Simvastatin Inhibits Angiotensin II–Induced Abdominal Aortic Aneurysm Formation in Apolipoprotein E–Knockout Mice
Possible Role of ERK

Yali Zhang, Jack C. Naggar, C. Michael Welzig, Debbie Beasley, Karen S. Moulton, Ho-Jin Park, Jonas B. Galper

Objective—Abdominal aortic aneurysm (AAA) is a life-threatening disease affecting almost 10% of the population over age 65. Generation of AAAs by infusion of angiotensin (Ang) II in apolipoprotein E–knockout (ApoE<sup>−/−</sup>) mice is an animal model which supports an imbalance of the renin–angiotensin system in the pathogenesis of AAA. The effect of statins on AngII-mediated AAA formation and the associated neovascularization is not known. Here we determined the effect of simvastatin and the ERK inhibitor, CI1040, on AngII-stimulated AAA formation.

Methods and Results—ApoE<sup>−/−</sup> mice infused for 28 days with AngII using osmotic minipumps were treated with placebo, 10 mg/kg/d simvastatin, or 100 mg/kg/d CI1040. 95% of AngII-treated mice developed AAA with neovascularization of the lesion, increased ERK phosphorylation, MCP-1 secretion, and MMP activity. These effects were markedly reversed by simvastatin and in part by CI1040. Furthermore, simvastatin and the ERK inhibitor U0126 reversed AngII-stimulated angiogenesis and MMP secretion by human umbilical vein endothelial cells.

Conclusions—These data support the conclusion that simvastatin interferes with AAA formation induced by AngII in ApoE<sup>−/−</sup> mice at least in part via ERK inhibition. (Arterioscler Thromb Vasc Biol. 2009;29:00-00.)

Key Words: angiotensin II  ■  abdominal aortic aneurysm  ■  statin  ■  ERK inhibition  ■  angiogenesis

Abdominal aortic aneurysm (AAA) is a potentially life-threatening degenerative vascular disease that affects 6% to 9% of men over the age of 65 years, claiming more than 15,000 lives annually.<sup>1</sup> Currently, surgical repair is the only effective method of AAA treatment.<sup>2</sup> Studies of the pathophysiology of AAA have demonstrated that human aneurysmal tissues are characterized by (1) chronic inflammation of the aortic wall with the accumulation of macrophages and MCP-1 secretion; (2) progressive degradation of extracellular matrix including elastin and collagen; (3) increased activity of matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9; (4) reendothelialization of the dilated luminal surface of the vessel wall and pronounced neovascularization of the media and adventitia.<sup>6,7</sup>

Several animal models of AAA have been developed in the mouse, including injury of the aortic wall with calcium chloride<sup>8</sup> or elastase.<sup>9</sup> Infusion of AngII via subcutaneous osmotic minipumps in ApoE<sup>−/−</sup> mice has also been shown to result in reproducible formation of suprarenal AAAs, which exhibit many characteristics of the human disease including secretion of MCP-1;<sup>5</sup> macrophage infiltration, secretion of MMPs, disruption of the media, rupture of the elastic layer, and neovascularization.<sup>10</sup> This is consistent with the finding that AngII not only plays a role in the control of cardiovascular and renal homeostasis but also affects vascular endothelial cell function, macrophage activation,<sup>11</sup> and the contraction, migration, and proliferation of vascular smooth muscle cells.<sup>12</sup> This model supports an imbalance of the renin-angiotensin system in the pathogenesis of AAA and is thus an important model for the study of mechanisms of AAA formation.

The mitogen-activated protein (MAP) kinase family, including ERK, c-Jun N-terminal kinase (JNK), and p38 MAPK, is essential for cellular growth and differentiation. ERK activation has been associated with infiltration and activation of macrophages in asthma and other inflammatory processes<sup>13</sup> and with the expression and activation of MMPs.<sup>14</sup> The role of MMPs in the degradation of extracellular matrix in AAA formation is supported by the finding that in mice targeted disruption of MMP expression<sup>9</sup> or treatment with a nonselective MMP inhibitor, doxycycline,<sup>15</sup> prevented the development of AAA in both the calcium-sensitive and calcium-insensitive models.<sup>16</sup>
chloride and elastase injury models. Although these data suggest that ERK might play a role in AAA formation, no data have been presented directly implicating ERK in the pathogenesis of AAA.

HMG-CoA reductase inhibitors, statins, have been shown to have both antiinflammatory and antiproliferative properties which appear to be independent of lipid lowering.\(^{16,17}\) These include the inhibition of MCP-1, IL-6, and IL-8 production by macrophages and endothelial cells.\(^{18,19}\) In the kidney of a double transgenic rat model overexpressing the human renin and angiotensinogen genes, statins interfere with the AngII-mediated inflammatory response.\(^{20}\) Finally, statins have been found to interfere with angiogenesis in both in vivo and in vitro models for VEGF- and FGF-stimulated new blood vessel formation.\(^{21}\)

Both inflammation and angiogenesis are involved in AAA formation suggesting that statins might protect the aorta from AngII-stimulated aneurysm formation in ApoE\(^{-/-}\) mice.

Here we demonstrate that the HMG-CoA reductase inhibitor, simvastatin, markedly decreased both the extent and severity of AngII-stimulated aneurysm formation and MCP-1 and MMP secretion and the level of ERK phosphorylation. Furthermore, treatment of mice with the ERK inhibitor CI1040\(^{22}\) mimicked the effect of simvastatin on AngII-induced AAA formation. These data suggest that statins inhibit AngII-stimulated aneurysm formation at least in part by the inhibition of ERK and that ERK activation might be a new therapeutic target for the treatment and prevention of AAA.

Methods
An expanded Methods section can be found in the supplemental materials (available online at http://atvb.ahajournals.org).

Drug Treatment
Alzet osmotic minipumps (Model 2004, Durect Corporation) were implanted into male ApoE\(^{-/-}\) mice at 5 to 6 months of age to deliver AngII subcutaneously at a dose of 1000 ng/kg/min or saline vehicle for 28 days, as described previously.\(^{23}\) AngII-treated mice were injected subcutaneously with either simvastatin (10 mg/kg/d, Merck), CI1040 (100 mg/kg/d, Pfizer), or vehicle 1 day before minipump implantation, and the treatment continued daily for 28 days. Simvastatin was activated by hydrolysis of the lactone ring as described.\(^{24}\) CI1040 was suspended in an 8:1:1 PBS:ethanol:cremophore solution.

Determination of Blood Pressure and Lipid Profile
Systolic blood pressures were measured in conscious mice using a computerized tail-cuff system (Kent Scientific Corporation). To avoid procedure-induced anxiety, mice were initially acclimated to the instrument for 3 consecutive days before the actual measurement. On the fourth day, pressures were measured in both the morning and afternoon. Moreover the first 5 of 30 blood pressure values at each session were disregarded, and the remaining 25 values were averaged and used for analysis. Total serum cholesterol was determined enzymatically (IDEXX Laboratories).

Classification of Aneurysms, Measurement of Aortic Diameter, and Whole-Mount Staining of Vasa Vasorum
After perfusion with 4% paraformaldehyde, the aorta was exposed under a dissecting microscope, and the periadventitial tissue carefully removed from the aortic wall. Aneurysm severity was rated from Type I to Type IV according to the method of Daugherty et al: Type I, dilated lumen without thrombus; Type II, remodeled aneurysmal tissue with little thrombus; Type III, a pronounced bulbous form of Type II with thrombus; Type IV, multiple, often overlapping aneurysms containing thrombus.\(^{25}\) The outer diameter of the suprarenal aorta was measured with a caliper. Whole-mount CD31 staining of vasa vasorum was carried out as described.\(^{26}\)

Western Blotting
Protein extracts were obtained from corresponding regions of aneurysmal tissue. Western blot analysis was performed as previously described.\(^{24}\) The protein bands were visualized using chemiluminescent substrates (Pierce). p-ERK and ERK antibodies were from Cell Signaling Technology.

Histology and Immunohistochemistry
Perfusion-fixed aortas were embedded in paraffin, cut in cross-section (5 to 10 μm), and stained with hematoxylin and eosin (H&E) and Verhoeff-van Geisen (VVG) for elastin, Mac-3 for macrophages, and CD31 for endothelial cells. Quantification of degree of inflammatory infiltration was performed using IP Laboratory, (Scanalitics) software, which permitted identification of regions with differences in the distribution of color. The region of brown Mac-3 staining in each section was determined and normalized to the total area of the aorta in that low-power field. CD31 staining was carried out as described\(^{26}\) and quantitated by counting microvessels per high-power field.

Measurement for MMP-2 and MMP-9 Activity and MCP-1 Secretion
MMP-2 and MMP-9 activities in homogenates of aortic tissue or conditioned media from human umbilical cord vein endothelial cell (HUVEC) cultures were determined by zymography as previously described.\(^{27}\) MCP-1 was measured by ELISA (Pierce). Equivalent samples were loaded based on protein content (Bradford assay, Bio-Rad).

In Vitro Angiogenesis Model
HUVECs were isolated by the method of Gimbrone\(^{28}\) and cultured as described previously.\(^{24}\) HUVECs grown to near confluence were pretreated either overnight with 1 μmol/L simvastatin or for 1 hour with 10 μmol/L U0126, an ERK inhibitor, followed by a 6-hour incubation with 200 mmol/L AngII. Conditioned medium was collected for analysis of MMPs. For the study of angiogenesis, HUVECs were cultured on Matrigel (Becton Dickinson) and the formation of capillary-like structures, honeycombs, quantitated.\(^{29}\) Specifically, HUVECs were cultured to 80% confluence in 6-well plates and pretreated as described. After pretreatment, HUVECs were trypsinized, plated onto Matrigel-coated 24-well plates (MatTek) at a density of 5×10\(^4\) cells per well, and incubated overnight with AngII and either simvastatin or U0126. After fixing and washing, honeycomb formation was determined by
photomicrography. Images were analyzed using Scion Image software and the total length of tube-like structures quantified. The total percent difference in tube formation relative to control was determined for each group. The mean values from 24 fields in each group from 3 independent experiments were calculated.

Statistical Analysis
To assess statistical significant dependence between treatment (AngII, AngII+simvastatin, AngII+CI1040) and aneurysm subtype variables (Daugherty classification), cross-tabulated contingency tables were constructed and likelihood ratio Chi-square statistics LR were computed. If LR demonstrated significant differences in the aneurysm severity type dependent on treatment, then Cramer’s V and Goodman and Kruskal tau tests were performed to assess the strength of the association. For measurements of aortic diameter, blood pressure, p-ERK, MMPs, total cholesterol, and cellular infiltrates the Kolmogorov-Smirnov test with Lilliefors correction and the Shapiro-Wilk test were used to test for normal distribution. Levene test was used to test for equality of variances. We compared the effect of AngII versus control and the effect of AngII plus simvastatin or AngII plus CI1040 to AngII alone using the Student t test. For multiple t tests comparing the different treatment groups we applied the Bonferroni adjustment to maintain an error rate at α=0.05 throughout the experiment. In all cases, 2-tailed significance levels of P<0.05 or the respectively adjusted levels were considered significant. The results are presented as mean±SE.

Results
Simvastatin Does Not Alter Systolic Blood Pressure and Lipid Profiles of AngII-Infused ApoE−/− Mice
Because AngII is a potent hypertensive agent, systolic blood pressure was measured in both control and drug treated conscious mice with a noninvasive tail-cuff system. AngII infusion for 4 weeks significantly increased systolic blood pressure from 105.9±1.97 mm Hg (n=8) in the control group to 154.2±2.35 mm Hg in the AngII-treated group (n=13, P<0.001), whereas systolic blood pressure in the AngII plus simvastatin–treated group was 155.9±3.82 mm Hg, (n=8, as compared to treatment with AngII alone, supplemental Table I). All ApoE−/− mice developed hyperlipidemia. However, there were no significant differences in total cholesterol between control mice, AngII-treated mice or mice treated with AngII plus simvastatin (supplemental Table I).

Simvastatin Reduces the Incidence and Severity of Aneurysms in AngII-Infused ApoE−/− Mice
To determine the effect of simvastatin on AngII-stimulated AAA formation, AngII-infused mice were treated subcutaneously with simvastatin (10 mg/kg/d) or vehicle. No aneurysms were present in saline-infused control mice (n=10). Nineteen of 20 (or 95%) of AngII-treated mice developed suprarenal aortic aneurysms (Figure 1A and 1B; supplemental Table II). This effect of AngII was markedly lower in mice treated with simvastatin where 8 out of 15, or 53% of AngII+simvastatin treated mice developed aneurysms (Figure 1B, Supplemental Table II). Furthermore, based on the classification system of Daugherty et al.,25 the aneurysms induced by AngII alone were more severe than those in the simvastatin-treated group. Thus, 12 of 20 (or 60%) of mice treated with AngII alone developed either Type III or Type IV aneurysms. Mice treated with AngII+simvastatin developed neither the Type III nor Type IV forms (Figure 1B). The distribution of aneurysms between no aneurysms (none), Type I, Type II, Type III, and Type IV was significantly different between the 2 treatment groups as determined by the Likelihood Ratio χ² test (P<0.001), the Goodman and Kruskal Tau test, and the Cramers V test, P<0.05 for each (supplemental Table II). Furthermore, ApoE−/− mice treated with AngII demonstrated a significantly larger suprarenal aortic diameter compared with the saline control group (1.926±0.140 mm, n=12 versus 1.015±0.008 mm, n=10, respectively, P<0.001). Mice treated with AngII plus simvastatin demonstrated a significantly smaller aortic diameter (1.319±0.088 mm, n=12, P<0.05 versus AngII alone; Figure 1C). Thus, simvastatin treatment markedly attenuated the incidence and severity of AngII-stimulated aneurysm formation.

Simvastatin Decreases Remodeling of the Aortic Wall and the Inflammatory Response in AngII-Induced AAA
H&E staining of cross sections taken from the suprarenal region of the aorta demonstrated that AngII treatment...
resulted in a thickening of the abdominal aortic wall, disruption of the intima with thrombus formation, and destruction of the media and adventitia (Figure 2A). VVG stained sections demonstrated the delicate organization of elastin fibers in control aortas and the disruption of the elastin fibers in the region of apparent discontinuity in the aortic wall (Figure 2A, middle, and Figure 2B). Abundant infiltration by Mac-3–positive macrophages was detected in the adventitia and media of the aortic aneurysms from the AngII-infused mice (Figure 2A, bottom). These histological changes were dramatically less marked in mice treated with AngII plus simvastatin (Figure 2A, right).

Quantitation of the area of Mac-3 staining as a ratio of the total aortic area per low power field demonstrated that both treatment groups were significantly different from control and that macrophage infiltration was significantly lower in mice treated with AngII plus simvastatin compared to the aortas of mice treated with AngII alone (19.9 ± 3.7%, n = 17 versus 47.8 ± 3.0%, n = 16, P < 0.001, Figure 2C).

Simvastatin Attenuates MMP-2 and MMP-9 Activity and MCP-1 Secretion in AngII-Induced AAA

AngII has been shown to stimulate the secretion of MCP-1 and the expression of MMPs during AAA formation. MCP-1 levels in homogenates of AAAs were significantly higher in AngII-treated mice compared to control (34.4 ± 5.16 pg/mg, n = 9, versus 3.72 pg/mg, n = 4, P < 0.001). In mice treated with AngII plus simvastatin, MCP-1 was significantly lower than in mice treated with AngII alone (13.6 ± 5.14 pg/mg, n = 5, P < 0.05; Figure 3A). AngII stimulated MMP-9 gelatinolytic activity in homogenates of aneurysms 4.6 ± 0.47 fold (n = 3, P < 0.05) and MMP-2 × 3.6 ± 0.53-fold (n = 3, P < 0.05) compared to aortas from control mice. Both MMP-2 and MMP-9 activities were significantly lower in mice treated with AngII plus simvastatin compared to mice treated with AngII alone (P < 0.05) and were not significantly different from level in aortas from control mice (Figure 3B and 3C). These data demonstrated that simvastatin decreased the extent and severity of AngII-stimulated aneurysm formation in association with a decrease in expression of MCP-1 and MMP secretion.

Simvastatin Attenuates ERK Phosphorylation in AngII-Induced AAA

Given the role of ERK in the regulation of MMP secretion, we determined the effect of AngII and simvastatin on ERK activation in homogenates of aneurysmal tissue. The relative levels of p-ERK in extracts of AAAs were significantly higher in AngII-treated mice compared to control (34.4 ± 5.16 pg/mg, n = 9, versus 3.72 pg/mg, n = 4, P < 0.001). In mice treated with AngII plus simvastatin, the ratio of p-ERK to ERK was 1.9 ± 0.13 fold higher (n = 3, P < 0.05) compared to aortas from control mice. In mice treated with AngII plus simvastatin, ERK phosphorylation was not significantly different from control, but was significantly lower than in mice treated with AngII alone (0.7 ± 0.07 fold, n = 3, P < 0.05, Figure 4A and 4B).

Inhibition of ERK Activation Interferes With AngII-Induced AAA

To determine whether ERK might play a role in AngII stimulation of AAA formation, mice infused with AngII were treated for 28 days with the MEK1 inhibitor, CI1040,
which specifically inhibits ERK phosphorylation. The administration of CI1040 had no effect on the increase in blood pressure in response to AngII treatment, 159.4 ± 1.36 mm Hg in mice treated with AngII alone (n = 13) compared to CI1040 (n = 13, ns) treated mice. CI1040 had no effect on total cholesterol (346.1 ± 14.4 mg/dL, supplemental Table I). Although 95% of the mice treated with AngII demonstrated AAA formation, only 9 of 14 mice (or 64% of the mice) treated with AngII plus CI1040 developed AAAs (Figure 4C and supplemental Table II). The aneurysms induced by AngII alone were more severe than those in the CI1040 treated group. None of the mice treated with AngII plus CI1040 developed Type IV aneurysms, whereas only 2 of 14 (or 14%) developed Type III aneurysm compared with 12 of 20 (or 60%) of the mice treated with AngII alone who developed either Type III or Type IV aneurysms (Figures 1B and 4C and supplemental Table II). The distribution of aneurysms classified by the 5 groups (none, Type I, Type II, Type III, and Type IV) was significantly different between the 2 treatment groups (P < 0.05, supplemental Table II). Hence CI1040 markedly attenuated the effect of AngII on aneurysm formation. Furthermore, in aortas from mice treated with AngII plus CI1040 the p-ERK/ERK ratio was 56.7 ± 8.6% compared to tissue from mice treated with AngII alone (n = 5, P < 0.055, supplemental Figure I). Finally, MMP-2 activity was 62.3 ± 1.2% in aneurysmal tissue of mice treated with AngII plus CI1040 compared to mice treated with AngII alone (n = 5, P < 0.001, Figure 4D). Surprisingly, CI1040 had no effect on MMP-9 activity. These findings suggest that ERK might play an important role in AAA formation and that the inhibitory effect of simvastatin on AAA formation might be due at least in part to inhibition of ERK activation.

Simvastatin Decreases AngII-Induced Neovascularization of Aneurysmal Tissue

Several studies have demonstrated that new capillary formation associated with AAA appears to be more localized to the site of AAA rupture which suggests that neovascularization is either a response to the inflammatory reaction in the aneurysmal tissue or might play a role in the pathogenesis of AAA. Whole-mount CD31 staining of aortas demonstrated the absence of vasa vasorum from control mice compared with extensive proliferation of the vasa vasorum in area of AAA formation in AngII-treated mice (Figure 5A, left 3 panels). Mice treated with AngII plus simvastatin demonstrated significantly less neovascularization of the aorta in the region of aneurysm formation compared with mice treated with AngII alone (Figure 5A, panel 4). CD31-positive microvessels were essentially undetectable in cross sections of aortas from control mice, whereas numerous capillary vessels could be observed in AngII-treated aortas (Figure 5B). In contrast, mice treated with simvastatin showed a marked decrease in capillary formation compared to mice treated with AngII alone (Figure 5B). Treatment of mice with AngII plus CI1040 also markedly decreased the neovascularization of the aorta in the region of aneurysm formation compared with mice treated with AngII alone (data not shown).

Both Simvastatin and ERK Inhibition Suppress AngII-Induced Tube Formation and MMP-2 Release by HUVECs

The finding that simvastatin and ERK inhibition decreased both the incidence, severity, and the neovascularization of AAAs suggested that the development of new capillaries might constitute either a response to the inflammatory process which accompanies aneurysm formation or might...
AngII has been shown to activate macrophages in the kidney\cite{34} and stimulate the secretion of MMPs in AAA.\cite{10} AngII has also been shown to stimulate neovascularization both in vitro and in vivo models for angiogenesis.\cite{35} Statins have been shown to interfere with the secretion of MMPs in atherosclerotic plaques\cite{36,37} and the migration of macrophages.\cite{38} Statins have also been shown to inhibit neovascularization in response to FGF and VEGF in the mouse corneal pocket and chick chorioallantoic membrane assays.\cite{39} However, direct effects of statins on AngII-stimulated macrophage migration, MMP secretion and neovascularization have not been described. Furthermore, although data suggest that statins reverse the effects of intraluminal infusion of elastase on AAA formation,\cite{39} the effect of statins on AngII-stimulated AAA formation has not been studied. Here we demonstrate that treatment of ApoE\textsuperscript{-/-} mice with simvastatin markedly attenuated the incidence and severity of AngII-stimulated aneurysm formation, MCP-1 secretion, macrophage infiltration, and MMP expression. Furthermore, not only did simvastatin inhibit AngII-mediated ERK activation in the aorta, but treatment of mice with AngII plus CI1040, an inhibitor of ERK activation, partially mimicked the effect of simvastatin on AAA formation by decreasing the incidence and severity of aneurysms and interfering with the increase in MMP-2 secretion and inhibiting AngII-stimulated proliferation of vasa vasorum. These data are consistent with the conclusion that simvastatin interferes with AngII-stimulated aneurysm formation and that inhibition of AngII-stimulated ERK activation in aneurysmal tissue also attenuates AAA formation suggesting that this effect of statins might be attributable at least in part to the inhibition of ERK activation. The extent to which ERK inhibition plays a role in statin inhibition of AAA formation is limited by the finding that CI1040 only partially inhibits ERK phosphorylation suggesting that a more potent ERK inhibitor might have a more significant effect. The limited efficacy of CI1040 has been attributed to its poor solubility and rapid clearance.\cite{40} 

One complication of this study is that the cellular composition of aortas from saline-treated control mice and AngII-treated mice with AAAs is different. To determine whether differences in MMPs, p-ERK, and MCP-1 in control and AngII-treated aortas might be related to these differences in cellular composition of the aneurysmal tissues, mice were treated with a submaximal dose of AngII. Levels of MMPs, p-ERK, and MCP-1 were compared in the suprarenal region of aortas of AngII-treated mice who did not develop aneurysms with those from control mice. Interestingly, levels of MCP-1 and p-ERK in AngII-treated mice who did not develop aneurysms were only modestly elevated and MMP levels were not significantly different from those in control mice. These data supported the conclusion that the markedly increased levels of MCP-1, p-ERK and MMP-2 and MMP-9 in AngII-treated mice that develop AAA are attributable to the cellular proliferation and intense infiltration of the aortic wall by inflammatory cells.
Although AngII increased blood pressure in ApoE<sup>−/−</sup> mice, we demonstrated that the attenuation of AngII-stimulated AAA formation by both simvastatin and CI1040 was independent of any effect on the AngII-stimulated hypertension in these mice. The conclusion that the development of AAA in response to AngII is independent of effects on blood pressure is further supported by the finding that increased blood pressure in response to nor-epinephrine infusion in ApoE<sup>−/−</sup> mice did not induce aneurysm formation<sup>9</sup> and that normalization of blood pressure by hydralazine treatment did not affect the incidence of AAA formation in AngII-treated ApoE<sup>−/−</sup> mice.<sup>41</sup> Furthermore our study showed that neither simvastatin nor CI1040 had an effect on the lipid profile in AngII-treated ApoE<sup>−/−</sup> mice.

The role of neovascularization and endothelial cells in aneurysm formation is unclear. Data have suggested that AngII-induced rupture of AAA is associated with increased medial neovascularization.<sup>7</sup> MMPs have been shown to stimulate inflammatory responses via the proteolytic activation of growth factors and other proteins.<sup>32</sup> Hence MMPs released by endothelial cells in response to AngII during neovascularization might play a role in both the inflammatory response in the wall of the aorta and the disruption of aortic tissue. Our data demonstrate that AngII stimulated a dramatic increase in neovascularization in the region of aneurysm formation in ApoE<sup>−/−</sup> mice. We previously demonstrated that in a mouse model for neovascularization simvastatin interfered with new blood vessel formation.<sup>31</sup> Neovascularization in response to AngII was markedly decreased in the aortas of mice treated with simvastatin and CI1040. Furthermore, in an in vitro model for angiogenesis, simvastatin, and the ERK inhibitor, U0126, both not only reversed AngII-stimulated tube formation by HUVECs, but also reversed the effect of AngII on MMP-2 secretion by HUVECs. Hence, the role of neovascularization in aneurysm formation remains controversial.

A recent study has demonstrated that SP600125, a specific JNK inhibitor, attenuated AAA formation induced by calcium chloride infusion. Furthermore, SP600125 decreased TNF-α stimulated MMP-2 and MMP-9 expression in rat vascular smooth muscle cells and MMP-2 expression was markedly decreased in vascular smooth muscle cells from Jnk2<sup>−/−</sup> mice. MMP-9 activity showed a high correlation with p-JNK but did not correlate with ERK activity. These data suggested that JNK may also play a role in the pathogenesis of AAA<sup>43</sup> and might explain our finding that ERK inhibition by CI1040 inhibited AngII-stimulated MMP-2 activity, while having no effect on the increase in MMP-9 activity. Our data demonstrating that both simvastatin and the ERK inhibitor CI1040 decreases the size and severity of AAA formation in response to AngII, while inhibiting MMP-2 release, support the hypothesis that ERK might play an important role in the pathogenesis of AAA and that simvastatin interferes with AngII stimulation of AAA at least in part via the inhibition of ERK activation. Furthermore, because statins inhibit the farnesylation and membrane localization of Ras, statins might attenuate AngII-mediated AAA formation via the inhibition of the Ras-Raf-MEK-ERK pathway. This pathway has been implicated in the control of events which play a role in tumor cell growth and survival. CI1040 is one of the earliest MEK inhibitors to reach clinical trials as an anticancer agent for ERK dependent tumors.<sup>44</sup> The potential role of statins in the treatment and prevention of AAA and the role of ERK as a therapeutic target in this disease are intriguing.

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**Disclosures**

None.

**References**


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Supplemental Material

Expanded Methods

Tissue Harvesting

Mice were anesthetized with 2.5% isoflurane in 100% oxygen and perfused via the left ventricle with cold saline followed by perfusion with 4% paraformaldehyde for histological studies or with cold RIPA buffer for western blot analysis.

MMP-2 and MMP-9 Activity Assay

MMP-2 and MMP-9 activities in homogenates of aortic tissue or conditioned media from HUVECs cultures were determined by zymography as previously described. Briefly, equal amounts of protein were electrophoresed under non-reducing conditions onto 10% SDS-polyacrylamide gels containing 3 mg/ml gelatin as a substrate. The gels were washed in a buffer containing 2.5% Triton X-100 and 50 mM Tris-HCl (pH7.2) for 1 hour to remove SDS, incubated overnight in a developing buffer: 50 mM Tris-HCl, pH7.2, 10 mM CaCl$_2$, 50 mM NaCl and 0.01% Triton X-100 at 37°C, stained with 0.5% Coomassie blue R-250 in 10% acetic acid and 50% methanol, and destained with the same solution without Coomassie blue. MMP activity was quantified by densitometry scanning.

Histology and Immunohistochemistry

New capillary formation and macrophage infiltration in the media and adventitia were determined by immunohistochemical analysis of frozen sections of aorta. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 30 minutes, and sections were blocked with diluted rabbit or goat serum. Slides were incubated overnight (4°C) with one of the following primary antibodies (Becton Dickinson/Pharmingen, CA, USA): 1µg/ml monoclonal rat
anti-mouse Mac3 for macrophages; monoclonal rat anti-mouse CD31 for endothelial cells.

Biotin-conjugated secondary antibodies were applied for 30 minutes at room temperature, and immune complexes were detected by avidin-biotin-peroxidase with 3,3′-diaminobenzidine (Vectastatin Elite kit; Vector laboratories Inc.). Slides were counterstained with hematoxylin.

Counts of CD31-positive microvessels in the medial and adventitial layers were made using light microscopy to quantify microvessels per high power field.

Whole-Mount CD31 Staining of Vasa Vasorum

Perfusion-fixed aortas were permeabilized overnight with buffered saline containing 1% BSA and 0.1% Triton X-100 and endogenous peroxidase activity blocked by incubation in 0.01% hydrogen peroxide for 1 hour. Aortas were then probed with a monoclonal rat anti-mouse CD31 antibody (0.5 μg/ml, Becton Dickinson/PharMingen) overnight (4°C) followed by an incubation with a biotinylated goat anti-rat IgG secondary antibody (1:500, Becton Dickinson/PharMingen) for 2 hours. Using an avidin-linked peroxidase complex kit (ABC kit, Vector Laboratories), aortas were further incubated with 0.05% diaminobenzidine substrate in 50 mM Tris, pH 7.4 followed by 0.01% hydrogen peroxide. The color was developed over 5 min in a shaded area and the aorta dissected to remove periaortic tissue while leaving vasa vasorum intact. In some cases aortas were subsequently processed for Sudan IV staining.

References


Supplemental Table I  
**Effect of simvastatin and AngII on systolic blood pressure and lipid profile in ApoE-/- mice**

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<th>Systolic blood pressure, mmHg</th>
<th>Total cholesterol, mg/dL</th>
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<tr>
<td>Control (n=8)</td>
<td>105.9±2.0</td>
<td>423.6±36.2</td>
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<tr>
<td>AngII (n=13)</td>
<td>154.2±2.4*</td>
<td>393.0±25.8</td>
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<tr>
<td>AngII+Simvastatin (n=8)</td>
<td>155.9±3.8*</td>
<td>405.4±21.6</td>
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<tr>
<td>AngII+CI1040 (n=7)</td>
<td>159.4±1.4*</td>
<td>346.1±14.4</td>
</tr>
</tbody>
</table>

Systolic blood pressure was measured using a tail-cuff system. Lipid analysis was performed at the end of the study. All results are expressed as mean±SEM. *P<0.001 compared with control.

Supplemental Table II  
**Crosstabulation Table used for Statistical Analysis of Aneurysm Severity by Treatment**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aneurysm Type</th>
<th>Count</th>
<th>%</th>
<th>Aneurysm Type</th>
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<th>Total</th>
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<td>Type II</td>
<td>Type III</td>
<td>Type IV</td>
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</tbody>
</table>

*AngII vs. AngII+Simvastatin 21.072 (p<0.001) 0.463 (p<0.05) 0.680 (p<0.05)
**AngII vs. AngII+CI1040 13.192 (p<0.05) 0.310 (p<0.05) 0.557 (p<0.05)
†Likelihood Ratio, ‡Goodman and Kruskal Tau, ††Cramer’s V
**Supplemental Figure S1**

**CI1040 reduces ERK phosphorylation in AngII treated ApoE-/- mice.** Western blots of p-ERK and ERK and quantitation of p-ERK/ERK ratio from suprarenal aneurysmal tissue from AngII and AngII+CI1040 treated mice. *P*<0.05 vs. AngII, n=5.