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# AMP-Activated Protein Kinase Is Involved in Endothelial NO Synthase Activation in Response to Shear Stress

Yingjia Zhang, Tzong-Shyuan Lee, Erik M. Kolb, Kai Sun, Xiao Lu, Frances M. Sladek, Ghassan S. Kassab, Theodore Garland Jr, John Y.-J. Shyy

**Objective**—The regulation of AMP-activated protein kinase (AMPK) is implicated in vascular biology because AMPK can phosphorylate endothelial NO synthase (eNOS). In this study, we investigate the regulation of the AMPK–eNOS pathway in vascular endothelial cells (ECs) by shear stress and the activation of aortic AMPK in a mouse model with a high level of voluntary running (High-Runner).

**Methods and Results**—By using flow channels with cultured ECs, AMPK Thr172 phosphorylation was increased with changes of flow rate or pulsatility. The activity of LKB1, the upstream kinase of AMPK, and the phosphorylation of eNOS at Ser1179 were concomitant with AMPK activation responding to changes in flow rate or pulsatility. The blockage of AMPK by a dominant-negative mutant of AMPK inhibited shear stress-induced eNOS Ser1179 phosphorylation and NO production. Furthermore, aortic AMPK activity and level of eNOS phosphorylation were significantly elevated in the aortas of High-Runner mice.

**Conclusions**—Our results suggest that shear stress activates AMPK in ECs, which contributes to elevated eNOS activity and subsequent NO production. Hence, AMPK, in addition to serving as an energy sensor, also plays an important role in regulating vascular tone. (*Arterioscler Thromb Vasc Biol.* 2006;26:1281-1287.)

**Key Words:** endothelium ■ AMPK ■ nitric oxide synthase ■ shear stress ■ exercise

Endothelium-derived NO can enhance vascular functions, including vessel relaxation, survival of vascular endothelial cells (ECs), inhibition of platelet aggregation, and attenuation of leukocyte infiltration.<sup>1,2</sup> Impaired NO bioavailability has been suggested as one of the earliest pathophysiological events preceding endothelial dysfunction and contributing to atherosclerosis.<sup>3,4</sup> Shear stress is an important physiological stimulus that enhances the production of NO by ECs.<sup>2,5</sup> An increase in shear stress such as in exercise augments the EC-mediated bioavailability of NO.<sup>6</sup>

Endothelial NO synthase (eNOS), the key enzyme for NO production in ECs, is tightly regulated not only at the transcriptional level but also by several post-translational mechanisms. The enhanced phosphorylation of Ser1179 of bovine eNOS (Ser1177 in humans) leads to increased eNOS activity. Mounting evidence has shown that shear stress enhances the phosphorylation of Ser1177/1179.<sup>7–9</sup> Use of the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin and LY 294002 has demonstrated that Akt phosphorylates eNOS Ser1177/1179 in response to shear stress.<sup>7,8</sup> However, dominant-negative mutants of Akt were unable to block the shear stress-stimulated Ser1179 phosphorylation.<sup>9</sup> Further, H89, a protein kinase A (PKA) inhibitor, and an adenovirus-

expressing PKA inhibitor (PKI) blocked the eNOS Ser1179 phosphorylation, which indicates the involvement of PKA.<sup>9–12</sup>

Functioning as a metabolic master switch, AMP-activated protein kinase (AMPK) senses and regulates the cellular energy status in various cell types. AMPK is activated by several physiological and pathological stresses such as exercise, hypoxia, and nutrient depletion that result in increased AMP-to-ATP ratio. Once activated, AMPK switches on the catabolic pathways that produce ATP while alleviating the ATP-consuming processes. LKB1, identified as a gene mutated in human Peutz-Jeghers syndrome, has been shown recently to be the upstream kinase phosphorylating AMPK.<sup>13–15</sup> In addition to phosphorylating multiple target proteins involved in energy regulation, such as acetyl coenzyme A carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, AMPK also phosphorylates eNOS at Ser1177/1179.<sup>16</sup> Indeed, AMPK is required for adiponectin-, thrombin-, and histamine-induced eNOS phosphorylation and subsequent NO production in ECs.<sup>17–19</sup> Activation of AMPK by the pharmacological activators 5'-aminoimidazole-4-carboxamide ribonucleoside or carbonyl cyanide m-chlorophenylhydrazone also results in eNOS activation in ECs.<sup>19</sup>

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Given the importance of AMPK in regulating energy balance and activation of eNOS, we examined the role of AMPK in eNOS phosphorylation in response to shear stress and its *in vivo* relevance in aorta of mouse with a high level of voluntary exercising (High-Runner). We demonstrated that AMPK is involved in shear stress-stimulated eNOS phosphorylation and NO production, particularly responding to changes in magnitude and pulsatility of shear stress. Further, High-Runners with high levels of voluntary exercise exhibit elevated AMPK and diaminofluorecein-2-diacetate (eNOS) phosphorylation in their aortas.

## Methods

### Materials

Antibodies against phospho-AMPK Thr172, LKB1, phospho-Src Tyr416, and Src were from Cell Signaling Technology. Anti-pan- $\alpha$ -AMPK, anti-phospho-ACC Ser79, and anti-phospho-Akt Ser473 antibodies were from Upstate Biotechnology. Anti-eNOS and anti-phospho-eNOS Ser1177/1179 were from BD Biosciences Pharmingen. Antibody against  $\alpha$ -tubulin and Akt were from Santa Cruz Biotechnology. Griess reagent and diaminofluorescein-2-diacetate (DAF-2 DA) were from Sigma and Calbiochem, respectively.

### Cell Culture and Fluid Shear Stress Experiments

Bovine aortic ECs (BAECs) isolated from bovine aortas were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. All experiments used cells within passage 2-5. A parallel-plate flow channel was used to impose laminar flow on confluent monolayer of BAECs as described.<sup>20</sup> The flow system was maintained at 37°C and ventilated with 95% humidified air and 5% CO<sub>2</sub>. The following 3 flow conditions were used: (1) step flow (instantaneous increasing shear stress from 0 to 10 dyne/cm<sup>2</sup>, followed by steady flow at 10 dyne/cm<sup>2</sup> for periods as indicated), (2) increased magnitude of shear stress (pre-exposure to shear stress at 5 dyne/cm<sup>2</sup> for 6 hours, followed by an increase in shear stress to 10 dyne/cm<sup>2</sup>), (3) increased pulsatility of shear stress (pre-exposure to 1-Hz pulsatile flow with shear stress at 10 dyne/cm<sup>2</sup>, followed by an increase of pulsatility to 2 Hz with the same magnitude of shear stress).

### Immunoblotting Analysis

BAEC lysates and mouse aortic extracts were resolved on SDS-PAGE according to standard protocols. After being transferred to nitrocellulose membranes, the samples were immunoblotted with primary antibodies as indicated, followed by secondary antibodies conjugated with horseradish peroxidase. The recognized bands were revealed by ECL detection kit (Amersham Biosciences) and quantified by Scion Image software (Scion Corp).

### Kinase Activity Assays

The kinase activities of LKB1 and AMPK were assayed as described.<sup>15,21</sup> Briefly, endogenous LKB1 or AMPK was immunoprecipitated from cell lysates by anti-LKB1 or anti-pan- $\alpha$ -AMPK, respectively. The phosphorylation of glutathione-S-transferase (GST)-AMPK by LKB1 was performed in a buffer containing 50 mmol/L Tris-HCl, pH 7.5, 10 mmol/L MgCl<sub>2</sub>, 1 mmol/L dithiothreitol (DTT), and 100  $\mu$ mol/L ATP at 30°C for 20 minutes. The phosphorylation of GST-eNOS by AMPK was performed in 40 mmol/L HEPES, pH 7.4, 80 mmol/L NaCl, 5 mmol/L MgCl<sub>2</sub>, 0.1 mmol/L DTT, and 200  $\mu$ mol/L ATP at 37°C for 10 minutes. After the addition of SDS loading buffer to terminate kinase reactions, the products were resolved by SDS-PAGE followed by immunoblotting with anti-phospho-AMPK Thr172 and anti-phospho-eNOS Ser1177 antibodies, respectively.

### Adenoviral Infection

Ad-AMPK-DN, a recombinant adenovirus expressing a dominant-negative mutant of AMPK (AMPK-DN), was generated by subclon-

ing the cDNA encoding AMPK $\alpha$ 1-DN-(D159A)<sup>22</sup> into an adenoviral vector pJM17. Confluent BAECs seeded on collagen I-coated slides or dishes were infected with recombinant adenoviruses at the indicated multiplicity of infection (MOI) and incubated for another 24 hours before experimentation. Ad-GFP-AMPK-CA was used to overexpress a constitutively active AMPK mutant.<sup>23</sup>

### Detection of NO

The adenovirus-infected BAECs were subjected to shear stress in Krebs' buffer containing 118 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl<sub>2</sub>, 1.2 mmol/L MgSO<sub>4</sub>, 1.2 mmol/L KH<sub>2</sub>PO<sub>4</sub>, 11 mmol/L glucose, 25 mmol/L NaHCO<sub>3</sub>, pH 7.4, and 0.1% BSA. Conditioned media were collected and mixed with an equal volume of methanol. After centrifugation at 10 000 rpm for 10 minutes, the supernatants were collected. Nitrite, the stable breakdown product of NO, was measured by ENO-20 NOx Analyser (Eicom).<sup>24</sup> In experiments with the constitutively active AMPK mutant (Ad-AMPK-CA), accumulated nitrite in culture media was measured by use of the Griess reagent.<sup>25</sup>

NO production was also measured by comparing DAF-2 DA fluorescence staining before and after the application of shear stress. Briefly, BAECs were incubated with DAF-2 DA (1  $\mu$ mol/L) at 37°C for 15 minutes. After being washed with PBS, the cells underwent fluorescent imaging with use of a Nikon TE300 fluorescence microscope. Cells were then subjected to shear stress for 1 minute, and images were recorded again with the same exposure time as that before shear stress experiments.<sup>26</sup>

### Detection of AMPK Phosphorylation in Mouse Aorta

The breeding protocols for High-Runners and their control counterparts are described in the online supplements (available at <http://atvb.ahajournals.org>). Eight High-Runners (4 families) and 8 control mice (4 families) were maintained at room temperature on an alternating 12-hour light/dark cycle and fed a standard chow diet *ad libitum*. Each animal was housed in a standard cage attached to a Wahman-type activity wheel, and the running activity was measured and analyzed daily.<sup>27,28</sup> After 52 days, mice were killed, and adventitia was removed from the isolated aortas. Aortas were then homogenized, and 2 aortic extracts from the same family were pooled to yield 8 samples, which were then analyzed by immunoblotting.

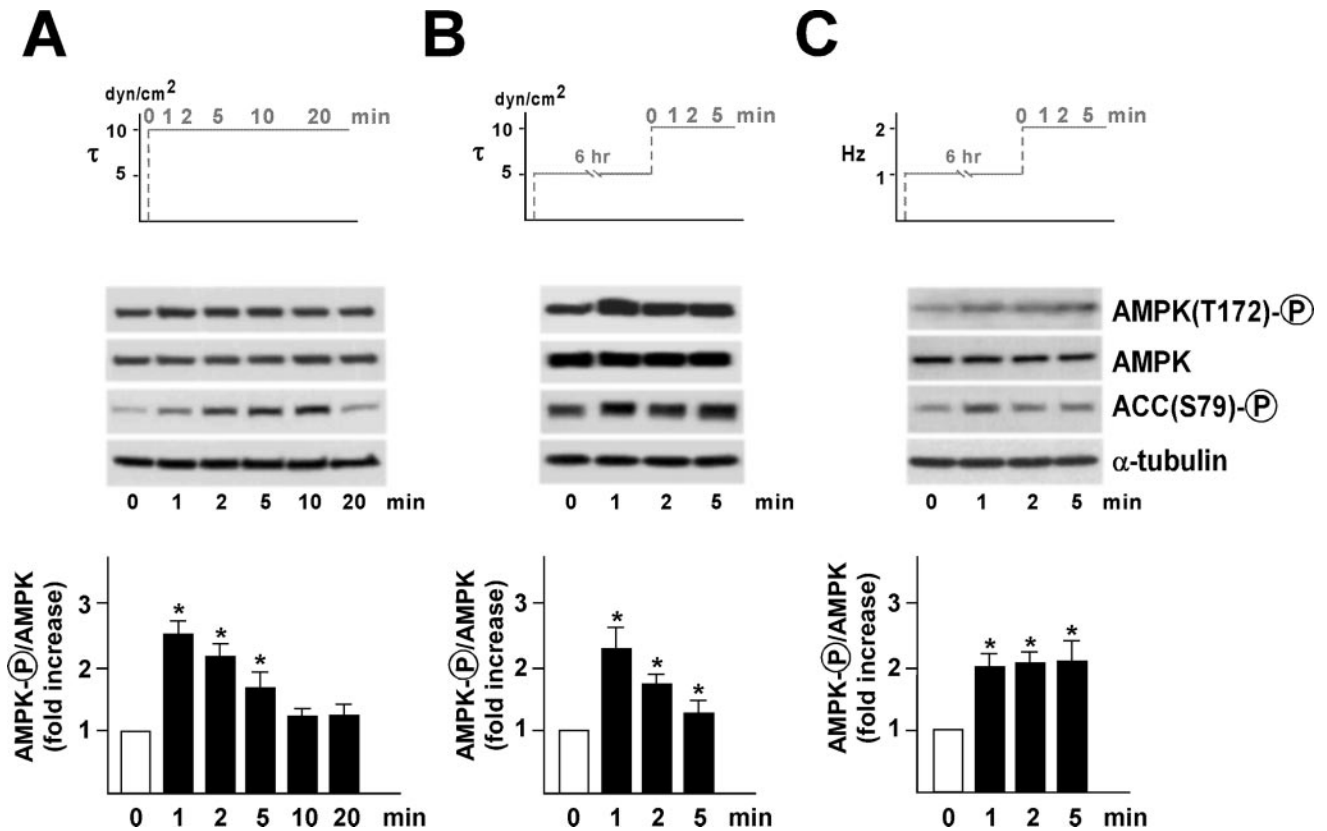
### Statistical Analyses

Unless otherwise indicated, results are reported as means $\pm$ SD from 3 independent experiments. Statistical analyses were performed by 1-way ANOVA followed by Student *t* test between various groups. In all cases, *P*<0.05 was considered statistically significant.

## Results

### Shear Stress Activates the LKB1-AMPK Pathway in ECs

To determine whether AMPK is activated in ECs in response to shear stress, BAECs were subjected to a laminar flow with shear stress of 10 dyne/cm<sup>2</sup> for various durations. Compared with that in the static control, the level of AMPK Thr172 phosphorylation was increased as early as 1 minute after the exposure to shear stress (Figure 1A). The phosphorylation of AMPK Thr172 led to its activation, as indicated by the corresponding phosphorylation of ACC Ser79, a substrate of AMPK (Figure 1A). To investigate whether changes in the magnitude of shear stress caused the AMPK activation, BAECs were pre-exposed to a laminar flow (5 dyne/cm<sup>2</sup>) for 6 hours, which was followed by a sudden increase to 10 dyne/cm<sup>2</sup>. Immunoblotting showed that the increase in shear stress caused a rapid phosphorylation of AMPK Thr172 and



**Figure 1.** Shear stress stimulates the phosphorylation of AMPK in BAECs. Confluent BAECs were subjected to a step flow (0 to 10 dyne/cm<sup>2</sup>) for the indicated times (A); pre-exposed to shear stress (5 dyne/cm<sup>2</sup>) for 6 hours followed by an increase to 10 dyne/cm<sup>2</sup> for up to 5 minutes (B); or pre-exposed to a pulsatile flow (1 Hz; 10 dyne/cm<sup>2</sup>) for 6 hours and increased frequency of pulsatility to 2 Hz for up to 5 minutes (C). Cell lysates were analyzed by immunoblotting. The bottom panels are densitometry quantification. Data show mean±SD from 3 independent experiments. \**P*<0.05.

ACC Ser79 (Figure 1B). Similarly, change in pulsatility from 1 to 2 Hz increased the AMPK and ACC phosphorylation (Figure 1C). In all conditions tested, the level of total AMPK protein did not change, which indicates that increased phosphorylation of Thr172 by shear stress was not attributable to increased expression of AMPK.

Because LKB1 can function as an AMPK kinase,<sup>13–15</sup> we explored whether LKB1 was also modulated by shear stress. BAECs were exposed to the same flow conditions as those in Figure 1, and LKB1 was immunoprecipitated for kinase activity assay with GST–AMPK fusion protein used as the substrate. As shown in Figure 2A, the phosphorylation of GST–AMPK was increased by shear stress in a time-dependent manner. Similarly, changes in both the magnitude and pulsatility of shear stress induced LKB1 kinase activity, as measured by the phosphorylation of GST–AMPK (Figure 2B and 2C).

### AMPK Phosphorylates eNOS in Response to Shear Stress

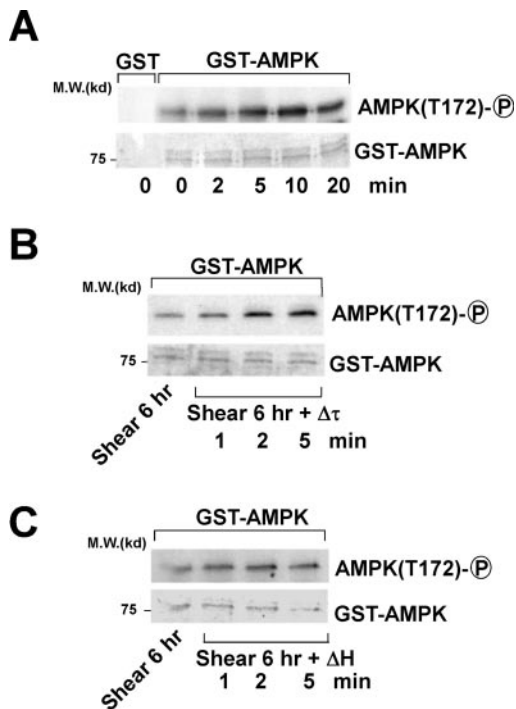
Shear stress is known to be a physiological stimulus of eNOS phosphorylation. As anticipated, the time course of eNOS Ser1179 phosphorylation paralleled that of AMPK (ie, occurred as early as 1 to 2 minutes after flow application or changes of magnitude/pulsatility; Figure 3A through 3C), suggesting that eNOS is a candidate substrate of AMPK. Because AMPK purified from the rat liver has been shown to phosphorylate recombinant eNOS,<sup>16</sup> we immunoprecipitated

AMPK from cell lysates collected from BAECs. The immunoprecipitated AMPK was then used in an *in vitro* activity assay, with GST–eNOS fusion protein used as the substrate. As shown in Figure 3D, activated AMPK by a step increase in shear stress (5 to 10 dyne/cm<sup>2</sup>) enhanced phosphorylation of eNOS Ser1179, suggesting that AMPK directly phosphorylated eNOS.

To further investigate the role of shear stress–activated AMPK in eNOS phosphorylation and the ensuing NO production in ECs, we used an adenoviral vector expressing the constitutively active form of AMPK (Ad-AMPK-CA)<sup>23</sup> to mimic the shear stress–activated AMPK. Expression of AMPK-CA in BAECs increased the phosphorylation of eNOS Ser1179 in a dose-dependent manner (Figure 4A). In contrast, the infection of Ad-null control virus had little, if any, effect on eNOS phosphorylation. Furthermore, the expression of AMPK-CA, but not the control virus, significantly increased NO production (Figure 4B).

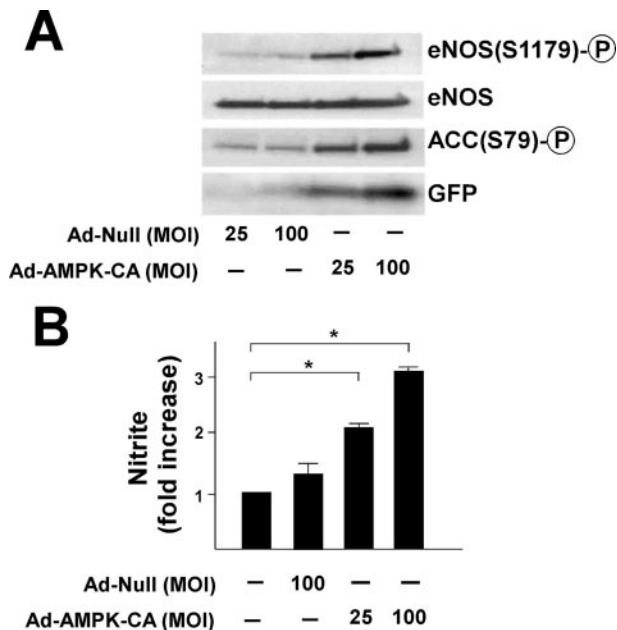
### Ablation of AMPK Attenuates Shear Stress–Stimulated eNOS Phosphorylation and NO Production

To examine whether AMPK is necessary for eNOS phosphorylation in response to shear stress, we constructed a recombinant adenovirus expressing a dominant-negative form of AMPK (Ad-AMPK-DN). With an MOI of 20, the constructed Ad-AMPK-DN blocked the AMPK activation in ECs treated



**Figure 2.** LKB1 phosphorylates AMPK in response to shear stress. BAECs were subjected to the same flow conditions as the correspondent A, B, and C in Figure 1. LKB1 was immunoprecipitated from the cell lysates, and GST-AMPK was used as the substrate in kinase activity assays. The bottom panels are results of Coomassie blue staining of GST-AMPK, indicating comparable loadings.

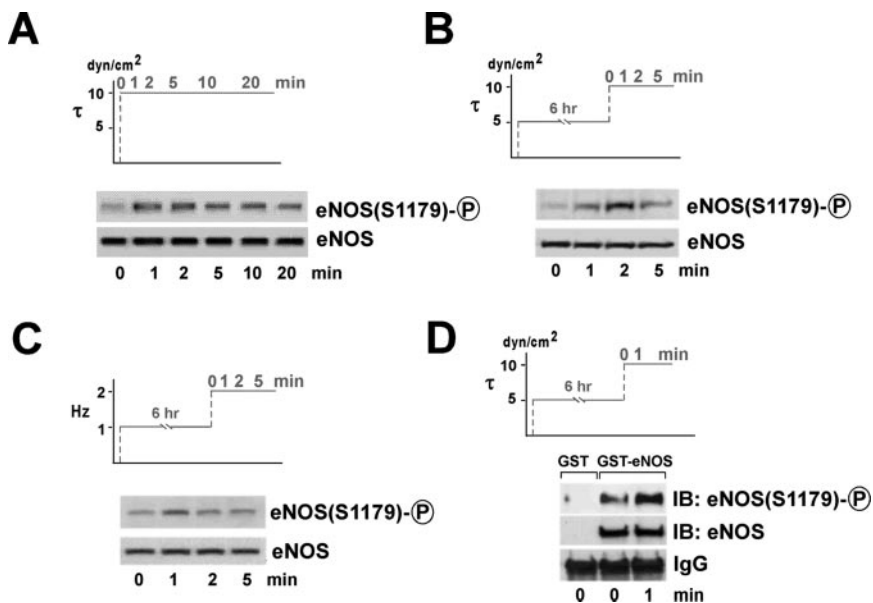
with H<sub>2</sub>O<sub>2</sub>, a treatment that has been shown to activate AMPK<sup>15</sup> (supplemental Figure I, available online at <http://atvb.ahajournals.org>). BAECs infected with Ad-null or Ad-AMPK-DN virus at an MOI of 20 were pre-exposed to shear stress at 5 dyne/cm<sup>2</sup> for 6 hours followed by a step increase to 10 dyne/cm<sup>2</sup> for 1, 2, and 5 minutes. Phosphorylation of AMPK Thr172 and eNOS Ser1179 was observed in Ad-null-infected cells but was attenuated in cells infected with



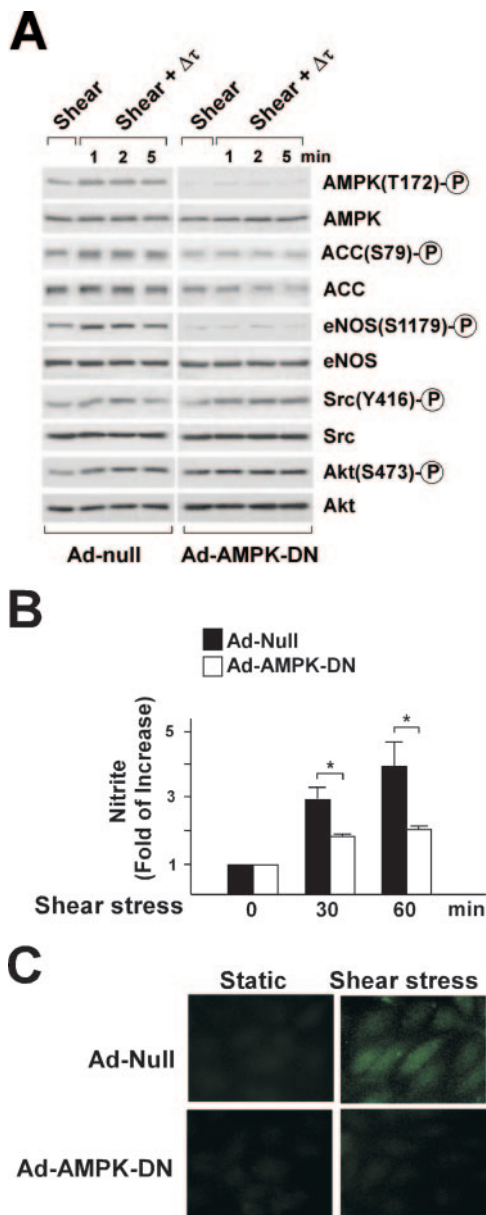
**Figure 4.** Constitutively active form of AMPK induces eNOS phosphorylation and NO production. BAECs were infected with Ad-null or Ad-AMPK-CA adenoviruses. A, One day after the infection, cells were lysed for immunoblotting with various antibodies as indicated. B, The production of nitrite in collected culture media was measured 1 day after the infection. The nitrite concentration in the nonvirus-infected cell culture medium was set as 1. \**P*<0.001.

Ad-AMPK-DN at the 3 time points (Figure 5A). These results demonstrate that AMPK contributes to the phosphorylation of eNOS, especially in response to the rapid changes of shear stress. Interestingly, the shear stress-induced Src and Akt phosphorylation<sup>29</sup> was not affected by the infection of Ad-AMPK-DN (Figure 5A).

We next examined the role of AMPK in shear stress-modulated NO production. As shown in Figure 5B, shear stress led to an increase in NO released into the medium,

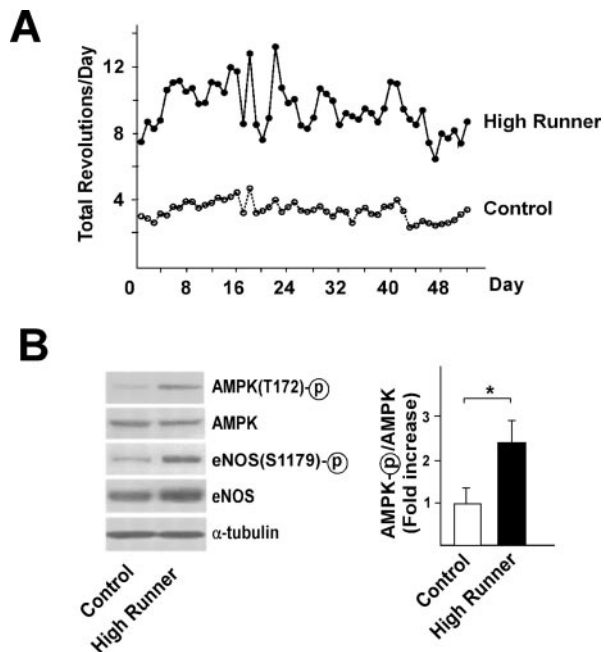


**Figure 3.** AMPK phosphorylates eNOS Ser1179 in response to shear stress. A through C, BAECs were subjected to the same flow conditions as in Figure 1. Cell lysates were subjected to immunoblotting. D, AMPK was immunoprecipitated from cell lysates, and AMPK kinase activity was assayed with recombinant GST-eNOS as the substrate. Phosphorylation of GST-eNOS was detected by anti-phospho-eNOS Ser1179.



**Figure 5.** Dominant-negative mutant of AMPK inhibits eNOS phosphorylation and NO production induced by shear stress. A, The Ad-null- or Ad-AMPK-DN-infected cells were pre-exposed to shear stress at 5 dyne/cm<sup>2</sup> for 6 hours, which was then increased to 10 dyne/cm<sup>2</sup> for 1, 2, or 5 minutes. Cell lysates were analyzed by immunoblotting with indicated primary antibodies. B, The infected cells were subjected to shear stress (10 dyne/cm<sup>2</sup>) for up to 60 minutes. The nitrite concentrations in shearing media were measured by an NOx analyzer. \**P*<0.05. C, DAF-2 DA staining was performed to confirm that Ad-AMPK-DN-infected cells exhibited attenuated NO production in response to shear stress.

whereas BAECs infected with Ad-AMPK-DN produced much less NO. To confirm results obtained from the measurement of NO byproducts, we stained the cells with DAF-2 DA, an NO-sensitive dye. The exposure of BAECs to shear stress for 1 minute resulted in an enhanced DAF-2 DA staining in Ad-null- but not Ad-AMPK-DN-infected cells (Figure 5C).



**Figure 6.** AMPK activity is increased in aortas of High-Runner mice. A, Mean daily wheel running of High-Runners and control mice. B shows representative immunoblotting with the use of various primary antibodies as indicated. The amount of increase is defined as the band intensity of phosphorylated AMPK Thr172 normalized to that of AMPK (n=8). \**P*<0.05.

### The Aortic AMPK Activity Is Increased in Mice With High Voluntary Running

We correlated the endothelial AMPK activity with physiological exercise in a mouse model with a high level of voluntary running, namely High-Runner. High-Runner mice or randomly bred controls were given access to running wheels for 52 days. As shown in Figure 6A, High-Runner mice ran  $\approx 2.5$  to  $3\times$  as many revolutions per day as did control mice. High-Runners also ran at higher average speeds and for more minutes each day (data not shown). Immunoblotting analysis revealed that the level of aortic AMPK in the High-Runner was comparable to that in control mice. However, the ratio of phosphorylated AMPK to total AMPK was  $\approx 2$ -fold higher in High-Runner mice compared with controls (Figure 6B). As well, levels of phosphorylated and expressed eNOS were increased in aortas of High-Runner mice.

### Discussion

Regarded as an energy sensor in muscles and liver, AMPK also plays important roles in vascular biology, such as in hypoxia-induced angiogenesis and adiponectin-activated eNOS.<sup>17,18,30</sup> In the current study, we showed that changes of shear stress, including magnitude and pulsatility, activate AMPK in ECs, that activation of AMPK is involved in the shear stress-stimulated eNOS phosphorylation and the consequent NO production, and that aortic AMPK and eNOS phosphorylation is elevated in mice with intensified voluntary running.

AMPK is activated by many stress conditions that deplete cellular ATP and hence increase the ratio of AMP to ATP. Although a change in AMP-to-ATP ratio in ECs subjected to

shear stress has not been reported, previous studies showed that shear stress induced a fast (<3 minutes) but transient release of ATP, which was proportional to the intensity of the applied shear stress.<sup>31,32</sup> Thus, changes in shear stress might cause a fast, transient depletion of intracellular ATP storage, which results in the rapid activation of AMPK. AMPK activation requires phosphorylation of Thr172 within the  $\alpha$ -subunit catalyzed by LKB1. The increased ratio of AMP to ATP seems to render AMPK a better substrate for LKB1. We showed that shear stress increases the LKB1 kinase activity, as revealed by the increased phosphorylation of GST-AMPK (Figure 2). While this manuscript was prepared, Fleming et al reported that platelet endothelial cell adhesion molecule-1 (PECAM-1) and c-Src are required for the shear stress–phosphorylated Akt and eNOS but not that of AMPK.<sup>29</sup> We also found that PI3K inhibitor wortmannin did not block the shear stress–activated AMPK and –phosphorylated eNOS (supplemental Figure II). Thereby, PECAM-1, Src, and PI3K are less likely to be the upstream of AMPK, at least not involved in the early phase of shear stress activation of AMPK. Together, our results suggest that alteration of shear stress may activate LKB1 or change the AMP-to-ATP ratio so that AMPK serves as a better substrate for an activated AMPK kinase, namely LKB1.

Several kinases seem to be involved in shear stress–activated eNOS. The PI3K-specific inhibitors wortmannin and LY 294002 have been shown to abolish the eNOS Ser1179 phosphorylation and subsequent NO production in response to shear stress,<sup>7,8</sup> which suggests that PI3K/Akt is critical for shear stress–activated eNOS. However, dominant-negative mutants of Akt were unable to inhibit eNOS Ser1179 phosphorylation, although these Akt mutants still inhibited shear-dependent NO production.<sup>9</sup> By using PKA inhibitor H89 and adenoviral PKI, Jo et al showed further that ablation of PKA decreased the phosphorylation of eNOS Ser1179 and NO production.<sup>9,10</sup> Thus, it was suggested that shear stress stimulates the phosphorylation of eNOS Ser1179 by PI3K- and PKA-dependent but Akt-independent mechanisms. The current study demonstrates that constitutively activated AMPK leads to a sustained increase in eNOS Ser1179 phosphorylation and NO production (Figure 4). In the reciprocal experiments, inhibition of AMPK by Ad-AMPK-DN attenuated drastically the phosphorylation of eNOS in response to shear stress. In contrast, such an inhibition of AMPK did not alter the shear stress–activated Akt and c-Src (Figure 5A). Of note, the duration of shear stress was 30 minutes in these previous studies in which various inhibitors (ie, wortmannin, H89, PKI, and PP1) were used.<sup>9–12,29</sup> We found that AMPK was phosphorylated as early as 1 to 2 minutes after the application of shear stress (Figure 1). The temporal response of eNOS phosphorylation was somewhat within the time frame of AMPK phosphorylation (Figure 3). Therefore, it is likely that AMPK may contribute to the rapid activation of eNOS induced by shear stress.

Exercise training increases cardiac output and augments blood flow and wall shear stress, which leads to vessel dilation. For example, lower limb exercise increases the blood flow rate in the human abdominal aorta by >2-fold, and the shear stress in the supraceliac and infrarenal aorta is

increased accordingly.<sup>33</sup> Hence, we used the High-Runner mice, with a much higher voluntary wheel-running trait compared with their controls, to investigate whether exercise activates AMPK in vivo. By comparing the aortic AMPK phosphorylation of the 2 types of mice, we showed that the level of activated AMPK was increased in the High-Runners. Moreover, the aortic eNOS phosphorylation and expression level were also increased in High-Runners.

In summary, our results show that AMPK activity is modulated by shear stress, which contributes to eNOS activation. Interestingly, elevated AMPK and eNOS phosphorylation are present in the conduit vessels of mice with high voluntary exercise. In the future, it will be of interest to examine the cardiovascular benefits of AMPK activation with the use of AMPK knockout mice in conjunction with hemodynamic measurements and the correlation underlying the elevated AMPK and eNOS activity in the High-Runners.

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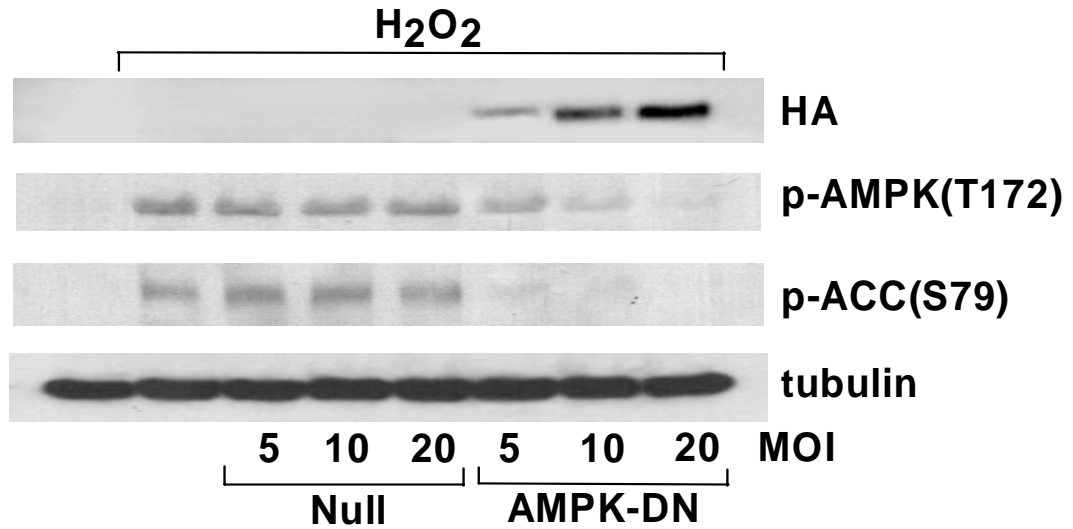
## Supplements

### Mouse Models and Voluntary Wheel-Running Protocols

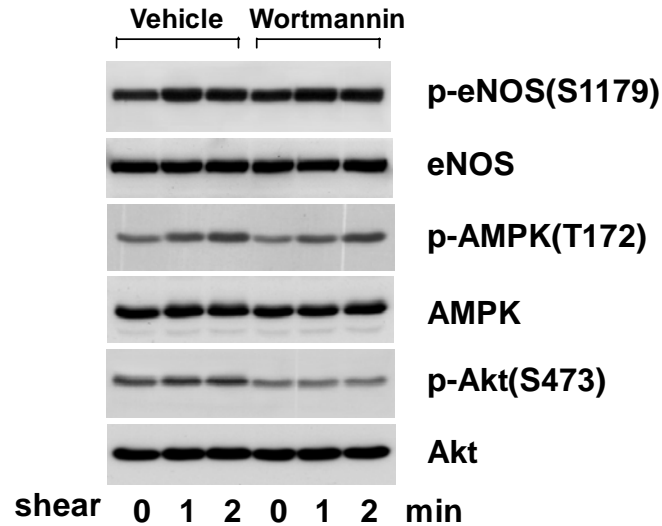
The experiment began in 1993 from a base population of outbred, genetically variable Hsd:ICR laboratory house mice. The base population was divided randomly into 8 genetic lines, each maintained by 10 mating pairs per generation. Four of the lines were bred regardless of their wheel-running trait and thus served as controls for random genetic drift. For the other 4 lines, referred to as ‘High-Runners’, the males and females with the highest running revolutions were selected as breeders (1,2).

Wheel running was measured individually with Wahman-type activity wheels (1) attached to standard housing cages by a stainless steel tunnel, so that each mouse had voluntary, continuous access to a wheel. A photocell counter was attached to each wheel, and customized software (San Diego Instruments, San Diego, CA) was used to record revolutions during 1-min intervals. Data were recorded continuously during wheel access and downloaded daily.

Mice running data from High-Runner and control lines were compared by one-way nested ANOVA, with replicate lines nested within line type and degrees of freedom of 1 and 6 (1,2), with use of SAS Version 8, Procedure Mixed. Because numerous previous studies have shown High-Runner males to run more total revolutions per day, at a higher average speed, and for more minutes per day as compared with control lines (1,2), we used 1-tailed tests to compare wheel-running traits.



**Supplement Figure 1.** Ad-AMPK-DN inhibits the H<sub>2</sub>O<sub>2</sub>-activated AMPK. BAECs were infected with Ad-null or Ad-AMPK-DN adenoviruses at the indicated multiplicity of infection (MOI). One day later, cells were treated with H<sub>2</sub>O<sub>2</sub> (250  $\mu$ M) for 5 min. Cell lysates were analyzed by immunoblotting with the use of antibodies recognizing phosphor-AMPK Thr172 and phosphor-ACC Ser79.  $\alpha$ -tubulin served as an internal control. The membrane was re probed with antibody against HA tag to show the expression of the exogenous AMPK-DN.



**Supplement Figure 2.** PI3K inhibitor wortmannin inhibits the shear stress-induced phosphorylation of Akt but not that of eNOS and AMPK. BAECs were pre-treated with wortmannin (100 nM) for 30 min followed by the application of shear stress (10 dyn/cm<sup>2</sup>) for 1 or 2 min. The level of various proteins were detected by immunoblotting with the use of indicated antibodies.

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