Fluid Shear Stress and NO Decrease the Activity of the Hydroxy-Methylglutaryl Coenzyme A Reductase in Endothelial Cells via the AMP-Activated Protein Kinase and FoxO1

Beate Fisslthaler,* Ingrid Fleming,* Benjamin Keserü, Kenneth Walsh, Rudi Busse

Abstract—The rate-limiting enzyme for cholesterol synthesis, the hydroxy-methylglutaryl coenzyme A reductase (HCR), is phosphorylated by the AMP-activated protein kinase (AMPK). As shear stress activates the AMPK in endothelial cells, we determined whether it affects HCR activity and subsequent HCR-dependent signaling. Shear stress (12 dynes cm⁻²) rapidly increased the phosphorylation and activity (6.5- and 4-fold, respectively) of the AMPK in cultured endothelial cells and the activated AMPK phosphorylated the HCR in vitro. Moreover, shear stress and the AMPK activator 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) attenuated endothelial HCR activity by 37% and 33%, respectively. Inhibition of NO production attenuated the acute shear stress–induced phosphorylation of the AMPK and the decrease in HCR activity. Prolonged shear stress (18 hours) led to a significant (50%) decrease in HCR mRNA expression that was dependent on NO, AMPK, and the subsequent phosphorylation and degradation of FoxO1a. Correspondingly, the downregulation of FoxO (small interfering RNA) decreased HCR expression. Prolonged shear stress also attenuated the bradykinin-induced activation of Ras and extracellular signal-regulated kinase 1/2, a phenomenon that was comparable to the effects of cerivastatin and that was reversed by mevalonate and thus attributed to HCR inhibition. A decrease (35%) in HCR expression was also detected in femoral arteries from mice following voluntary exercise, and the bradykinin-induced vasodilatation of the mouse hindlimb was attenuated by both exercise and the HCR inhibitor cerivastatin. These data indicate that fluid shear stress regulates the activity and expression of the HCR in endothelial cells and determines responsiveness to stimuli, such as bradykinin via a mechanism involving NO, AMPK, FoxO1a, and p21Ras. (Circ Res. 2007;100:e12-e21.)

Key Words: endothelial cells ■ HMG-CoA reductase ■ nitric oxide ■ shear stress ■ signal transduction

The adenosine-monophosphate–activated protein kinase (AMPK) was initially identified as the kinase that phosphorylates the 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase (HCR), the rate-limiting enzyme of cholesterol biosynthesis.¹ The AMPK-dependent phosphorylation of the HCR on Ser871 inactivates the enzyme and thus attenuates cellular cholesterol synthesis.² As the name suggests, the AMPK is activated by increased intracellular concentrations of AMP and is generally described as a “metabolite-sensing kinase.” As a consequence, AMPK is activated following heat shock, vigorous exercise, hypoxia/ischemia, and starvation and appears to be a metabolic master switch, phosphorylating key target proteins that control flux through metabolic pathways of hepatic ketogenesis, cholesterol synthesis, lipogenesis, triglyceride synthesis, adipocyte lipolysis, and skeletal muscle fatty acid oxidation (reviewed elsewhere¹⁴). Initially, the AMPK was simply thought to be an indicator of intracellular energy demands and to modulate intracellular energy levels as well as to initiate events that maintain intracellular ATP levels and prevent its depletion.⁵ However, it is now apparent that the AMPK can be activated by additional intracellular signals, including upstream kinases such as LKB1 and Ca²⁺/calmodulin-dependent kinase kinase,⁶ as well as by reactive oxygen species.⁷-⁹ Relatively little is known about the role of the AMPK in endothelial cells, although its activation has been linked to the phosphorylation and activation of the endothelial nitric oxide synthase (eNOS)⁸,¹⁰,¹¹ and implicated in hypoxia-induced angiogenesis.¹²

We recently reported that fluid shear stress activates the AMPK in cultured endothelial cells,¹³ therefore, the aim of the present investigation was to determine whether fluid shear...
stress affects HCR activity and/or expression in endothelial cells via an AMPK-dependent mechanism and to identify additional signaling molecules involved in this process. Furthermore, given the prominent consequences of HCR inhibition by substances such as cerivastatin on the activation of small G proteins and associated endothelial cell signaling, we assessed the consequences of fluid shear stress on the bradykinin-induced, Ras-dependent activation of extracellular signal-regulated kinase 1/2 (ERK1/2).

Materials and Methods

Materials

Antibodies for Western blotting directed against AMPK, phospho-AMPK, ERK1/2, phospho-Thr202/Tyr204 ERK1/2, FoxO1, and phosphory-Thr32 FoxO1/Thr32 FoxO3A were from Cell Signalling (New England Biolabs, Frankfurt, Germany); the antibodies against platelet endothelial cell adhesion molecule (PECAM)-1 were from Santa Cruz (Heidelberg, Germany). NS2028 was from Merck (Darmstadt, Germany), cerivastatin was provided by Bayer AG (Wuppertal, Germany). N'-nitro-L-arginine (L-NA), 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR), iodothecurcidin, and all other substances were from Sigma (Deisenhofen, Germany).

Endothelial Cells

Human umbilical vein and porcine aortic endothelial cells as well as murine lung endothelial cells were isolated and cultured as described. Primary and first passage cultures of human and porcine endothelial cells were used throughout, whereas murine cells were used between passages 3 and 5. Confluent endothelial cell cultures were incubated in culture medium containing 2% FCS and were used as a control. Flow cytometry analysis demonstrated that >90% of the isolated endothelial cells were positive for PECAM-1. Confluent cultures were treated with 0.25% trypsin and 0.02% EDTA (3 min), pelleted, and resuspended in a buffer containing 200 mmol/L NaCl, 1 mmol/L EDTA (pH 7.4), and 0.1% Nonidet P-40.

Immunoblotting

Cells were lysed in buffer containing Tris/HCl (pH 7.5; 50 mmol/L), NaCl (150 mmol/L), NaF (100 mmol/L), Na2VO4 (2 mmol/L), leupeptin (2 μg/mL), pepstatin A (2 μg/mL), trypsin inhibitor (10 μg/mL), phenylmethylsulfonyl fluoride (44 μg/mL) and Triton X-100 (1% vol/vol), left on ice for 10 minutes and centrifuged at 10,000 g for 10 minutes. Cell supernatants or immunoprecipitates were heated with SDS-PAGE sample buffer and separated by SDS-PAGE as described.

AMPK Activity Assay

AMPK-α1 was immunoprecipitated from Triton X-100–soluble cell supernatants, and AMPK activity was assessed by determining the incorporation of 32P into the synthetic SAMS peptide (H2N-HMRSAMSGLHLVKRR-COOH) over 30 minutes, as described. The 32P incorporation was exposed to an X-ray film.

HCR Activity Assay

Endothelial cells were subjected to shear stress (12 dynes cm−2) in the absence and presence of cerivastatin (10 mmol/L) and snap frozen in liquid nitrogen. HCR activity was assayed as described with minor modifications. Briefly, microsomal proteins from liver or endothelial cells were resuspended in a buffer containing K2HPO4 (pH 7.4, 50 mmol/L), diithiothreitol (5 mmol/L), EDTA (0.5 mmol/L), and KCl (0.2 mol/L), and the activity of the HCR was assessed in the presence of 5–14C-mevalonolactone and 3-hydroxy-3 methyl[3-14C]-glutaryl-coenzyme A. The reaction mixture was separated using instant thin layer chromatography monosil glass fiber sheets (Pall, Dreieich, Germany) with acetone/benzene (1:1, vol/vol) as liquid phase. After drying, the region containing the mevalonolactone was excised and the radioactivity assessed by liquid scintillation counting. The 14C-mevalonolactone synthesized was normalized to the recovery of the 14H-mevalonolactone. HCR activity is presented as cerivastatin-sensitive (10 mmol/L) activity relative to that assessed in untreated cells.

Ras Activity Assay

Activated Ras protein was quantified by affinity purification with a glutathione S-transferase (GST) fusion protein, corresponding to residues 1 to 149 of the Ras-binding domain (RBD) of human Ras-1, bound to glutathione agarose beads (Upstate, Dundee, UK). Briefly, after stimulation cells were snap frozen and lysed in a buffer containing HEPES (25 mmol/L, pH 7.5), NaCl (250 mmol/L), Nonidet P-40 1%, MgCl2 (10 mmol/L), EDTA (5 mmol/L), and glycerol (10%). Lysates were then incubated with the beads (1 hour, 4°C), which were subsequently recovered by centrifugation, washed twice with lysis buffer, boiled in SDS sample buffer, and subjected to SDS-PAGE (14% acrylamide). Ras was detected using a specific polyclonal antibody (Upstate). In some experiments, activated Ras was detected by an ELISA using glutathione-coated plates coated with GST-Raf-RBD (Active Motif, Rixensart, Belgium).

Adenoviral Transduction of Endothelial Cells

Subconfluent (60% to 80% confluent) endothelial cells were infected with adenoviruses to overexpress constitutively active AMPK, dominant-negative AMPK (mutation of Lys45 to Arg), wild-type eNOS, or an inactive eNOS mutant (Y657E), as described.

Small Interfering RNA

To silence FoxO gene expression, transfection of a small interfering RNA (siRNA) duplex was performed using TransGene II transfection reagent (Mo Bi Tec, Goettingen, Germany) using the primers listed in Table 1. The primers and the siRNA also targeted the green fluorescent protein DNA sequence CCACTACCTGAGCACCCAG, sequence for FoxO3a. A nonrelated siRNA, which targeted the green fluorescent protein DNA sequence CCACTACCTGAGCACCCAG, was used as a control.

Real-Time RT-PCR

Total cellular RNA was isolated using TRIzol (Sigma, Heidelberg, Germany) or an Absolutely RNA Miniprep kit (Stratagene, Amsterdam, The Netherlands), and equal amounts (1 μg) of total RNA were reverse transcribed (Superscript III, Invitrogen, Heidelberg, Germany). HCR, eNOS, and PECAM-1 were detected using SYBR Green (Absolute QPCR SYBR Green Mix; Abgene, Hamburg, Germany) using the primers listed in Table 1. The primers and the TaqMan probe used for the detection of the 18S RNA were purchased from Applied Biosystems (Darmstadt, Germany). PCR product sizes and specificity were verified by separation of the fragments on an agarose gel and visualized by staining with ethidium bromide. Cycle threshold (Ct) values obtained by real-time PCR on a MX4000 (Stratagene) were converted into relative amounts on the basis of a standard curve, and HCR and eNOS levels were normalized with respect to 18S RNA or PECAM-1.
Voluntary Exercise
C57 black 6 mice (Charles Rivers, Sulzfeld, Germany) and eNOS−/− mice (generated by Axel Gödecke, Düsseldorf,24 and bred in the animal facility of the University Hospital, Frankfurt) were either kept in standard cages or supplied with a calibrated exercise wheel for 3 or 7 days.

Perfused Hindlimb
C57 black 6 mice either received daily subcutaneous injections of cerivastatin (0.25 or 2.5 mg/kg per day), an equal volume of vehicle (0.9% NaCl), or were placed in cages containing a calibrated exercise wheel. After 3 days, the animals were euthanized and vasodilatation to acetylcholine, bradykinin, and sodium nitroprusside was assessed in the hindlimb, as described.25

Statistics
Data are expressed as mean±SEM, and statistical evaluation was performed using Student’s t test for unpaired data, 1-way analysis of variance (ANOVA) followed by a Bonferroni t test, or ANOVA for repeated measures where appropriate. Values of P<0.05 were considered statistically significant.

Results
Effect of Fluid Shear Stress on AMPK and HCR Activity
In confluent cultures of human endothelial cells, a low level of AMPK phosphorylation on Thr172 was detected (Figure 1). The application of fluid shear stress (12 dynes cm−2) elicited a marked and time-dependent phosphorylation of the AMPK that was evident as early as 2 minutes after the application of the stimulus (Figure 1A) and was maintained as long as the stimulus was applied. The phosphorylation of AMPK on Thr172 correlated with the activation of the kinase, assessed as its ability to phosphorylate the SAMS peptide. The kinase inhibitor iodotubercidin (10 μmol/L), prevented the shear stress–induced phosphorylation and activation of the AMPK (Figure 1). Identical results were obtained in human endothelial cells preconditioned to fluid shear stress for 18 hours to induce endothelial cell remodeling as well as in porcine endothelial cells (data not shown).

As the AMPK was initially identified as an HCR kinase,2 we next determined the effects of shear stress on the ability of the AMPK to phosphorylate the enzyme. It was not possible to demonstrate the phosphorylation of the endogenous HCR by Western blotting as the endogenous protein is rapidly degraded. Therefore, the AMPK was immunoprecipitated from unstimulated cells and the phosphorylation of the purified catalytic subunit of the HCR was assessed in vitro (Figure 2A). The AMPK immunoprecipitated from unstimulated cells phosphorylated the purified HCR protein but phosphorylation was markedly increased when the peptide was incubated with the AMPK immunoprecipitated from cells exposed to shear stress (12 dynes cm−2; 30 minutes). As expected, no phosphorylation was detectable in the absence of the HCR.

Phosphorylation of the HCR has been linked to its inhibition26 and exposure of endothelial cells to shear stress (12 dynes cm−2; 2 hours) significantly reduced endogenous HCR activity (Figure 2B). A similar effect was observed in cells treated with the AMPK activator AICAR (1 mmol/L). It was not possible to determine whether the effects of either fluid shear stress or AICAR were sensitive to iodotubercidin as the kinase inhibitor directly inhibited HCR activity to the same extent as cerivastatin (data not shown). Because of the large cell number required for this assay, porcine rather than human endothelial cells (first passage) were used. However, in preliminary experiments, both cell types responded to fluid shear stress with a comparable decrease in HCR activity.

Role of NO in the Shear Stress–Induced Activation of AMPK and Inhibition of HCR Activity
Because shear stress is the physiologically most important stimulus for the continuous production of NO, we assessed the consequences of NOS inhibition on the activation of the AMPK. As mentioned above, shear stress (5 minutes to 24 hours) stimulated the phosphorylation of the AMPK (Figure 3A). The addition of L-NA (300 μmol/L) slightly attenuated the initial activation of the AMPK, ie, during the first 15 minutes of cell stimulation, and significantly reduced the phosphorylation of the kinase at all other time points (Figure 3A). Moreover, the shear stress–induced phosphorylation of the AMPK was evident in cultured lung endothelial cells from wild-type mice but was not observed in endothelial cells from eNOS−/− mice (Figure 3B). The shear stress–induced (2 hours) decrease in endothelial HCR activity was also mark-
thelial cells from eNOS−/− mice was associated with a significant decrease in HCR activity (Figure 3D). A decrease in HCR activity was also observed in endothelial cells from wild-type mice overexpressing the constitutively active AMPK (Figure 3E).

Regulation of HCR Expression by Shear Stress and FoxO Transcription Factors

To determine whether a change in HCR expression could account for the decrease in its overall activity in endothelial cells exposed to shear stress, particularly for longer periods, we assessed HCR mRNA levels. Fluid shear stress induced a significant time-dependent decrease in HCR mRNA expression in human umbilical vein endothelial cells, so that expression was reduced by approximately 70% after 24 hours (Figure 4B). Whereas Nω-nitro-D-arginine did not affect the shear stress–induced decrease in HCR mRNA, L-NA prevented this effect (Figure 4B). L-NA methyl ester (300 μmol/L) and the guanylyl cyclase inhibitor NS2028 (10 μmol/L) also prevented the shear stress–induced decrease in HCR/18S RNA mRNA expression, so that expression was comparable in cells maintained under static conditions (100%) and cells exposed to fluid shear stress in the presence of L-NA methyl ester and NS2028 (134 ± 48% and 164 ± 50%, respectively; n = 4). On the other hand, the NO donor diethylenetriamine-NO2,2,2-(Hydroxynitrosohydrazono)bis-ethanamine (DETA-NO) (10 and 100 μmol/L) significantly attenuated HCR expression (Figure 4C), a stimulus that also elicited the phosphorylation of the AMPK (Figure 4D).

The expression of the HMG-CoA synthase gene, which is coregulated with the HCR gene, is reported to depend on the activity of a number of transcription factors, including FoxO3a/FKHRL1.28 FoxO factors are generally classified as downstream targets of the protein kinase Akt, which phosphorylates and renders them transcriptionally inactive by promoting their nuclear exclusion and subsequent proteasomal degradation.29 However, FoxOs can also be activated by

![Figure 2](http://circres.ahajournals.org/)

**Figure 2.** Effect of AMPK from shear stress–stimulated endothelial cells on the phosphorylation of the purified HCR. A, Representative autoradiograph showing the effect of AMPK immunoprecipitated from human endothelial cells either maintained under static conditions (Static) or exposed to fluid shear stress (12 dynes cm−2, 30 minutes) on the in vitro phosphorylation of the catalytic subunit of HCR. The lower panels show AMPK phosphorylation in the same cell lysates. Similar results were obtained in 3 additional experiments. B, Effect of shear stress (12 dynes cm−2, 2 hours) and AICAR (1 mmol/L, 2 hours) on the cerivastatin-sensitive activity of microsomal HCR in porcine endothelial cells. The data presented summarize the results of 4 to 7 independent experiments. *P<0.01 vs the respective control (CTL).

![Figure 3](http://circres.ahajournals.org/)

**Figure 3.** Role of eNOS in the shear stress–induced activation of AMPK and inhibition of HCR activity. Endothelial cells were exposed to shear stress (12 dynes cm−2) for up to 24 hours in the presence of either solvent (culture medium) or L-NA (300 μmol/L). The phosphorylation of AMPK (pAMPK) was assessed by Western blotting and quantified with respect to total AMPK levels in human umbilical vein endothelial cells (A) and lung endothelial cells from wild-type (WT) or eNOS−/− mice (B). C through E, The cerivastatin-sensitive activity of microsomal HCR was assessed in microsomes prepared from porcine endothelial cells exposed to shear stress (2 hours) in the absence and presence of L-NA (C), lung endothelial cells isolated from eNOS−/− mice with either wild-type eNOS or an inactive eNOS mutant (Y657E; inset demonstrates equal expression of eNOS) (D), or lung endothelial cells isolated from wild-type mice infected with either a control (CTL) virus or with the constitutively active AMPK (CA-AMPK) adenovirus (E). The data presented summarize the results of 3 to 7 independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs the respective control.
AMPK-activating stimuli, such as glucose starvation and AICAR. We therefore determined whether or not shear stress affected the phosphorylation and degradation of FoxO transcription factors and whether this was linked to the activation of AMPK.

Shear stress elicited the rapid phosphorylation of FoxO1a in endothelial cells, a phenomenon that was associated with a concomitant decrease in FoxO1a protein and is consistent with the rapid degradation of the phosphorylated protein (Figure 5A). The phosphatidylinositol 3-kinase inhibitors wortmannin (20 nmol/L) and LY294002 (10 nmol/L) attenuated the shear stress–induced activation of Akt but failed to prevent the phosphorylation of FoxO1a (data not shown). However, overexpression of a constitutively active AMPK resulted in a decrease in FoxO1a expression (Figure 5B).

To demonstrate a link between FoxO transcription factors and HCR expression, we assessed the effects of siRNA targeted against FoxO1a and FoxO3a. The siRNA approach resulted in a significant decrease in HCR mRNA expression as detected by real-time RT-PCR, whereas a control siRNA was without effect on either FoxO protein or on HCR mRNA levels (Figure 5C).

Regulation of HCR Expression in Exercising Mice
To assess the effect of shear stress on HCR expression in vivo, mice were housed in standard cages and half of them were provided with a running wheel. After 1 week of voluntary exercise (3838±348 m/d, n=6) HCR expression in the femoral arteries was decreased relative to that of the static group (Figure 6A). In contrast, eNOS expression was significantly higher in the same arteries from the exercised than the sedentary group of animals (Figure 6B). We were unable to perform the same series of experiments with eNOS knockout animals as they ran significantly less than the wild-type mice (eNOS−/− mice ran 1197±404 m/d; n=6, P<0.01). However, HCR activity was significantly greater in livers from wild-type than from eNOS−/− mice maintained under static conditions, indicating that the lack of endothelium-derived NO is associated with an increase in HCR activity in this organ (Figure 6C). The latter difference in activity is most probably related to changes in proteins expression and/or post translational protein modification as HCR mRNA levels were not significantly different between the 2 strains of mice (Figure 6D).

Functional Consequences of the Inhibition of HCR
One functional consequence of the inhibition of the HCR is the attenuation of intracellular signaling events caused by the attenuation of isoprenylation of Ras. We therefore assessed the effects of shear stress on Ras activation, as well as on the ability of bradykinin (a Ras-activating agonist) to elicit the activation of ERK1/2 in endothelial cells either maintained under static conditions or exposed to fluid shear stress (18 hours) or pretreated with cerivastatin.

Activated Ras could be detected in human endothelial cells maintained under static conditions, indicating a basal level of activity. Ras activity was however significantly attenuated in cells preexposed to fluid shear stress or to cerivastatin (Figure 7A). In cells maintained under static conditions, bradykinin (100 nmol/L, 2 minutes) activated Ras, a response that was markedly attenuated in cells exposed to fluid shear stress or to cerivastatin. Moreover, although bradykinin increased the content of p21Ras in the microsomal protein fraction by 75%, the agonist failed to elicit marked Ras translocation in cells preexposed to shear stress (membrane Ras levels were increased by only 12% versus the solvent treatment; values are
the mean of 2 independent experiments). Consistent with the link between the activation of the AMPK, inhibition of the HCR and Ras activity, the AMPK activator AICAR (1 mmol/L, 18 hours) decreased basal Ras activity in endothelial cells by 22.7% versus solvent-treated cells (P<0.01, n=4), as assessed using a specific ELISA for activated p21Ras.

Effects similar to those on the activation of Ras were observed on the basal and bradykinin-induced phosphorylation of ERK1/2. The pretreatment of endothelial cells with shear stress attenuated both the basal and the bradykinin-induced phosphorylation of ERK1/2 without affecting the expression of the kinases (Figure 7B). Similar effects were observed following pretreatment of the cells with cerivastatin (Figure 7B) but not in cells exposed to shear stress or cerivastatin in the presence of mevalonate (0.5 mmol/L) to rescue Ras isoprenylation (Figure 7C).

To determine whether or not the effects observed in vitro were of physiological relevance, we next assessed the vasodilator response to bradykinin in the hindlimbs of mice receiving cerivastatin or performing voluntary exercise. In these experiments, exercise was limited to 3 days to avoid effects associated with training such as angiogenesis and increased eNOS expression. We observed that although cerivastatin (0.25 and 2.5 mg/kg/d) and exercise did not affect the maximal vasodilator responses to acetylcholine and sodium nitroprusside (Table 2), responses to bradykinin were significantly attenuated (Table 2 and Figure 8).

**Discussion**

The results of the present investigation indicate that the activity and expression of the HCR in endothelial cells is modulated by fluid shear stress. Two distinct mechanisms appear to be involved: an initial NO-dependent activation of the AMPK that phosphorylates the HCR to decrease its activity and a more prolonged effect linking NO and the AMPK to the phosphorylation and degradation of FoxO1a and a decrease in HCR mRNA expression (Figure 9). This mechanism also appears to be relevant in vivo as the expression of the HCR was attenuated in femoral arteries from mice removed performing voluntary exercise.

We and others have recently reported that the AMPK in endothelial cells can be activated by fluid shear stress. It is not entirely clear how the application of fluid shear stress to the endothelial cell surface can activate the kinase, but the signaling cascade does appear to be distinct from that involving PECAM-1 and leading to the activation of the phosphatidylinositol 3-kinase and Akt. A role for mitochondria-derived reactive oxygen species has been suggested, and there is more than circumstantial evidence for a
role for eNOS in the activation of the AMPK. Indeed, NO facilitates the generation of mitochondrial reactive oxygen species and the subsequent phosphorylation of the AMPK and peroxynitrite, the reaction product of superoxide anions and NO, activates the kinase in endothelial cells. Moreover, activation of the AMPK by the antidiabetic drug metformin is prevented by a NOS inhibitor and metformin fails to activate the AMPK in eNOS−/− mice. Our results agree with these observations, as the shear stress–induced activation of the AMPK was largely dependent on the production of NO and was markedly attenuated in endothelial cells from eNOS−/− mice. Although it has been suggested that the shear stress–induced activation of the AMPK affects eNOS phosphorylation and activation, we have consistently failed to detect a significant link between AMPK and eNOS (unpublished observations, 2005). Our results clearly indicate that the activation of the AMPK is downstream and not upstream of the activation of eNOS by fluid shear stress.

Because the AMPK was initially characterized as the acetyl-CoA carboxylase kinase 3 and the HCR kinase, we assessed the effects of the AMPK immunoprecipitated from endothelial cells exposed to fluid shear stress on the in vitro phosphorylation of the purified catalytic subunit of the HCR. Consistent with the results of the AMPK activity assay, the extracts from endothelial cells exposed to fluid shear stress phosphorylated the reductase. However, as it was not possible to demonstrate the phosphorylation and degradation of the endogenous enzyme using either commercially available or custom-made antibodies (unpublished observations, 2006), we assessed the effect of shear stress on HCR activity. Fluid shear stress significantly attenuated the production of 14C-mevalonolactone in endothelial cells via a mechanism that was sensitive to NOS inhibition. Moreover, the AMPK activator AICAR and a constitutively active AMPK attenuated HCR activity to a similar extent. Although the degree of inhibition of HCR activity was significant, the effect was less pronounced than that achieved using cerivastatin.
The turnover of the HCR protein is rapid and the phosphorylation of HCR enhances its cleavage by calpain-2 in vitro, resulting in the disappearance of the 97-kDa holoenzyme and the appearance of a soluble 52- to 56-kDa reductase fragment contain the active site. In addition, HCR activity declines rapidly in cycloheximide-treated cells, highlighting the importance of continual HCR gene expression in maintaining protein levels. As the effects of shear stress on the activity of the HCR were prolonged, we hypothesized that hemodynamic stimuli may affect the expression of the enzyme in addition to its phosphorylation. Indeed, HCR mRNA levels were time-dependently decreased in endothelial cells exposed to fluid shear stress. This response could be reproduced by incubating cells with a NO donor and was not observed in shear-stress-stimulated cells treated with a NO inhibitor or an inhibitor of the guanylyl cyclase. Voluntary exercise in mice results in increased blood flow and the shear stress–induced activation of eNOS and was associated with a decrease in femoral artery HCR expression. Although the differences in voluntary running distance precluded a direct comparison between HCR expression in arteries from wild-type and eNOS–/– mice, HCR activity was significantly greater in the livers from eNOS–/– mice than that from wild-type animals in the absence of a significant effect on expression. Moreover, reconstitution of endothelial cells from eNOS–/– mice with the wild-type enzyme resulted in a significant decrease in HCR activity.

The regulation of HCR expression is relatively complex, and the mevalonate pathway is modulated in large part by feedback control of the amount of HCR protein. The expression of the HCR is coregulated with that of the HMG CoA synthase gene, which is regulated by a number of transcription factors, including FoxO transcription factors. The latter are generally classified as downstream targets of the protein kinase Akt, which phosphorylates and renders them transcriptionally inactive by promoting their nuclear exclusion and subsequent proteasomal degradation. Although the activation of Akt often seems to be inextricably linked to that of the AMPK, our results suggest that the latter kinase is an important regulator of FoxO transcription factors and of the HCR in cells stimulated by fluid shear stress. Indeed, a constitutively active (but not a dominant-negative) AMPK mutant enhanced the degradation of FoxO1a and downregulating FoxO1a using as siRNA approach also decreased HCR expression in endothelial cells. Moreover, there is a precedent for the regulation of FoxO by the AMPK as AMPK-activating stimuli such as glucose starvation and AICAR also activate FoxO1a.

Much interest has been expressed in the pleiotropic effects of HCR inhibitors such as cerivastatin and it is highly likely that the beneficial effects of the statins on vascular homeostasis can largely be attributed to the consequences of HCR inhibition on intracellular signaling. Many of these pleiotropic effects are mediated by inhibition of isoprenoids, which serve as lipid attachments for intracellular signaling molecules, in particular, inhibition of the small guanosine triphosphate–binding proteins Rho, Ras, and Rac, whose membrane localization and function are dependent on isoprenylation. There are many reports demonstrating the effects of statins on Ras and Rac activation and the subsequent

### Table 2. Maximal Decrease in Perfusion Pressure in the Perfused Murine Hindlimb in Animals

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Solvent</th>
<th>Cerivastatin (0.25 mg/kg)</th>
<th>Cerivastatin (2.5 mg/kg)</th>
<th>Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>31.7±3.7</td>
<td>29.1±3.4</td>
<td>29.8±2.5</td>
<td>29.9±2.9</td>
</tr>
<tr>
<td>Bradykinin</td>
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<td>36.2±3.1</td>
<td>39.3±4.9*</td>
<td>34.6±2.04*</td>
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<tr>
<td>SNP</td>
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<td>29.0±2.6</td>
<td>30.7±1.7</td>
<td>34.5±2.9</td>
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</tbody>
</table>

Data show maximal decrease in perfusion pressure (mm Hg) in the perfused murine hindlimb elicited by acetylcholine (1 μmol/L), bradykinin (1 μmol/L), and sodium nitroprusside (SNP) (10 μmol/L) in animals receiving solvent or cerivastatin (0.25 or 2.5 mg/kg per day) or performing voluntary exercise for 3 days (n=6–9 animals per group). *P<0.05 vs the static, solvent-treated control group.

### Figure 8. Effect of HCR inhibition and voluntary exercise on the bradykinin-induced vasodilatation of the mouse hindlimb.

C57 black 6 mice either received subcutaneous injections of cerivastatin (0.25 or 2.5 mg/kg per day) or an equal volume of vehicle (0.9% NaCl), or were placed in cages containing a running wheel. After 3 days, animals were euthanized and the vasodilator response to bradykinin (0.1 nmol/L to 1 μmol/L) was assessed in the buffer-perfused hindlimb as described in Materials and Methods. The graph summarizes the results obtained using 6 to 9 animals per group. P<0.05 vs the solvent-treated group.

### Figure 9. Scheme showing the proposed link between shear stress, the AMPK-dependent decrease in HCR activity and expression, and attenuated Ras and ERK1/2 signaling in endothelial cells.

The beneficial effects of the statins on vascular homeostasis can largely be attributed to the consequences of HCR inhibition on intracellular signaling. Many of these pleiotropic effects are mediated by inhibition of isoprenoids, which serve as lipid attachments for intracellular signaling molecules, in particular, inhibition of the small guanosine triphosphate–binding proteins Rho, Ras, and Rac, whose membrane localization and function are dependent on isoprenylation. There are many reports demonstrating the effects of statins on Ras and Rac activation and the subsequent...
Our findings demonstrate that fluid shear stress, in addition to decreasing HCR activity and expression, also attenuates Ras activation and the phosphorylation of ERK1/2 in response to bradykinin to the same extent as cerivastatin. The effects of shear stress on Ras and ERK1/2 activity can be linked to the decrease in HCR activity/expression inasmuch as the inclusion of mevalonate, to rescue protein isoprenylation, prevented the shear stress–induced attenuation of bradykinin-induced Ras activation.

In addition to the inhibitory effects of shear stress and cerivastatin on bradykinin-induced signaling in vitro we found that 2 concentrations of the HCR inhibitor as well as voluntary exercise modestly reduced the vasodilator response to bradykinin in the mouse hindlimb. Although, at first glance, this finding appear to contradict previous observations that “statins” improve endothelial function, it is important to note that such observations have generally been made in isolated aortic or carotid artery rings in which NO is the primary endothelium-derived relaxing factor. An endothelium-derived hyperpolarizing factor (EDHF), however, seems to play a major role in the bradykinin-induced vasodilatation of the mouse hindlimb, and statins do not improve EDHF-mediated responses. Unfortunately, little is currently known about the role of small G proteins in EDHF-mediated relaxations; but one candidate EDHF, 11,12-epoxyeicosatrienoic acid, has been linked with the activation of Rac. It is also important to bear in mind that unlike many other endothelium-dependent vasodilators, bradykinin is an inflammatory mediator that enhances vascular permeability and adhesion molecule expression. Therefore, the inhibition of bradykinin-activated Ras-dependent signaling may contribute to the anti-inflammatory effects of statin therapy, as well as the beneficial effects of exercise in vivo. Although these data correlate well with the findings obtained in vitro, we are aware that these studies do not show a direct link between exercise, the activation of AMPK and inhibition of Ras signaling. This is currently not possible to address directly, as the inhibitors of the AMPK that are currently available are poorly tolerated in vivo, and even in vitro prolonged incubation frequently results in cell death; furthermore, as both of the AMPKα isoforms (AMPKα1 and α2) can potentially phosphorylate the HCR, it is likely that studies in AMPKα−/− mice will be confounded by compensation by the remaining isoform.

Taken together, the results of the present investigation indicate that fluid shear stress regulates HCR activity and Ras-dependent signaling in endothelial cells via short- and long-term mechanisms involving the phosphorylation of the enzyme by AMPK, the AMPK-dependent phosphorylation and degradation of FoxO1a and a subsequent decrease in HCR expression, respectively. Moreover, the shear stress– and exercise-induced decrease in HCR activity and Ras-dependent signaling may account for many of the beneficial effects of exercise and NO production on endothelial cell function.

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Disclosures

None.

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Fluid Shear Stress and NO Decrease the Activity of the Hydroxy-Methylglutaryl Coenzyme A Reductase in Endothelial Cells via the AMP-Activated Protein Kinase and FoxO1

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