Strain-induced vascular endothelial cell proliferation requires PI3K-dependent mTOR-4E-BP1 signal pathway

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Li, Wei, and Bauer E. Sumpio. Strain-induced vascular endothelial cell proliferation requires PI3K-dependent mTOR-4E-BP1 signal pathway. Am J Physiol Heart Circ Physiol 288: H1591–H1597, 2005.—The aim of this study was to determine whether the phosphatidylinositol 3-kinase (PI3K)-dependent mammalian target of rapamycin (mTOR)-eukaryotic initiation factor 1 (4E-BP1) signal pathway and S6 kinase (S6K), the major element of the mTOR pathway, play a role in the enhanced vascular endothelial cell (EC) proliferation induced by cyclic strain. Bovine aortic ECs were subjected to an average of 10% strain at a rate of 60 cycles/min for 24 h. Cyclic strain-induced EC proliferation was reduced by pretreatment with rapamycin but not the MEK1 inhibitor PD-98059. The PI3K inhibitors wortmannin and LY-294002 also attenuated strain-induced EC proliferation and strain-induced activation of S6K. Rapamycin but not PD-98059 prevented strain-induced S6K activation, and PD-98059 but not rapamycin prevented strain-induced activation of extracellular signal-regulated kinases 1 and 2. Cyclic strain also activated 4E-BP1, which could be inhibited by PI3K inhibitors. These data suggest that the PI3K-dependent S6K-mTOR-4E-BP1 signal pathway may be critically involved in strain-induced bovine EC proliferation.

VASCULAR ENDOTHELIAL CELLS (ECs), which comprise the inner lining of blood vessels, are subjected not only to a continually changing chemical environment but also to the hemodynamic forces of the circulation. These forces include cyclic strain, the repetitive deformation that occurs as the vessel wall rhythmically distends and relaxes with the cardiac cycle (54). The hemodynamic forces recognized by ECs have been shown to play an important role in the modulation of vessel function and structure. Cyclic strain stimulates the production of prostacyclin (55), endothelin (56), tissue plasminogen activator (28), and nitric oxide (5). We have previously shown that in ECs, cyclic strain induces cells to proliferate (47), elongate, and align perpendicularly to the vector force (62). Focal adhesion kinase pp125FAK and paxillin are tyrosine phosphorylated (62) and regulate the morphological change and migration induced by cyclic strain.

Protein kinase C (PKC) is activated in ECs subjected to cyclic strain and has been previously reported to be a critical mediator of the cyclic strain effect on EC proliferation (48). The mitogen-activated protein kinase family of ubiquitous intracellular serine/threonine kinases including extracellular signal-regulated kinases 1 and 2 (ERK1/2) also plays an important role in the transduction of mitogenic and differentiation signals from cytoplasm to cell nucleus (12). An important role for ERK1/2 activation in cell migration has been demonstrated (33), and ERK2 has been shown to be involved in the wound repair process, which requires cell migration and proliferation (13). However, ERK1/2 activation does not seem to be essential for strain-induced EC proliferation and alignment (30).

It is evident that activation of different signal pathways targeting the nucleus and cytoplasm can stimulate cell proliferation. However, the commitment to mitogenesis imposes a significant demand on a cell’s metabolic reserve, as all essential intracellular structures must be duplicated before division (23). Translational control is an important element in the critical processes of growth and proliferation. Multiple effector proteins contribute to translation initiation of specially modified mRNAs that modulate these processes (42). The mammalian target of rapamycin (mTOR) is an intermediate in a key translational control pathway that regulates the cell cycle, proliferation, and growth (45). Activation of this pathway by growth factors has been demonstrated to induce the phosphorylation of S6 kinase (S6K; Ref. 10). Signals from the S6K-mTOR pathway converge with mitogenic inputs from the phosphatidylinositol 3-kinase (PI3K) pathway through proteins such as PDK-1 (Ref. 53) and Akt (Ref. 52). Akt activation in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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maintained in a 1:1 mixture of DMEM and high-glucose Ham’s F-12 solution (GIBCO-BRL; Gaithersburg, MD) supplemented with 10% heat-inactivated FCS (HyClone Laboratories; Logan, UT), 5 μg/ml deoxytocytidin-thymidine (Sigma Chemical; St. Louis, MO), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 250 ng/ml amphotericin B (GIBCO-BRL) and were grown to confluence at 37°C in a humidified incubator with 5% CO2. ECs were identified by their typical cobblestone appearance under phase-contrast microscopy (model IMT-2; Olympus Optical; Tokyo, Japan). Cells used in this study were between passages 3 and 6.

Application of cyclic strain. ECs were seeded onto collagen I precoated flexible membranes (Flex I; Flexcell International; Hillsborough, NC) and then subjected to repetitive mechanical deformation using a Flexercell strain unit (model FX-4000; Flexcell International) for an average 10% strain at 60 cycles/min (0.5 s of deformation alternating with 0.5 s of neutral conformation) as previously described (11, 39, 40). Briefly, the strain unit consists of a vacuum regulated by computer-controlled solenoid valves. The valves apply and release a

ECs were seeded to confluence, synchronized for 24 h before study (11, 39, 40). All proliferation studies were done in two 6-well plates (12 wells/group), and cell counts from each of the 12 wells were obtained independently in triplicate. Triplicate counts were averaged to yield a mean cell count per well, and data from each experiment were thus analyzed with 12 observations in each group. We used specific inhibitors to probe the cell proliferation and attenuation effects of phosphorylation of each protein and kinase. Although this procedure has limitations, the inhibitors we adapted are all well established and have been broadly used by others and by us in previous studies (11, 17, 39–41, 57, 64, 65). ECs were then subjected to cyclic strain for 24 h in the presence and absence of the MEK1 inhibitor PD-98059 (10 μM; Calbiochem; La Jolla, CA), the mTOR-S6K pathway inhibitor rapamycin (10 nM; Sigma Chemical; Ref. 51), and the PI3K inhibitors wortmannin (25 nM; Calbiochem) and LY-294002 (10 μM; Calbiochem). All inhibitors were preincubated with the ECs 1 h before the initiation of cyclic strain (36). Because inhibitors were dissolved in DMSO, control cells in these studies were similarly treated with DMSO to a final concentration of 0.2%.

Western blot analysis. ECs were seeded to confluence, synchronized by incubation in serum-free media for 24 h, and then subjected to cyclic strain for 24 h in the presence and absence of the specific inhibitors. After experimentation, ECs were lysed in lysis buffer that contained 25 mmol/l HEPES, pH 7.4, 500 mmol/l NaCl, 1% Triton X-100, 0.1% SDS, 1% deoxycholate, 5 mmol/l EDTA, 50 mmol/l sodium fluoride, 1 mmol/l PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mmol/l sodium orthovanadate. ECs were then assayed for protein using the Bio-Rad protein assay method according to the manufacturer’s protocol (Bio-Rad Laboratories; Hercules, CA) and diluted to equal protein concentrations. The samples were resolved by 10% SDS-PAGE and transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech; Piscataway, NJ). The membranes were incubated with the following phospho-specific antibodies: phospho-specific PKD-1, Akt(Ser473), Akt(Thr308), ERK1/2, 4E-BP1, and S6K antibody (Cell Signaling Technology; Beverly, MA) with the appropriate secondary antibodies and were visualized by the enhanced chemiluminescence technique according to the manufacturer’s protocol. Equal protein loading was determined by stripping the membranes and reprobing with total nonphosphorylated antibodies (Cell Signaling Technology) for each protein separately.

Band densities on immunoblots were measured with a densitometer (ImageQuant; Molecular Dynamics; Sunnyvale, CA). All densitometry was performed on exposures within the linear range of the film and the densitometer.

Statistical analysis. Data are presented as means ± SE and were analyzed by Student’s unpaired t-test. Proliferation studies were performed in six wells per condition with two plates to yield 12 independent total observations for statistical analysis. We performed each study at least three times with similar results. Each experiment in itself yielded statistically significant results, and we have therefore chosen to show raw data from a single representative study for the proliferation data, thereby accepting a somewhat higher standard error, rather than pooling data from multiple studies, which would have decreased our standard error but would have required data transformation to normalize the data against controls before pooling.

RESULTS

Bovine aortic EC proliferation was stimulated by cyclic strain and significantly reduced by rapamycin, wortmannin, and LY-294002 but not PD-98059. Data presented are from one of at least three separate experiments, each of which yielded statistically significant and similar results. In a typical experiment, bovine aortic EC monolayers maintained under cyclic strain conditions increased in number from 199,627 ± 13,211 to 258,822 ± 11,714 cells/well over 24 h, which represents an average 29.7% increase in cell number. Similarly, identical monolayers incubated with 10 μM PD-98059 subjected to cyclic strain for 24 h increased in number from 190,743 ± 13,211 to 234,938 ± 7,335 cells/well, which is an average 23.1% increase in cell proliferation in response to strain (P < 0.05; n = 12; Fig. 1). However, addition of the mTOR inhibitor rapamycin to the culture medium during the 24-h incubation blocked strain-induced proliferation with even slight decreases in each static group for wortmannin (25 nM), LY-294002 (10 μM), or rapamycin (10 nM).

Cyclic strain on bovine aortic ECs phosphorylated S6K in a time-dependent manner. To determine whether cyclic strain induces S6K activation in ECs exposed to cyclic strain, we assessed for phosphorylation of S6K in time-course experiments using phospho-specific antibodies (15). Figure 2A shows that strain-induced S6K phosphorylation occurred in a time-dependent manner. Activation increased to 140 ± 13.6% by the 30-min time point and peaked at 60 min to 168.6 ± 11.0% (P < 0.05; n = 5), after which S6K activation declined; however, it remained above basal levels 240 min after the initiation of strain.

Rapamycin, wortmannin, and LY-294002 but not PD-98059 prevent strain-induced S6K activation; however, PD-98059 but not rapamycin prevents strain-induced ERK1/2 activation. Rapamycin has been reported to prevent S6K activation in many other cell types (51). The MEK1 inhibitor PD-98059 has been demonstrated to prevent growth factor and mechanical force-induced ERK1/2 phosphorylation (30, 31). Figure 2A demonstrates strain-induced S6K activation. Rapamycin (10 nM; Fig. 2B), wortmannin (25 nM), and LY-294002 (10 μM; Fig. 2C) completely prevented S6K activation. PD-98059 (10 μM) slightly diminished but could not prevent cyclic strain-induced S6K activation (P < 0.05; n = 3; Fig. 2B).
Similar to our previous results (30), Fig. 2D shows that 1 h of cyclic strain phosphorylated ERK1/2, and this response could be prevented by PD-98059. However, although the fold increases of ERK1/2 activation in the rapamycin groups were lower than those of the DMSO control group (Fig. 2D), rapamycin could not completely block the strain-induced ERK1/2 activation.

**PI3K inhibitors attenuated the time-dependent phosphorylation of 4E-BP1 by cyclic strain.** Figure 3 demonstrates that strain-induced 4E-BP1 phosphorylation occurred in a time-dependent manner beginning at 30 min and was sustained to 120 min. Phosphorylation of 4E-BP1 was starting to diminish by 240 min after the initiation of strain. Wortmannin (25 nM) and LY-294002 (10 μM) largely prevented the phosphorylation of 4E-BP1 (Fig. 3B).

Cyclic strain phosphorylated PDK-1 and Akt in a time-dependent manner. To further determine whether cyclic strain also activates PDK-1 and Akt, which are two major putative intermediary components between PI3K and mTOR, we measured the phosphorylation of PDK-1 and Akt [both phospho-Akt(Ser473) and phospho-Akt(Thr308)] in ECs exposed to cyclic strain. In a previous study, to achieve a similar concentration of collagen I substrate on both flexible plates for cyclic strain and glass slides for shear stress, 62/2 g/ml collagen I was applied to both the plates and the slides (26). In one of our present studies, we did not add any extra collagen. The exact amount of collagen I and the coating method cannot be disclosed by the company for proprietary reasons, but we assume that the concentration is much lower than what we applied in our previous study (26). Figure 4 shows that by 30 min, strain had induced the phosphorylation of both the Thr308 and the Ser473 sites of Akt; activation peaked at 60 min and was...
sustained for \( \leq 240 \) min. Activation of PDK-1 followed a similar time course as the Akt's.

**DISCUSSION**

The phenotype of vascular cells such as ECs becomes apparent when information stored in the nuclei are converted to functional proteins through the processes of transcription and translation (23, 36, 63). It has long been recognized that transcriptional events are tightly controlled by many signaling pathways and regulatory systems (35). Each cell also separately controls translation of mRNA into protein by mechanisms that resemble those that regulate transcription (7, 50).

Phosphorylation cascades such as the S6K-mTOR pathway and its downstream protein 4E-BP1 transmit extracellular signals to the translational apparatus similar to the way signals are communicated to the nucleus (1, 40). In other instances, transacting proteins either promote or inhibit translation through sequence-specific interactions such as phosphorylation and dephosphorylation with regulatory regions in the mRNA molecule. We have previously described (26, 29) that the involvement of PI3K in the signal transduction pathway leads to the strain-induced activation of kinases and proteins. Others have reported a potential relationship between PDK-1 and PI3K through Akt and other proteins (14, 49). In this study, we began to characterize S6K activation to evaluate whether it...
plays a role in the mitogenic effects of strain and to determine whether PDK-1 and Akt subunits might be upstream of PI3K activation.

The S6K-mTOR pathway was originally characterized in lymphocytes (6) and fibroblasts (8) and is at the center of the pathway that controls translation (9). One of the mTOR pathway elements is S6K, which is an essential component of translation initiation complexes. Recent studies have indicated that the S6K signaling pathway is involved in the regulation of cell growth and proliferation, and it has been implicated in the translational upregulation of mRNAs that code for the components of protein synthetic apparatus (20). Our previous results showed that although cyclic strain caused strain- and time-dependent phosphorylation and activation of ERK1/2 in ECs (22, 30), the MEK1 inhibitor PD-98059 could not inhibit the increased cell proliferation and altered cell alignment induced by strain (30). In this study, we demonstrated that the cyclic strain-induced proliferation was largely prevented by rapamycin, thereby implicating the involvement of the mTOR pathway. We also showed that the downstream mTOR targets S6K and 4E-BP1 were phosphorylated by cyclic strain, and this could be prevented by rapamycin but not PD-98059. The protein 4E-BP1 normally binds eukaryotic initiation factor 4E and inhibits translation (25). The phosphorylation (activation) of 4E-BP1 disrupts this binding and activates cap-dependent translation (16). Our results are consistent with the premise that mTOR-S6K directly regulates 4E-BP1 (16, 25), which is a critical protein factor for cell proliferation.

In our studies, treatment with rapamycin had no effect on ERK1/2 phosphorylation in ECs exposed to cyclic strain (see Fig. 2D). These results indicate that although strain activates ERK1/2 in parallel with S6K, these pathways do not seem to interact in bovine aortic ECs. Interestingly, other groups have reported ERK1/2 and S6K activation in human umbilical vein ECs exposed to disturbed flow (36). In those studies, the shear-induced activation of S6K also occurred independently of ERK1/2 activation (36). Unfortunately, those studies did not examine the relationship between S6K activation and cell proliferation.

We have previously reported that PI3K activation is upstream of ERK1/2 activation in ECs exposed to cyclic strain (30). The results of the present study indicate that PI3K may also be upstream of the mTOR pathway, because the PI3K inhibitors wortmannin and LY-294002 significantly attenuated strain-induced cell proliferation (see Fig. 2). Besides demonstrating phosphorylation of 4E-BP1 in ECs exposed to strain (see Fig. 3A), we also found that the phosphorylation of 4E-BP1 was abolished by the PI3K inhibitors wortmannin and LY-294002 (see Fig. 3B). Taken together, our data suggest that strain-induced EC proliferation requires a PI3K-dependent mTOR-4E-BP1 signaling pathway. In addition to this study, we are also investigating a dominant negative approach in which the dominant negative kinases are transfected into ECs. Our preliminary results indicate that the results from both approaches confirm one another (data not shown).

Furthermore, we have also noted a similar temporal activation of PDK-1 and Akt, which are two important downstream effectors of PI3K that are implicated in both cell transcription and translation pathways (21, 26, 37). For example, Akt is reported to be rapidly activated by insulin in adipocytes, the liver, or skeletal muscle by a mechanism that requires both PI3K and PDK-1 (61). PI3K generates specific inositol lipids that have been implicated to regulate cell behaviors such as growth, proliferation, survival, differentiation, and cytoskeletal change (58). One of the best-characterized targets of PI3K lipid products is the protein kinase Akt, which is also called protein kinase B (PKB; Ref. 58). Others have reported that PKB resides in the cytosol in a low-activity conformation. Upon cellular stimulation, PKB is activated through recruitment to cellular membranes by PI3K lipid products and phosphorylation by PDK-1, which suggests that PKB is activated and, as a multifunctional kinase, induces downstream actions (2, 3, 58).

In our previous studies, we found that shear stress and cyclic strain stimulate vascular cell growth and intimal hyperplasia (24, 26, 38, 46, 60), possibly by promotion of cell survival through inhibition of apoptosis (26). We also found that the effect of cyclic strain is to inhibit apoptosis and phosphorylation of Akt and its downstream target Bad in ECs in vitro (26). Phosphorylation of Akt in ECs exposed to shear stress and cyclic strain on the lower (62 μg/ml) concentration of collagen I substrate-precoated matrix was time dependent but showed maximal stimulation at 30 or 5 min (26). Our previous study suggests an additional mechanism by which hemodynamic forces such as cyclic strain can differentially regulate translation in ECs and thereby possibly maintain the viability of normal endothelium. In our present study, we not only detected similar temporal activation of PDK-1 and both Akts (Ser473 and Thr308) on a low and close-to-normal in vivo concentration of collagen I substrate-precoated matrix (Fig. 4), but we also found that these activations occurred in a coordinate manner with S6K and 4E-BP1 (Fig. 5). All of the proteins studied tended to achieve peak activation by 60 min after the initiation of cyclic strain. However, the proteins upstream of mTOR (PDK-1 and Akt) tended to have sustained activations after reaching a maximum level of activation. The proteins downstream of mTOR (S6K and 4E-BP1), however, started to return to basal levels after an initial activation. Other investigators have reported that expression of certain genes requires the activation of the PI3K-PKB-Akt-S6K pathway (27, 34) and have suggested that PDK-1 is a central mediator of the cell signaling between PI3K and Akt-S6K (32). Although we have
not confirmed that either Akt or PDK-1 is critical for activation of mTOR from P3K in ECs, our preliminary results (data not shown) with vascular smooth muscle cells indicate that the addition of P3K inhibitors could attenuate the strain-induced activation of Akt. As an additional finding, we found that the higher collagen I concentration induces earlier and stronger Akt(Ser473) phosphorylation than does the lower concentration. Others have reported that in fetal rat calvarial cells, elevating the Ca2+ concentration resulted in phosphorylation of Akt, which is consistent with signals of cell survival and proliferation. Meanwhile, the expression of collagen I was increased by a high Ca2+ concentration (18). Our results indicate that a relatively low collagen I concentration could accelerate and amplify the strain-induced Akt activation. This finding is consistent with one of our previous results with human Caco cells, whereby certain cellular matrices influence the proteins in cell signal transduction, which may in turn induce cell proliferation, differentiation, and intracellular signaling (65). The P3K pathway reveals a major path of cell signaling including P3K, Akt, and phosphatase and tensin homolog (PTEN). The mTOR pathway dominates the downstream outcome of P3K signaling with interdependence of these two pathways made clear by the discovery of rapamycin (4, 44). Another finding that a link between mitogenic signals and mTOR via the lipid second-messenger phosphatidic acid suggests that mTOR is critical in the integration of mitogen signals and nutrients (19). Our preliminary study on the role of PTEN and its interactions with Akt shows that PTEN is activated by cyclic strain and is related to Akt (data not shown). Future studies utilizing specific inhibitors of these proteins or dominant-negative transfection strategies will be required to definitively confirm this hypothesis.

In summary, these results indicate the importance of the P3K-dependent mTOR-4E-BP1 signal translational control pathway in determining the ultimate phenotype of a blood vessel. Our study also suggests that regulating translation may allow a cell to respond to external stimuli more quickly by producing protein from existing pools of mRNA rather than employing new transcription. The rapid activation in response to strain is an example of this feature. As a means of regulating vascular cell phenotype, translational control is as important as cellular transcription. Given the diversity of transcription control mechanisms already characterized and the similar mechanisms used to control translation, it is likely that numerous additional control pathways will be identified and shown to be active in blood vessel walls. However, transcription and translation pathways are not necessarily independent. As we reported, rapamycin did not prevent strain-induced ERK1/2 activation, although the magnitudes of activation were not as high as the strain control (see Fig. 2D). Thus these two pathways might at some points cross over, and this is an area of further investigation. In addition, additional study of cyclic strain-induced changes in translational efficiency of specific mRNA species that might be handled by mTOR is another novel approach for investigating this pathway.

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PI3K-DEPENDENT EC PROLIFERATION

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