vs corresponding control conditions.
We have recently reported that hsp90 binding to eNOS promoted the vascular endothelial growth factor–induced PI(3)K-dependent phosphorylation of Ser1177 eNOS.40 We therefore examined the modulation of eNOS phosphorylation by statins using anti-phospho-Ser1177 eNOS antibodies. Figure 3 (bottom) shows that exposure of CMECs to atorvastatin did produce eNOS phosphorylation in a PI(3)K-dependent manner because the PI(3)K inhibitor LY294002 completely blocked the statin-induced phosphorylation of eNOS. Interestingly, although LY294002 did not prevent the hsp90/eNOS interaction, geldanamycin blocked the phosphorylation of eNOS, suggesting that in CMECs, hsp90 binding to eNOS is a prerequisite for statin-induced phosphorylation of eNOS.

In AoECs, we did not observe any effect of statin treatment on the phosphorylation of eNOS. However, this appeared to be dependent on the experimental model; the 48-hour period in the absence of atorvastatin (after the 5-day treatment with the drug) precluded the IB detection of the phosphorylated form of eNOS. We verified, indeed, that an uninterrupted exposure of outgrowing AoECs (or isolated AECs) to the statin did produce a significant increase in the amounts of phospho-eNOS and hsp90/eNOS interaction (not shown).

**Hsp90 Is Required for an Efficient Phosphorylation of eNOS by Akt Upon Statin Exposure**

We next characterized the molecular mechanisms governing the statin-induced phosphorylation of Akt and eNOS and the subsequent tube formation by modulating hsp90 and caveolin abundance. The need for time course studies and various pharmacological treatments prompted us to use the more easily isolatable and tractable HUVECs (instead of CMECs).

We first verified that statins produced the rapid phosphorylation of Ser1177 eNOS in HUVECs and that this process was PI(3)K/Akt-dependent using LY294002 (see Figure 4A). Interestingly, whereas IB with anti-phospho-Ser473 Akt antibodies revealed only a weak signal on statin treatment, we identified the presence of a robust phospho-Akt signal in the eNOS immunoprecipitate (Figure 4A).

To further characterize the role of hsp90 in mediating statin-induced Akt-mediated angiogenic effects in HUVECs, we next combined the use of geldanamycin and okadaic acid, a phosphatase (PP2) inhibitor which has been reported to prevent Akt dephosphorylation.12 We first documented that the statin-induced Akt-dependent phosphorylation of eNOS was hsp90-mediated because it was inhibited by geldanamycin (Figure 4A). Furthermore, in the presence of okadaic acid, the (basal) level of Akt phosphorylation was dramatically increased and the statin-induced phosphorylation of eNOS was increased by ~3-fold (compare the okadaic acid versus okadaic acid+statin conditions in Figure 4A). Importantly, the simultaneous preincubation with geldanamycin and okadaic acid did not change the extent of Akt phosphorylation but completely repressed the phosphorylation of eNOS, indicating that the phosphorylation of eNOS is not strictly dependent on the level of Akt phosphorylation but instead on the level of hsp90 bound to eNOS. To confirm this finding, we quantified the interaction between phosphorylated Akt

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**Atorvastatin Increases the Extent of the hsp90/eNOS Interaction and eNOS Phosphorylation in ECs**

The analysis of the amount of hsp90 immunoprecipitated by eNOS antibodies revealed that the hsp90/eNOS interaction was increased by ~5-fold in CMECs exposed for 3 hours to atorvastatin (Figure 3, top). Furthermore, a 30-minute preincubation with 1 μg/mL geldanamycin (known to block the hsp90 chaperone function) led to a nearly complete blockade of both the recruitment of hsp90 to eNOS, at least under our detergent conditions (Figure 3, top), and the reorganization of CMECs in tubes (not shown).
Atorvastatin Independently Promotes the Akt/hsp90 and eNOS/hsp90 Interactions and Stimulates the hsp90 Tyrosine Phosphorylation

Although the data presented in Figure 4A documented that the phosphorylation of eNOS on Ser1177 depends on the ability of Hsp90 to recruit Akt in the eNOS complex, the exact nature of the eNOS multicomplex induced by statins was not clear. Therefore, we examined the time course of the Akt/hsp90, eNOS/Akt, and eNOS/hsp90 interactions in co-IP experiments. As shown in Figure 4B (top), we found that a 5-minute exposure to atorvastatin was sufficient to promote the interaction of hsp90 with both Akt and eNOS but insufficient to promote the association of Akt with eNOS in HUVECs. In agreement with these observations, the 5-minute incubation with atorvastatin did not promote eNOS phosphorylation, whereas it was sufficient to induce Akt phosphorylation as shown in IB experiments (Figure 4B, bottom). As expected, longer (30 minutes) exposure to the statin produced eNOS phosphorylation and the association of Akt-hsp90-eNOS in a trimeric complex, as detected by mutual co-IP (Figure 4B, last lanes). Of note, the extent of this mutual interaction reached its maximum after 1 to 2 hour(s) of atorvastatin treatment.

The direct effect of statins on the ability of hsp90 to act as a scaffold (independently for Akt and eNOS) incited us to examine whether hsp90 was itself modified by phosphorylation. Hsp90 is, indeed, likely to correspond to the 90-kDa protein ENAP-1 originally identified by Venema et al.13 as a tyrosine-phosphorylated protein. Figure 4C shows that, indeed, atorvastatin rapidly and dose-dependently induced the tyrosine phosphorylation of hsp90.

Hsp90 Overexpression Potentiates the Akt-Mediated Phosphorylation of eNOS in Response to Statins

Having documented that modulating the integrity of the hsp90/eNOS interaction directly affected the statin-induced phosphorylation of Ser1177 eNOS, we further examined the role of hsp90 by increasing the abundance of this chaperone protein in HUVECs by heterologous expression. IB experiments revealed that hsp90 overexpression and statin exposure separately led to a slight increase in eNOS phosphorylation (Figures 5 [left] and 6B [open bars]). When both hsp90 overexpression and the statin treatment were combined, the level of eNOS phosphorylation was synergistically increased together with the extent of the eNOS/hsp90 association (see Figures 5 and 6B).

Figure 5 (left) also shows that a phospho-Akt signal could be detected in the eNOS IP from statin-treated cells, and importantly that the enrichment in phospho-Akt was increased >3-fold when statin-treated cells also expressed recombinant hsp90 (see also Figure 6B, hatched bars). This potentiation of the effect of statin treatment by hsp90 overexpression was observed, whereas the level of phospho-Akt was not significantly higher than under the conditions of hsp90 overexpression alone (ie, in the absence of atorvastatin), thereby emphasizing the critical role of hsp90 in recruiting Akt in the vicinity of eNOS to allow enzyme phosphorylation. Of note, the stimulatory effect of hsp90 overexpression on Akt phosphorylation is very likely to be explained by the recently documented inhibitory effect of hsp90 on PP2 phosphatase activity.14

Hsp90-Dependent Phosphorylation of eNOS After Statin Treatment Is Sensitive to Mevalonate and Ca2+ Chelation and Is Blocked by the CSD Peptide

We further dissected the mechanism by which statins impact on the hsp90-dependent regulation of eNOS activation. First, the addition of mevalonate was sufficient to completely reverse the stimulatory effect of atorvastatin on eNOS/hsp90 interaction as well as on the phosphorylation of both eNOS and the fraction of Akt associated with eNOS (Figure 5, right).

We also examined the effects of synthetic peptides derived from the CSD introduced by reversible permeabilization in HUVECs before plating on Matrigel. Immunoblot analyses revealed that cell loading with CSD peptides prevented the association of eNOS with hsp90 and Akt and blocked the phosphorylation of eNOS, even though it did not influence the phosphorylation of Akt (Figure 5, right). Because we showed previously that CSD peptides competitively inhibit the Ca2+/calmodulin-dependent activation of eNOS,9,10 we next examined whether changes in intracellular calcium were critical in the statin-induced Akt/eNOS activation pathway. Figure 5 shows that when the calcium chelator BAPTA-AM was added to the cells 30 minutes before the treatment with statin, the interaction of eNOS with hsp90 and its phosphorylation were abrogated. Interestingly, the phosphorylation of Akt was also blocked by BAPTA treatment, suggesting that under our experimental conditions, Akt phosphorylation by statins is a calcium-dependent mechanism or, at least, is sensitive to changes in [Ca2+]i.

Hsp90 and Caveolin Oppositely Modulate the Atorvastatin-Induced Endothelial Network Organization

We next sought to verify whether the observed modulation of the hsp90/eNOS interaction by hsp90 overexpression or CSD peptide loading (see Figure 5) could increase or decrease the proangiogenic effects of statins, respectively, in the model of HUVECs cultured on Matrigel.

Figure 6A shows that atorvastatin (0.1 μmol/L) stimulated tube formation in HUVECs with kinetics similar to those observed with CMECs (see Figure 1) and, importantly, that the heterologous expression of hsp90 significantly potentiated this proangiogenic effect for at least 24 hours under the conditions of our assay (see Figures 6A and 6B [solid bars]). Of note, as already observed with the CMECs, the use of LY294002 prevented HUVEC reorganization induced by
Conversely, the phosphatase inhibitor okadaic acid stimulated basal and statin-induced tube formation (not shown). Furthermore, when HUVECs were first loaded with CSD peptides, atorvastatin treatment was unable to stimulate tube formation (see Figures 6A and 6B), whereas the use of a scrambled CSD peptide was without effect on the proangiogenic effects of statins (not shown).

Additional results can be found in an online data supplement available at http://www.circresaha.org.

Discussion

This study identifies hsp90 as a key player in the proangiogenic action of statins and underscores the importance of caveolin as a regulator of NO-dependent angiogenesis.

One of the major findings of this study is that the capacity of statins to reduce the extent of the inhibitory caveolin/eNOS interaction, and thereby to stimulate NO-mediated angiogenesis, is dependent on the EC type. Indeed, although caveolin overexpression or caveolin peptide leads to the inhibition of NO-dependent processes in virtually any type of cells expressing eNOS9,15 (see also this study), we report here that the effects of statins on the caveolin/eNOS interaction vary from one EC type to another.

Our data support the notion that the reduction in caveolin abundance induced by statin treatment accounts for (part of) the proangiogenic effects of these hypolipidemic drugs exclusively in ECs with a low pool of caveolin. Accordingly, we showed that in aortic ECs, the low cellular pool of caveolin is very sensitive to the statin treatment, whereas in microvascular ECs (either isolated or outgrowing), the 6-
8-fold higher pool of caveolin is not responsive to the statin exposure. Likewise, the effects of statins on the rate of FC efflux from serum-stimulated ECs appear dependent on the abundance of caveolin. Atorvastatin failed, indeed, to modulate the FC efflux in CMECs (which have a high caveolin pool), whereas in statin-exposed aortic ECs, a nearly complete blockade of the cholesterol efflux was observed under the conditions of our assay. Interestingly, this observation, ie, the much greater abundance of caveolin in the microcirculation relative to macrovessels, is consistent with NO being the major vasodilator in the conduit arteries compared with the microvessels in which endothelium-derived hyperpolarizing factors may predominate.

Moreover, whereas in aortic ECs a high proportion of eNOS interacts with caveolin, in the coronary microvascular ECs, the caveolin/eNOS interaction is limited to a few percent of total eNOS, and the activity of eNOS is therefore intrinsically less conditional on the statin effects on caveolin abundance (eNOS is known to be mostly located in the Golgi network in those cells and not in the caveolae). We propose therefore that the conjunction of this parameter, eg, the extent of the interaction between eNOS and caveolin, with the extent of the caveolin pool, determines the effects of statins on the stoichiometry of the caveolin/eNOS interaction and the subsequent NO-mediated angiogenic effects.

The second major finding of this study is that the capacity of statins to stimulate the formation of capillary-like structures can be blocked by inhibiting the function of hsp90 (by the use of geldanamycin) and conversely, promoted after hsp90 overexpression.

Indeed, we provide evidence that although Akt-dependent phosphorylation of Ser1177 eNOS ultimately accounts for the NO-mediated proangiogenic effects of statins, hsp90 is the control point for this effect. Thus, we have documented that overexpression of hsp90 by itself (without statin treatment) led to an increased phosphorylation of Akt and eNOS, and to the formation of capillary-like structures. Also, we showed that statin treatment and hsp90 overexpression synergistically increased both the level of Ser1177 eNOS phosphorylation and the stability of the endothelial tube reorganization (see Figure 5); under our experimental conditions, changes in the phosphorylation status of Thr495 (another eNOS phosphor- ylatable residue) do not appear to account for the statin effects (see online Figure 1, available at http://www.circresaha.org).

Finally, we showed that whereas the phosphatase inhibitor okadaic acid (and calyculin) stimulated Akt phosphorylation, the blockade of the hsp90/eNOS interaction by geldanamycin suppressed the ability of statins to promote eNOS phosphorylation and tube formation (whereas it had no effect on Akt phosphorylation [see Figure 4A]).

Furthermore, our data are consistent with the following sequence of events: (1) statin treatment stimulates the interactions of hsp90 with eNOS and Akt (independently), (2) hsp90 acts as a scaffold bridging activated Akt and eNOS, and (3) eNOS is phosphorylated on Ser1177 by neorecruited Akt. In fact, our data revealed that statins by targeting hsp90 have a double beneficial effect leading to the potentiation of the activation of both eNOS and Akt. Indeed, the binding of hsp90 to eNOS is known to facilitate the further binding of calmodulin and contributes also to preventing the uncoupling of eNOS activity, thereby leading to an increase in eNOS activation and NO bioavailability. As for the interaction between hsp90 and Akt, it was recently shown to repress the phosphatase PP2A activity and therefore promote the activation of Akt. Altogether, these findings emphasize the critical role of hsp90 in driving the statin effects and particularly those leading to angiogenesis.
Our data also shed some light on the upstream mechanisms leading to the hsp90 binding to eNOS and Akt after statin exposure. Indeed, the promoting effects of statins on the eNOS/hsp90 interaction were reversed by mevalonate, thereby attesting to the implication of the HMGCoA reductase and probably of the isoprenoid synthesis. Moreover, the effect of statins on the recruitment of hsp90/phospho-Akt to form a multicomplex with eNOS appeared closely dependent on changes in [Ca^{2+}], as revealed by the inhibitory effects of the calcium chelator BAPTA in our study (see Figure 5). Also, we showed that by antagonizing the Ca^{2+}/calmodulin-mediated activation of eNOS by loading HUVECs with synthetic peptides corresponding to the CSD sequence, both the statin-induced tube organization and the eNOS multicomplex formation could be completely prevented. Of interest, a calcium-dependent activation of the PI(3)K/Akt pathway has been recently observed after stimulation of the G protein–coupled EDG receptor by sphingosine 1-phosphate (also known to exert angiogenic effects). Finally, we also documented that hsp90 was rapidly and dose-dependently tyrosine phosphorylated on statin exposure (Figure 4C), confirming the direct effect of the statin on the chaperone protein. Further studies are under way to examine whether the alteration in calcium homeostasis and the tyrosine phosphorylation of hsp90 are linked and to identify the determinants of these alterations after the statin exposure.

In summary, we have identified the caveolin/eNOS and hsp90/eNOS interactions as key pharmacological targets to modulate NO-dependent angiogenesis. Together with the direct stimulatory effects of statins on eNOS mRNA stability (not observed at the concentrations of statins used in this study, eg, 0.1 and 1 μmol/L), our data support the therapeutic potential of statins but also of other modulators of hsp90 and/or caveolin to promote neovascularization, particularly in the context of ischemic diseases.

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Hsp90 and caveolin are key targets for the pro-angiogenic nitric oxide-mediated effects of statins.

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**Expanded Methods.**

**Free cholesterol efflux.** AEC and CMEC were seeded in 6-well plates at a density of $2.10^4$ cells/well. After 48h, the medium was replaced with EGM and 5µCi/ml $[^3]$H]-cholesterol dispersed in 0.1% ethanol for 48 hours. Before each efflux experiment, cells were washed 3 times with EBM and then incubated with EBM containing 20% fetal calf serum. Media aliquots were taken at different times of incubation. At the end of the experiment, cells were solubilized in 0.5 M NaOH to determine protein and $[^3]$H]-cholesterol content. Results are expressed as the percentage of labeled cholesterol remaining in the cells as a function of time.

**Transfection.** COS-7 cells were transfected with the S1177A mutant and wild-type eNOS constructs (gifts from Dr S. Dimmeler) using Lipofectamine (Invitrogen) according to the manufacturer’s protocol.

**Nitrite detection.** Quantitative analysis of nitrite ($NO_2^-$) was used as an index of NO production. Acidic iodide was used to convert $NO_2^-$ to NO that was electrochemically measured with a NO-selective microsensor (WPI), as recommended by the manufacturer; adequate controls using either vehicle or NOS inhibitors were routinely performed in parallel. Data are normalized for the amount of protein in the dish, and are presented for convenience as mean ± SEM. Statistical analyses were made using Student’s t test.
Results.

Different authors have very recently reported the existence of another phosphorylatable residue within eNOS sequence, namely Thr 495 (human sequence), which contrary to Ser 1177, appears to be constitutively phosphorylated (in basal conditions) and needs to be de-phosphorylated to lead to Ca\(^{2+}\)/CaM activation of eNOS\(^{1-3}\).

To ascertain the identity of Ser 1177 in the eNOS sequence as the target of activated Akt upon statin exposure, we used COS cells expressing either the non-phosphorylatable S1177A eNOS mutant (in which the serine 1177 is mutated in alanine) or wild-type eNOS. Cells were treated with 1µM atorvastatin for 3 hours and nitrite accumulation was determined electrochemically. Online Figure 1 shows that a 75 ± 7% increase (P<0.01, n =3) in nitrite production (over basal level) was observed in the WT-eNOS expressing cells whereas the S1177A mutant did not respond to atorvastatin treatment; this stimulatory effect of statins was completely blocked by LY294002 (see online Figure 1). Note also that two other reasons allow to preclude a role of the phosphorylation of the residue Thr 495 in our experiments: (i) The phosphorylation of Thr 495 was shown not to be mediated by Akt (but probably by PKC\(^{1,3}\)) whereas the promoting effects of statins on tube formation (and Ser 1177 eNOS phosphorylation) were Akt-dependent as shown by the inhibitory effect of the PI(3)K inhibitor LY294002 (this study). (ii.) Calyculin, which is known to block the de-phosphorylation of Thr 495\(^3\), did not prevent the pro-angiogenic effects of statins in our study (not shown).
Evidence that Ser 1177 functionally mediates the statin-dependent increase in NO production in transfected COS cells. COS cells were transfected with the constructs encoding for wild-type eNOS (left) or S1177A eNOS mutant (right). NO$_2^-$ accumulation in the extracellular medium (collected in the 45-48h interval after transfection) was determined by electrochemical detection. Data are expressed as percentages (± s.e.m) of the NO$_2^-$ production in cells expressing WT-eNOS; *P<0.01 vs control condition, n=3. The expression of wild-type and mutant eNOS was controlled by IB using anti-eNOS antibodies.
REFERENCES.

