Regulation of Endothelial Cell Cycle by Laminar Versus Oscillatory Flow

Distinct Modes of Interactions of AMP-Activated Protein Kinase and Akt Pathways

Deliang Guo, Shu Chien, John Y.-J. Shyy

Abstract—Steady laminar flow in the straight parts of the arterial tree is atheroprotective, whereas disturbed flow with oscillation in branch points and the aortic root are athero-prone, in part, because of the distinct roles of the flow patterns in regulating the cell cycle of vascular endothelial cells (ECs). To elucidate the molecular basis underlying the endothelial cell cycle regulated by distinct flow patterns, we conducted flow-channel experiments to investigate the effects of laminar versus oscillatory flows on activation of AMP-activated protein kinase (AMPK) and Akt in ECs. Laminar flow caused a transient activation of both AMPK and Akt, but oscillatory flow activated only Akt, with AMPK being maintained at its basal level. Constitutively active and dominant-negative mutants of AMPK and Akt were used to elucidate further the positive effect of Akt and negative role of AMPK in mediating mTOR (mammalian target of rapamycin) and its target p70S6 kinase (S6K) in response to laminar and oscillatory flows. Measurements of phosphorylation of mTOR Ser2448 and S6K Thr389 showed that AMPK, by counteracting Akt under laminar flow, resulted in a transient activation of S6K. Under oscillatory flow, because of the lack of AMPK activation to effect negative regulation, S6K was activated in a sustained manner. As a functional consequence, AMPK activation attenuated cell cycle progression in response to both laminar and oscillatory flows. In contrast, AMPK inhibition promoted EC cycle progression by decreasing the cell population in the G_0/G_1 phase and increasing it in the $S+G_2/M$ phase. In vivo, phosphorylation of the promitotic S6K in mouse thoracic aorta was much less than that in mouse aortic root. In contrast, AMPK phosphorylation was higher in the thoracic aorta. These results provide a molecular mechanism by which laminar versus oscillatory flow regulates the endothelial cell cycle. (***Circ Res*****. 2007;100:564-571.)**

Key Words: Akt ■ AMPK ■ cell cycle ■ endothelial cells ■ shear stress ■ vascular biology

The vascular endothelium plays an important role in the dynamic regulation of vascular functions, rather than being simply an inert cell lining covering the luminal surface of blood vessels. Vascular endothelial cells (ECs) are exposed to shear stress, the tangential component of hemodynamic forces acting on the vessel wall.[1] The magnitude and spatial and temporal features of shear stress are determined by local flow patterns, which, in turn, are affected by the geometry of the vessels. Increasing evidence suggests that laminar flow in straight parts of the arterial tree is atheroprotective, whereas disturbed flow in the curvatures and bifurcations, partially because of its proliferative effect, is proatherogenic.[2] Light microscopic examination of the luminal surface of the entire rabbit thoracic aorta indeed revealed that ECs under disturbed flow patterns exhibited higher mitotic rates.[3] Laminar flow applied to ECs cultured in a flow channel is known to arrest ECs in the G_0/G_1 phase, but disturbed flow promotes cell cycle progression, particularly an increased EC population in the $S+G_2/M$ phase.[4,5] The increased mitotic rate of ECs under disturbed flow may contribute to the increased leaky spots in the curvatures and bifurcations, thus enhancing the uptake of low density lipoprotein (LDL) from the circulation.[6]

At the level of intracellular signaling, laminar flow applied to ECs in a flow channel activates the phosphatidylinositol 3 kinase (PI3K)-Akt/protein kinase B (PKB) pathway.[7] One of the downstream targets of mechanosensitive Akt is the phosphorylation of endothelial nitric oxide synthase (eNOS) Ser1177, leading to increased NO bioavailability, including vasodilation, antiinflammation, and antiapoptosis.[8] The activation of PI3K-Akt should result in proliferative/antiapoptotic signaling by Akt phosphorylating and inactivating the tuberous sclerosis complex (TSC-2).[9,10] TSC-2 forms a complex with TSC-1, which ultimately regulates the mammalian target of rapamycin (mTOR).[11–13] Activated mTOR phosphorylates p70S6 kinase (S6K), which is engaged in ribosome biogenesis and modification of S6, essential for enter-

Original received September 7, 2006; revision received December 18, 2006; accepted January 22, 2007.

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Circulation Research **is available at http://circres.ahajournals.org** **DOI: 10.1161/01.RES.0000259561.23876.c5**

ing the mitotic phase.[14,15] In addition, mTOR phosphorylates 4E binding protein-1 (4EBP1), a eukaryotic initiation factor. As a result of 4EBP1 phosphorylation, the eukaryotic initiation factor 4E (eIF4E) is released for cap-dependent initiation of translation and thus promotes cell growth.[16]

AMP-activated protein kinase (AMPK) is activated by physiological and pathological stresses, such as exercise, hypoxia, and nutrient shortage that deplete cellular ATP in many metabolically related organs.[17] Several recent reports indicate that the proliferation-promoting mTOR pathway is negatively regulated by AMPK. Under energy deprivation, AMPK also phosphorylates TSC-2 and enhances its activity. Phosphorylation of TSC-2 by AMPK in turn leads to an inhibition of mTOR, which causes upregulation of 4EBP1 and downregulation of S6K.[12] Interestingly, shear stress activates AMPK in ECs,[18,19] which phosphorylates 2 AMPK-targeted proteins, eNOS and acetyl–coenzyme A carboxylase (ACC), in ECs. A dominant-negative mutant of AMPK (AMPK-DN) was able to block the shear stress–induced eNOS and ACC activation.[19]

It seems enigmatic that laminar flow activates the proliferation-promoting Akt pathway in the flow channel; yet EC turnover rate is low at the straight parts of the vessels. Here, we show that laminar flow activates both Akt and AMPK. By converging on S6K in an antagonistic manner, these 2 pathways maintain endothelial homeostasis, namely EC arrested in the G_0/G_1 phase. Under oscillatory flow, AMPK is inactive, but the Akt/mTOR/S6K pathway remains activated. Our results may help to interpret the respective physiological and pathophysiological roles of laminar versus disturbed flow in the arterial tree.

Materials and Methods

Antibodies and Reagents

Anti-S6K monoclonal antibody (mAb) was obtained from BD Biosciences Pharmingen (San Diego, Calif). Polyclonal anti–phospho-Akt Ser473 and polyclonal anti-ACC were from Upstate Biotechnology (Lake Placid, NY). Anti-Akt and anti–α-tubulin mAb were from Santa Cruz Biotechnology (Santa Cruz, Calif). Polyclonal anti–phospho-AMPK Thr172, anti–phospho-S6K Thr389, anti–α-AMPK, anti–phospho-ACC Ser-79, anti–phospho-mTOR Ser2448, and anti-mTOR were from Cell Signaling Technology (Beverly, Mass). Compound C was from Calbiochem (La Jolla, Calif). Wortmannin and propidium iodide were from Sigma (St Louis, Mo). Rapamycin was from LC Laboratories (Woburn, Mass). The recombinant adenovirus expressing a dominant-negative mutant of AMPK (Ad-AMPK-DN) was constructed as described previously.[12] The parental adenoviral vector was referred to as null, when used as a control. Ad-AMPK-CA, expressing the constitutively active mutant of AMPK, was provided by Dr B. Viollet (Cochin Hospital, University of Paris V, France).

Cell Culture and Flow Experiments

Bovine aortic endothelial cells (BAECs) were isolated from bovine aortas and cultured in DMEM (Invitrogen, Carlsbad, Calif) supplemented with 10% FBS (Omega, Tarzana, Calif), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were pretreated with 100 nmol/L wortmannin, 20 nmol/L rapamycin, or 40 μmol/L compound C in various experiments before shear stress application. Confluent ECs were infected with various recombinant adenoviruses at the indicated multiplicity of infection (moi) and incubated for 24 hours before shear stress experiments.

The shear stress experiments were performed as previously described.[20] In brief, confluent monolayers of BAECs seeded on a glass plate were assembled into a parallel-plate flow channel. The flow system was kept at 37°C and ventilated with 95% humidified air with 5% CO_2. The imposed laminar flow had a shear stress of 12 dyne/cm^2 without oscillation. An oscillatory flow was generated by the addition of an oscillator to create a shear stress of 1 ± 5 dyne/cm^2 with a frequency of 1 Hz.

Immunoblotting Analysis

Static or sheared BAECs were washed with ice-cold PBS twice and scraped into a lysis buffer containing 10 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L NaF, 20 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3VO_4, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, 10% glycerol, 10 μg/mL leupeptin, 60 μg/mL aprotinin, and 1 mmol/L phenylmethanesulfonyl fluoride. Equal amounts of protein extracts were separated by using 8% or 10% SDS-PAGE, and then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories Inc, Hercules, Calif). After blocking for 1 hour in a Tris-buffered saline containing 0.1% Tween 20 and 5% nonfat milk, the membrane was probed with various primary antibodies, followed by secondary antibodies conjugated to horseradish peroxidase. The immunoreactivity was revealed by use of an ECL kit (Amersham Biosciences Co, Piscataway, NJ), and the densities of the protein bands were quantified by Scion Image software (Scion Corp, Frederick, Md).

Flow Cytometric Assays

A fluorescence-activated cell sorter (FACS) with Cell Quest software was used to determine the DNA content in BAECs. Cells exposed to shear stress for 10 hours or static controls were washed twice with ice-cold PBS and trypsinized with 2 mL of trypsin-EDTA. After several washes with 1% FBS in PBS, the resuspended cells were fixed with 70% EtOH at 4°C overnight. Immediately before FACS analysis, the fixed cells were centrifuged, washed twice with PBS, and then incubated at 37°C for 20 minutes in 200 μL of PBS containing 0.1% Triton X-100 and 250 μg/mL RNase A. The cells were stained with 50 mg/mL propidium iodide, and 5×10^4 cells were used for FACS analysis to determine the cell cycle.

Animal Experiments and En Face Staining

The animal experimental protocols were approved by University of California, Riverside, institutional Animal Care and Use Committee. Thoracic aorta and aortic arch from 10-week-old male C57BL/6 mice were isolated for immunoblotting detection of mTOR, S6K, AMPK, and Akt phosphorylation.

En face immunostaining for phosphorylated S6K in mouse endothelium was also performed in 10-week-old male C57BL/6 mice. Aortas were perfused with 2% paraformaldehyde in PBS. After dissection, the specimens were incubated with 0.3% H_2O_2 in PBS to block the peroxidase activity according to procedures described previously.[21] The tissues were then permeabilized with 0.2% Triton X-100 and blocked with 3% BSA before incubation with rabbit anti–phospho-S6K Thr389 primary antibody (Abcam, Cambridge, Mass) and Alexa 488–labeled goat anti-rabbit IgG (Invitrogen). After washing 3 times with PBS, aortas were opened and mounted on slides with mounting medium containing 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, Calif). The distal arch served as the negative control and was incubated with nonimmune rabbit IgG (10 μg/mL) instead of anti–phospho-S6K Thr389. Images of the immunostaining were obtained with a Leica SP2 confocal microscope. In every mouse, 3 or 4 images were obtained from 3 to 4 regions in the aortic arch and the thoracic aorta.

Statistical Analyses

Results are expressed as mean±SD from at least 3 independent experiments. The data were analyzed by 2-tailed Student's t test. $P<0.05$ was considered statistically significant.

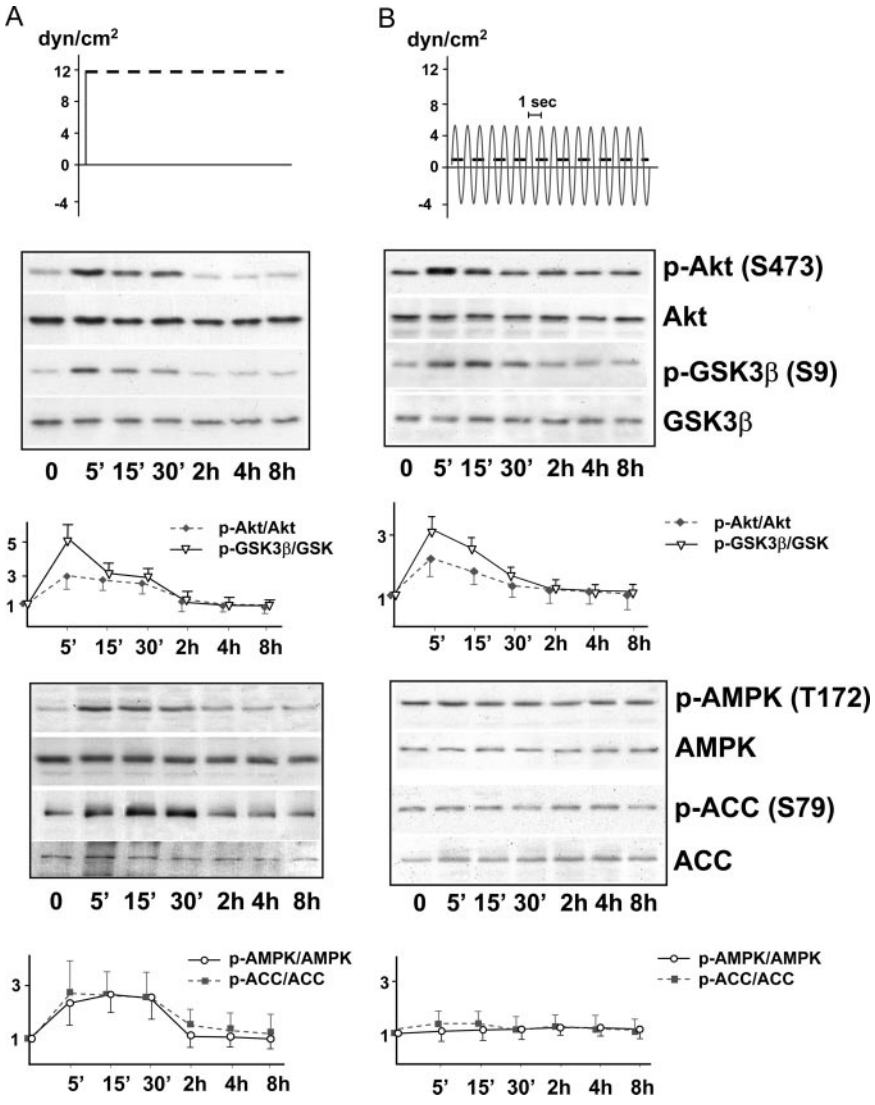

Figure 1. Laminar flow activates both Akt and AMPK in BAECs, but oscillatory flow activates only Akt. Confluent monolayers of BAECs were subjected to laminar flow (12 dyne/cm^2) or oscillatory flow (1 ± 5 dyne/cm^2) for different durations, up to 8 hours, or were kept as static controls (represented by time 0). Shown at the top are schematic representations of the flow patterns, with dashed lines indicating the mean shear stress. Cells were lysed and the phosphorylation of Akt S473, GSK S9, AMPK Thr172, and ACC S79 was analyzed by Western blotting. As loading controls, membranes were stripped and reprobed with antibody against Akt, GSK, AMPK, and ACC. The graphs below indicate the ratio of phosphorylated to total proteins for each time point, with that of static cells set to 1. All data are from 3 independent experiments.

Results

Laminar Flow Activates Both Akt and AMPK in ECs, but Oscillatory Flow Activates Only Akt

We examined first the activation of Akt and AMPK in response to laminar and oscillatory flows applied to BAECs in a flow channel. Laminar flow (at 12 dyne/cm^2) increased the phosphorylation of Akt Ser473 as early as 5 minutes, which then declined to the basal level at 2 hours (Figure 1A, top). The phosphorylation of glycogen synthase kinase-3β (GSK3β) at Ser9, catalyzed by Akt, was also increased in a transient manner. The same transient increase in phosphorylation was observed for AMPK Thr172 and its target ACC Ser79 (Figure 1A, bottom) induced by laminar flow. With an oscillatory flow at 1 ± 5 dyne/cm^2 (1 Hz), the phosphorylation of Akt Ser473 and GSK3β Ser9 was increased transiently (Figure 1B, top), similar to that under laminar flow, but oscillatory flow produced little if any changes in the level of AMPK Thr172 phosphorylation. The lack of an effect of oscillatory flow on AMPK activity was confirmed by the constant level of ACC Ser79 phosphorylation (Figure 1B, bottom).

Because mTOR Ser2448 is specifically phosphorylated by Akt, which reveals proliferation and growth signaling, we examined the effect of laminar and oscillatory flows on mTOR Ser2448 phosphorylation. As shown in Figure 2, both laminar and oscillatory flow caused a rapid increase in phosphorylation of mTOR Ser2448. This peak level lasted for 30 minutes before a gradual decline. mTOR 2448 phosphorylation at 8 hours in cells under laminar or oscillatory flow was still higher than that in static controls. The putative downstream target of mTOR, S6K Thr389, showed transient phosphorylation in response to laminar flow, whereas that under oscillatory flow was sustained for up to 8 hours.

AMPK Counteracts Akt in Response to Laminar Flow

Because laminar flow activated both Akt and AMPK, which have opposite effects on S6K phosphorylation,[12] we studied the effect of inhibition of AMPK on S6K phosphorylation by comparing Ad-AMPK-DN–infected cells versus Ad-null–infected control cells under laminar flow. As shown in Figure 3, the expression of AMPK-DN, indicated by the positive hemagglutinin blotting, attenuated phosphorylation of AMPK

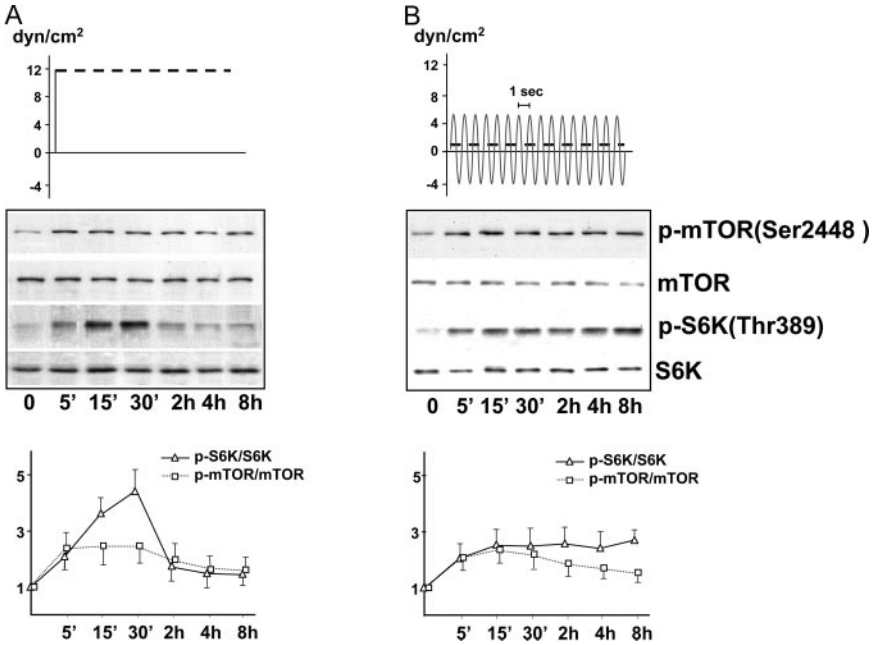

Figure 2. Activation of S6K, as indicated by Thr389 phosphorylation, was transient in response to laminar flow but sustained after oscillatory flow. BAECs were subjected to laminar or oscillatory flow under the same conditions as those described in Figure 1, and Western blotting was used to analyze the phosphorylation of mTOR Ser2448 and S6K Thr389. The graphs below indicate the ratio of phosphorylated to total proteins for each time point, with that of static cells set as 1. All data are from 3 independent experiments.

Thr172 and ACC Ser79 but increased that of S6K Thr389. At 8 hours postflow, the level of S6K Thr389 phosphorylation in Ad-AMPK-DN–infected cells was still significantly higher than that in Ad-null–infected and control static cells. The laminar flow-induced phosphorylation of Akt Ser473 and its downstream mTOR Ser2448 were not affected by AMPK inhibition with Ad-AMPK-DN. Inhibition of AMPK by treating BAECs with compound C, a pharmacological AMPK inhibitor,[22,23] also enhanced S6K Thr389 phosphorylation after laminar flow (Figure I in the online data supplement, available at http://circres.ahajournals.org), with no change in Akt Ser473 and mTOR Ser2448 phosphorylation (data not shown).

In parallel experiments, BAECs were infected with Ad-Akt-DN, expressing a dominant-negative mutant of Akt. Compared with Ad-null–infected controls, Ad-Akt-DN–infected cells showed marked reduction of phosphorylated mTOR Ser2448 and S6K Thr389 (Figure 4A). Treating BAECs with wortmannin, a PI3K inhibitor, similarly reduced mTOR and S6K phosphorylation (Figure 4B). The phosphorylation of AMPK and ACC was not affected by Akt inhibition. Furthermore, inhibition of mTOR by rapamycin reduced S6K Thr389 phosphorylation in response to laminar flow (Figure 4C). Together, the results from Figures 1 through 4 suggest that the S6K activity is positively regulated by Akt and negatively regulated by AMPK in an antagonistic fashion in response to laminar low.

AMPK Negatively Regulates S6K in Response to Oscillatory Flow

Because oscillatory flow activated Akt but not AMPK (Figure 1), we infected BAECs with Ad-AMPK-CA encoding a constitutively active mutant of AMPK to investigate whether AMPK activation prevented the activation of S6K in response to oscillatory flow. As shown in Figure 5A, S6K phosphorylation/activation was lower in cells infected with Ad-AMPK-CA, compared with control cells infected with Ad-null, which indicates that AMPK negatively regulates S6K under oscillatory flow. Of note, phosphorylation of mTOR Ser2448 was not affected by Ad-AMPK-CA. However, Ad-Akt-DN inhibited the phosphorylation of both mTOR Ser2448 and S6K Thr389 (Figure 5A). The effect of Ad-Akt-DN could be mimicked by treating cells with wortmannin (Figure 5B). In addition, the blockade of mTOR by rapamycin decreased S6K Thr389 phosphorylation in response to oscillatory flow (Figure 5C). Thus, the data presented in Figure 1 and 5 suggest that oscillatory flow activated the PI3K-Akt pathway without affecting the AMPK pathway. The opposite activation status of AMPK and Akt contributed to upregulated mTOR-S6K activity.

EC Cycle Is Regulated by Akt-AMPK Crosstalk in Response to Laminar Versus Oscillatory Flow

We used FACS to analyze the BAEC cell cycle to confirm the proliferative or antiproliferative roles of Akt and AMPK under the 2 flow patterns. The populations of Ad-null–infected cells in the G_0/G_1 phase under laminar and oscillatory flow were $58.1\pm5.3\%$ and $49.9\pm5.2\%$, respectively, and that in the $S+G_2/M$ phase were $41.2\pm3.5\%$ and $49.7\pm4.1\%$, respectively (Figure 6). Thus, laminar flow led to a greater cell population in the quiescent state, whereas oscillatory flow increased the mitotic rate; these results are consistent with previous findings.[4,5] Because laminar flow activated both Akt and AMPK, we infected BAECs with either Ad-Akt-DN or Ad-AMPK-DN to study cell cycle regulation under laminar flow following inhibition of Akt or AMPK, respectively. As shown in Figure 6A, Ad-Akt-DN increased the EC population in the G_1/G_0 phase ($71.3\pm4.3\%$), with a complementary decrease in the $S+G_2/M$ phase ($27.8\pm2.8\%$). In contrast, Ad-AMPK-DN decreased the EC population in the G_1/G_0 phase ($50.1\pm4.9\%$), with a complementary increase in the $S+G_2/M$ phase ($48.1\pm2.8\%$), suggesting that EC proliferation is increased under laminar flow following the inhibition of the antiproliferative effect of AMPK.

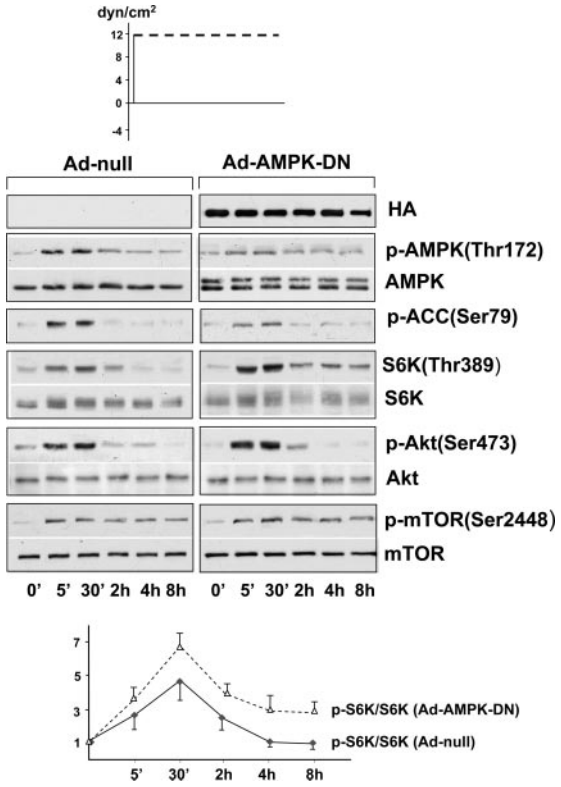

Figure 3. AMPK negatively regulates S6K in ECs in response to laminar flow. BAECs were infected with Ad-null or Ad-AMPK-DN (100 mois), and the confluent infected cells were exposed to laminar flow (12 dyne/cm^2) for the times indicated or kept as static controls (time 0). Cell lysates underwent Western blot analyses with various antibodies as indicated. The data represent results from 3 independent experiments. The graph below demonstrates that AMPK inhibition with Ad-AMPK-DN increased the laminar flow-induced S6K phosphorylation.

Under oscillatory flow, BAECs infected with Ad-Akt-DN showed a marked increase in the G_0/G_1 phase (72.9±3.4%), with a decrease in the $S+G_2/M$ phase (25.9±2.4%), which indicates the blockade of the proliferative role of Akt in cells exposed to oscillatory flow (Figure 6B). Infection with Ad-AMPK-CA led to proportions similar to that with Ad-Akt-DN infection among different phases of the cell cycle, ie, increased quiescence and decreased mitotic rate.

ECs in the Athero-Prone Areas Exhibit a High Level of S6K Activation

Because ECs in the bends and bifurcations of the arterial tree show an increased mitotic rate,[3] we compared the activation of mTOR and its downstream target S6K in the mouse aortic arch, where flow patterns are disturbed and the thoracic aorta is exposed to undisturbed steady flow. In particular, we assessed phosphorylation of mTOR Ser2448 and its downstream target S6K Thr389 by Western blotting. As shown in Figure 7A, tissue from the aortic arch exhibited elevated phosphorylation of S6K (Thr389), as compared with that from the thoracic aorta. However, mTOR Ser2448 phosphorylation was comparable in both regions. In terms of upstream signaling, AMPK phosphorylation was greater in the thoracic aorta than in the aortic arch. The higher activity of AMPK was confirmed by the elevated phosphorylation of ACC in the thoracic aorta. However, Akt phosphorylation was similar in the 2 regions. Because AMPK negatively regulated S6K, as suggested by in vitro flow experiments (Figure 5A), the lower S6K activity in the thoracic aorta would result from the higher AMPK activity.

Because the aortic specimens used for Western blotting consisted of both ECs and vascular smooth muscle cells (VSMCs), we thus used en face immunostaining to examine the phosphorylation of S6K in the endothelium of different regions of the mouse aorta. The proximal region of the aortic arch represents a high probability area for atherogenesis.[24] Confocal microscopy revealed increased endothelial S6K phosphorylation in the aortic arch, as compared with the straight part of thoracic aorta (Figure 7B). We were unable to detect mTOR phosphorylation in both areas, owing to the lack of appropriate anti–phospho-mTOR for immunostaining.

Discussion

Earlier studies with cultured ECs in flow channels showed that steady laminar flow reduced EC proliferation, whereas disturbed flow patterns increased EC turnover.[25,26] In accordance with these observations, laminar flow has been shown to reduce the number of cells entering the cell cycle, with most cells arrested in the G_0/G_1 phase.[4,5] Results from our study, together with previous knowledge, have led us to propose that AMPK and Akt respond differentially to different flow patterns. Owing to the distinct modes of AMPK/Akt interaction, the activity of downstream mTOR-S6K is crucial for EC cycle regulation (see Figure 8 for a proposed model). Our results suggest that laminar flow, as the physiologically relevant stimulus of the endothelium, activates both the antiproliferative AMPK and promitotic Akt in ECs (Figure 1). The offset actions of AMPK and Akt in the mTOR-S6K pathway would mitigate the proliferative effect on the EC cell cycle, as shown in our FACS analyses (Figure 6).

The proliferative mTOR-S6K pathway is tightly regulated by mitotic and stress signaling. Mitotic stimuli such as insulin activate Akt, which in turn phosphorylates several sites in TSC-2 to lead to the inactivation of TSC-2.[9] Hence, Akt augments mTOR signaling via a negative regulation of TSC-2.[27] Under energy depletion (in the form of ATP), TSC-2 is also phosphorylated at several other residues by AMPK, particularly Ser1345,[12] causing the activation of TSC-2 and the ensuing inactivation of mTOR. Because Akt and AMPK are concurrently activated by laminar flow, the downstream effects of the promitotic Akt and antiproliferative AMPK could converge at the level of TSC-2. If so, laminar flow would cause phosphorylation on multiple sites of TSC-2; the phosphorylation events causing activation of TSC-2 apparently offset those leading to inhibition, thus maintaining ECs in a quiescent state.

In contrast to laminar flow, oscillatory flow activates only Akt, with AMPK remaining at its basal level of activity. The exclusive activation of Akt leads to a sustained phosphorylation of S6K Thr389 (Figure 2B), possibly via phosphorylation of TSC-2 at Ser939, Ser1086/Ser1088, and Thr1462 but not Ser1345. This phosphorylation/dephosphorylation status of TSC-2 would result in mitotic signaling.[27–29] The opposite effects of Akt versus AMPK in regulating S6K was

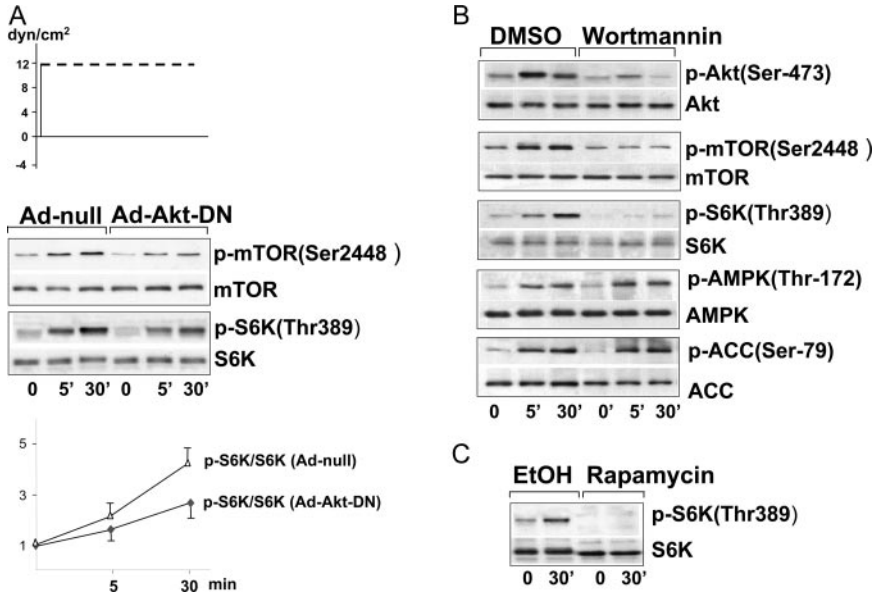

Figure 4. Akt positively regulates S6K in ECs in response to laminar flow. BAECs were infected with Ad-null or Ad-Akt-DN (100 mois) (A), treated with DMSO or wortmannin (100 nmol/L) for 30 minutes (B), or treated with vehicle (EtOH) or rapamycin (20 nmol/L) for 1 hour (C). The cells were then exposed to laminar flow (12 dyne/cm^2) for the times indicated or kept as static controls. Cell lysates underwent Western blotting with various antibodies as indicated. The graph in the bottom of A demonstrates that Akt inhibition with Ad-Akt-DN decreased the laminar flow–induced S6K phosphorylation.

demonstrated by the inhibitory effects of constitutively active AMPK on the mTOR-S6K pathway under oscillatory flow (Figure 5A). In our experimental system, both laminar and oscillatory flows induced a higher level of mTOR Ser2448 phosphorylation for up to 8 hours, compared with that in static cells (Figure 2). However, S6K Thr389 phosphorylation was transient in response to laminar flow, which would be the result of AMPK counteracting Akt.

An increased mTOR activity depends on not only phosphorylation of Ser2448 but also its association with small GTPase Rheb.[9,12] Because Akt was transiently activated by both types of flows, phosphorylation of mTOR Ser2448 by Akt would be necessary but not sufficient for S6K activation. It is interesting to note that mitotic signaling mainly results in the phosphorylation of mTOR Ser2448, which is attenuated by amino acid and nutrient starvation[30,31] and that AMPK is responsible for Ser2446 phosphorylation induced by nutrient deprivation.[32] The decreased mTOR Ser2448 phosphorylation in Ad-Akt-DN–infected but not Ad-AMPK-CA—infected cells indicates that Ser2448 is regulated by Akt but not AMPK. Although Ad-AMPK-CA did not alter the mTOR Ser2448 phosphorylation, it did, similar to Ad-Akt-DN, inhibit S6K T389 phosphorylation in response to oscillatory flow (Figure 5). We thus postulate that the greater population of ECs in the mitotic state in response to oscillatory flow, and possibly also disturbed flow, is attributable, at least in part, to a lack of the "checks and balances" by AMPK.

Laminar flow is a major physiological stimulation that activates eNOS, and phosphorylation of eNOS Ser1177 by the PI3K-Akt pathway has been proposed as the key step of this modulation.[7,33] Our recent work demonstrated that eNOS Ser1177 can also be phosphorylated by AMPK.[19,34] The concurrent activation of Akt and AMPK by laminar flow may also help explain the synergistic phosphorylation of eNOS Ser1177. Interestingly, eNOS Ser1177 phosphorylation by oscillatory flow was not as strong as that by laminar flow (D.G. and J.Y.-J.S., unpublished results, 2006), which is consistent with our finding that oscillatory flow activates only Akt.

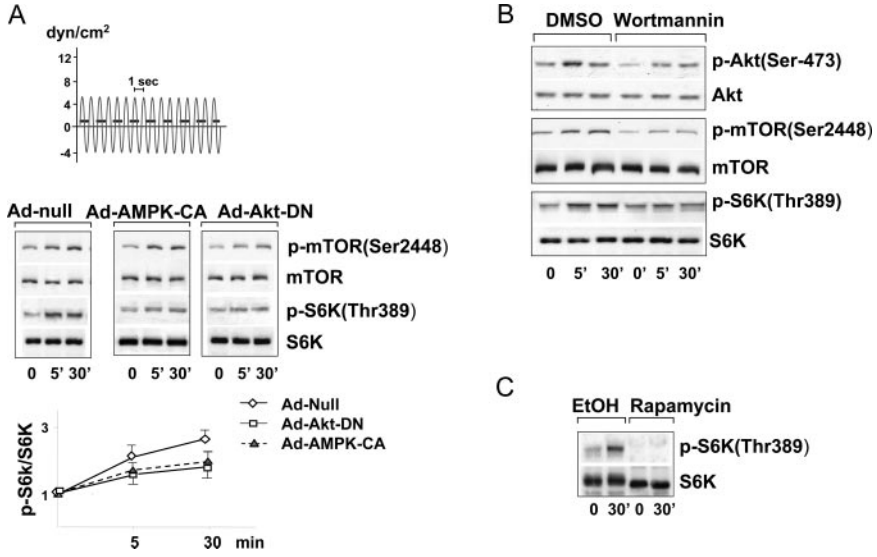

Figure 5. AMPK and Akt regulate oscillatory flow–activated S6K in an opposite manner. BAECs were infected with Ad-null, Ad-Akt-DN, or Ad-AMPK-CA (100 mois) (A), treated with DMSO or wortmannin (100 nmol/L) for 30 minutes (B), or treated with EtOH or rapamycin (20 nmol/L) for 1 hour (C). Cells were then exposed to oscillatory flow for different times or kept as static controls. Cell lysates underwent Western blotting with various antibodies as indicated. The graph at the bottom of A demonstrates that AMPK activation with AMPK-CA was similar to that of Akt inhibition with Ad-Akt-DN in attenuating the oscillatory flow-induced S6K phosphorylation.

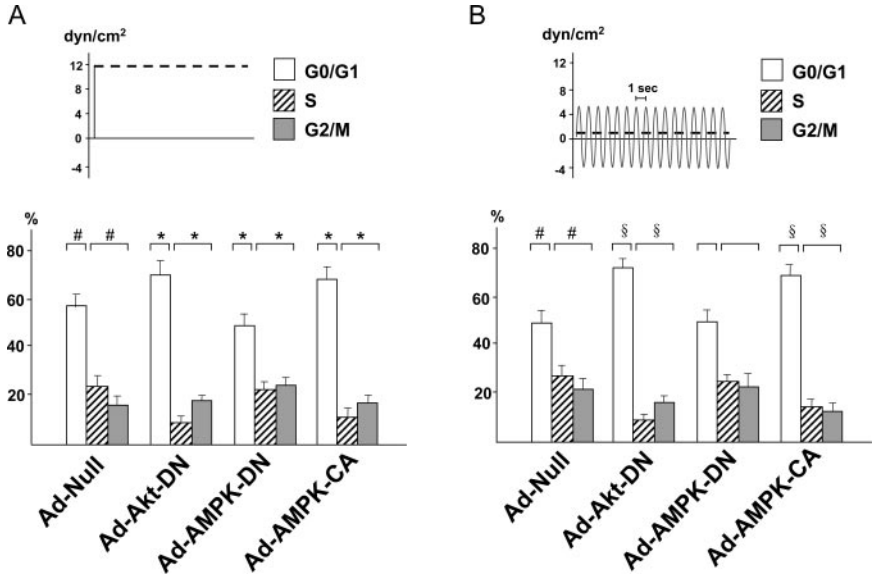

Figure 6. Akt positively but AMPK negatively regulates EC cycle progression in response to shear flow. BAECs were infected with Ad-null, Ad-Akt-DN, Ad-AMPK-DN, or Ad-AMPK-CA (100 mois). Infected cells were then exposed to laminar flow (A) or oscillatory flow (B) for 10 hours. FACS analysis of cell cycle status showed a higher proportion of mitotic ECs ($S+G_2/M$) under oscillatory flow than under laminar flow. The positive and negative effects of Akt and AMPK, respectively, were demonstrated by the use of various adenoviruses. #$P<0.05$ for Ad-null–infected cells subjected to laminar vs oscillatory flows; *$P<0.05$ and §$P<0.05$ between the Ad-mutant–infected groups and Ad-null–infected control cells subjected to laminar or oscillatory flow.

The principal difference between the 2 types of flow in our study is that the laminar flow produced a significant mean shear stress (12 ± 0 dyne/cm^2), whereas oscillatory flow produced a low mean shear stress and relatively large amplitude of oscillation (1 ± 5 dyne/cm^2). We have experimentally addressed whether Akt and AMPK activation depends on changes in mean shear stress and/or amplitude by applying shear stress at 12 ± 1 or 12 ± 5 dyne/cm^2 to cultured ECs. As shown in supplemental Figure II, mean shear stress at 12 dyne/cm^2 activated both Akt and AMPK, whether the amplitude of oscillation was 1 or 5 dyne/cm^2. Thus, flow patterns without a significant reverse component (ie, mean$\pm$amplitude of oscillation >0) seem to activate both Akt and AMPK. In contrast, low mean shear stress with high amplitude of oscillation resulting in a reverse component (ie, mean$\pm$amplitude of oscillation <0) may activate Akt but not AMPK. If true, flow patterns with distinct mean$\pm$amplitude of oscillation have significant physiological and/or pathophysiological implications in EC biology. In straight parts of the vessels, where blood flow patterns are laminar, with a high mean shear stress, the flow pattern likely activates both Akt and AMPK, resulting in EC quiescence. Indeed, in the present study, higher S6K phosphorylation was found in the mouse aortic arch than in the thoracic aorta, and these findings are consistent with increased mitosis of rabbit ECs in areas of disturbed flow or transition of flow direction.[3] In addition to the higher mitotic rate and lower NO bioavailability, disturbed flow patterns would also cause EC inflammation, as demonstrated by increased nuclear factor κB activation,[35] and vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression.[21] Thus, the differential effects because of Akt and AMPK activation by laminar and oscillatory flows provide an example of how endothelial homeostasis is maintained in relation to atherogenesis.

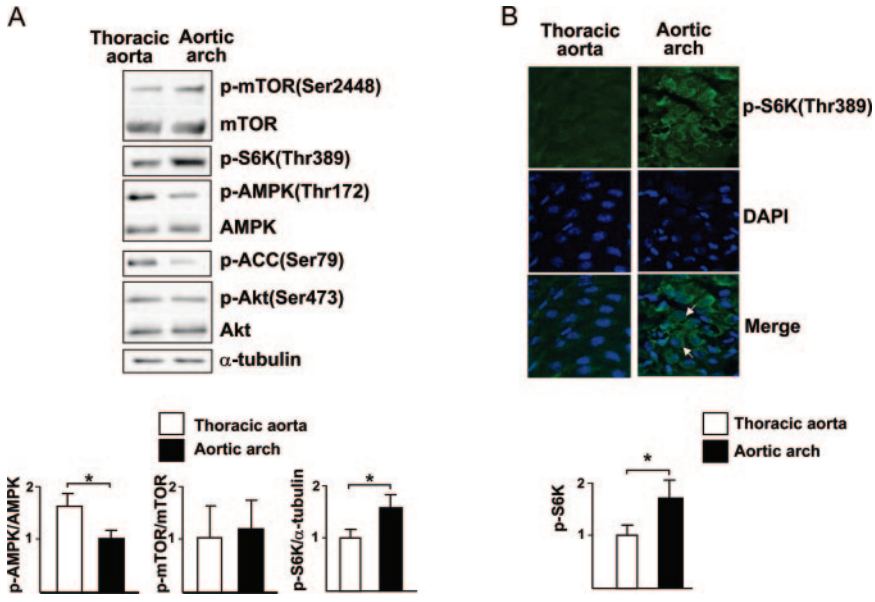

Figure 7. S6K activity is differentially regulated in the mouse aortic arch and thoracic aorta. A, Tissue extracts from the aortic arch and thoracic aorta of C57BL/6J mice (n=24) were analyzed by Western blotting with various antibodies as indicated. Each lane represents pooled specimens from 3 animals. *$P<0.05$ between aortic arch and thoracic aorta. B, The aortic arch and the thoracic aorta from C57BL/6J mice were immunostained with anti–phospho-S6K Thr389 and Alexa 488–conjugated secondary antibody. Nuclei were counterstained with DAPI. The immunostained images of anti–phospho-S6K Thr389 and DAPI were obtained by confocal microscopy. Shown are representative images from 8 animals. Arrows in the merge image indicate the positive staining of phosphorylated S6K around the peripheral nucleus areas.

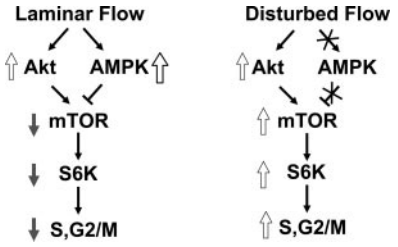

Figure 8. A proposed model for AMPK interacting with Akt in response to laminar vs disturbed shear stress. Laminar shear stress activates both AMPK and Akt in ECs. The antimitotic AMPK counteracts proliferative Akt, resulting in relatively undisturbed mTOR-S6K. Thus, ECs entering the mitotic S and G_2/M phases are attenuated, maintaining endothelial homeostasis. In contrast, disturbed flow activates only Akt. The lack of antagonistic effect because of AMPK activation leads to a sustained activation of S6K and, consequently, a high proportion of ECs in the mitotic state.

Sources of Funding

This study was supported in part by NIH grants HL77448 (to J.Y.-J.S.), HL80518, and HL85159 (to S.C.).

Disclosures

None.

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