Role of AKT in cyclic strain-induced endothelial cell proliferation and survival

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AORTIC ENDOTHELIAL CELLS (ECs) are exposed to repetitive cyclic strain (CS) in vivo by the beating heart. The aim of this study was to assess the influence of CS amplitude and/or frequency on EC proliferation and survival and to determine the role of AKT in CS-induced EC proliferation and survival. Cultured bovine aortic ECs were exposed to 10% strain at a frequency of 60 (60 cpm-10%) or 100 (100 cpm-10%) cycles/min or 15.6% strain at a frequency of 60 cycles/min (60 cpm-15.6%). AKT, glycogen synthase kinase (GSK)-3β, BAD, and cleaved caspase-3 were activated by CS in ECs. Increasing the magnitude or frequency of strain resulted in an earlier phosphorylation of GSK-3β, although the magnitude of phosphorylation was similar. After CS at 60 cpm-10% for 24 h, the number of nontransfected ECs was significantly increased by 8.5% (P < 0.05). We found that the number of apoptotic ECs was slightly decreased with exposure to CS. ECs transfected with kinase-dead AKT (KA179) as well as plasmids containing a point mutation in the pleckstrin homology domain of AKT (RC25) not only prevented AKT, GSK-3β, and BAD phosphorylation but also inhibited the CS-induced increase in cell number as well as the CS-induced protection against apoptosis (both P < 0.05). The ratio of 5′-bromo-2′-deoxyuridine-positive cells was increased when ECs transfected with RC25 and KA179 as well as nontransfected ECs and ECs transfected with Lipofectamine 2000 were exposed to CS. We conclude that AKT is important in enhancing the survival of ECs exposed to CS but is not involved in EC proliferation.

apoptosis; glycogen synthase kinase

The serine/threonine AKT/PKB regulates multiple biological processes, including cell survival, proliferation, growth, and glycogen metabolism (23, 28, 62). AKT was identified as a downstream component of survival signaling through phosphatidylinositol 3-kinase (PI3-kinase) (21, 37, 38, 40, 44). AKT consists of a pleckstrin homology (PH) domain, a kinase domain, and a regulatory domain. In unstimulated cells, AKT protein exists in cytoplasm, and the two regulatory phosphorylation sites at Thr308 and Ser473 are in an unphosphorylated state. With growth factor stimulation, the PH domain binds to the lipid products of PI3-kinase and AKT is recruited to the plasma membrane. AKT is then sequentially phosphorylated at Thr308 and Ser473 by upstream kinases referred to as phosphoinositide-dependent protein kinases 1 and 2, respectively, to yield a fully activated kinase (1, 20, 30, 55, 60). Fully activated AKT becomes available to phosphorylate its downstream substrates, and a portion of these molecules detach from the plasma membrane and translocate to various subcellular locations, including the nucleus (8). AKT is then dephosphorylated and inactivated by protein phosphatases such as protein phosphatase 2A (3). We previously reported (9, 26, 27) that both SS and CS increase AKT phosphorylation through a PI3-kinase-dependent mechanism.

Phosphorylated AKT activates several downstream pathways, including metabolic and antiapoptotic pathways, that activate glycogen synthase kinase (GSK)-3, glucose transporter-4, phosphofructokinase-2, P70 S6 kinase, e4-binding protein-1, caspase-9, nitric oxide synthase, and forkhead (11, 15, 35). GSK-3 not only regulates glycogen synthesis through phosphorylation and inactivation by AKT (14, 28) but also has been implicated as a mediator of the PI3-kinase survival signal in Rat-1 and PC-12 cells (50).

Hemodynamic forces have been demonstrated to prevent vascular smooth muscle cell and EC apoptosis (9, 18, 19, 26, 31). However, the effect of the amplitude and/or frequency of CS on EC intracellular signal transduction pathways is not well defined. The aim of this study was to assess the influence of CS magnitude and/or frequency on AKT activation and cell proliferation. In addition, we sought to investigate the role of AKT in CS-induced protection against apoptosis and CS-induced proliferation.

MATERIALS AND METHODS

Cell culture. ECs were harvested from bovine thoracic aortas as previously described (49). Cells were maintained in Dulbecco’s modified Eagle’s medium-Ham’s F-12 (GIBCO-BRL/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gemini

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Bio-products, Woodland, CA), 5 μg/ml deoxycytidine-thymidine (Sigma-Aldrich, St. Louis, MO), and antibiotics (100 U/l penicillin, 100 μg/ml streptomycin, 250 ng/ml amphotericin B; all from GIBCO-BRL) at 37°C in a humidified incubator with 5% CO₂. Cells used in this study were from passages 6–15. Cells were synchronized in serum-free medium for 24 h before each experiment.

**Application of CS.** ECs were seeded onto type I collagen-coated flexible membranes (Flex 1 plate; Flexcell, McKeesport, PA), and when they attained 70–80% confluence, they were subjected to repetitive mechanical deformation with a Flexercell Strain Unit (FX-4000; Flexcell International) as previously described (5, 26, 33, 45). When a vacuum of 225 mmHg was applied, the cells at the periphery showed <15% strain, with the majority receiving no strain, and the average strain was 10%. When a vacuum of 150 mmHg was applied, the cells at the periphery showed a 15–35% strain, cells grown in the inner circumference demonstrated <15% strain, with the majority receiving no strain, and the average strain was 15.6%. ECs were subjected to three different regimens at an average 10% strain at 60 cycles/min (60 cpm-10%), an average 15.6% strain at 60 cycles/min (60 cpm-15.6%), or an average 10% strain at 100 cycles/min (100 cpm-10%). The duration of exposure ranged from 0 to 4 h for acute strain response (i.e., Western blot measurement for caspase-3 and BAD (a downstream of AKT substrate)) and up to 24 h for chronic strain responses (i.e., Western blot of caspase-3 and BAD). The % of HA-tagged forms of AKT, a point mutation in a PH domain AKT (RC25), and a kinase-dead AKT (KA179) were kind gifts from Anke Klippel (Atugen, Berlin, Germany; Refs. 30, 42, 43). According to the manufacturer’s protocol, 0.5 μg of dominant-negative AKT plasmid diluted in 100 μl of Opti-MEM (Invitrogen, Carlsbad, CA) and 2.5 μl of Lipofectamine 2000 (Invitrogen) diluted in 100 μl of Opti-MEM were gently mixed and incubated for 20 min at room temperature. One milliliter of medium in the Flex 1 plate was mixed with two hundred microliters of DNA-Lipofectamine 2000 and incubated at 37°C in a CO₂ incubator for 4 h. After the incubation, culture medium was replaced and cells were incubated for 24 h to allow protein expression. To confirm the transfection efficiency, the cells were assessed by staining with anti-HA-tag antibody (Cell Signaling Technology, Beverly, MA) and a staining kit (R&D Systems, Minneapolis, MN). Cells stained with anti-HA-tag antibody were considered transfection positive. The percentage of HA-tag-positive cells was counted using phase-contrast microscopy (Olympus IMT-2; Olympus Optical, Tokyo, Japan) at ×400 magnification. Figure 1 demonstrates that the efficiency of RC25 and KA179 transfection was 70–80%. On the other hand, no transfection-positive cells were observed using control or Lipofectamine 2000 transfection reagent.

**Cell number.** We seeded ECs at 50,000 cells/well with 1 ml of medium onto the flexible plates and allowed the cells to recover for 24 h. After synchronization by serum starving for 24 h, we transfected 70–80% confluent ECs with RC25, KA179, or Lipofectamine 2000 as described above. To determine the effect of the amplitude and/or frequency of CS on EC proliferation, the cells were exposed to the static condition or to 60 cpm-10%, 60 cpm-15.6%, or 100 cpm-10% for up to 24 h. Cell number was assessed by counting with a Coulter cell counter (model ZM; Coulter Electronics, Hialeah, FL) both before application of CS and after 24 h of exposure to static or CS conditions.

**Immunoblot technique.** ECs were synchronized in serum-free medium for 24 h before exposure to CS. After exposure to CS, cells were washed with ice-cold PBS and scraped in lysis buffer containing (in mM) 50 HEPES, 150 sodium chloride, 1.5 sodium orthovanadate, and 1 phenylmethylsulfonyl fluoride, with 10% glycerol, 1% Triton X-100, and 10 μg/ml leupeptin. Cell lysate was centrifuged to collect supernatant, and equal amounts of protein (30 μg/lane, Bio-Rad protein assay system; Bio-Rad Laboratories, Hercules, CA) were separated with 10% or 15% (for caspase-3 studies) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories) and transferred onto a nitrocellulose membrane (Amersham Life Science, Arlington Heights, IL). The membranes were incubated with primary antibody: anti-AKT antibody, anti-phospho-AKT (Ser473, Thr308) antibody, anti-GSK-3β antibody, anti-phospho-GSK-3β (Ser9) antibody, anti-BAD antibody, anti-phospho-BAD (Ser136) antibody, anti-caspase-3 antibody (Cell Signaling Technology), or an anti-β-tubulin antibody (Sigma-Aldrich) as a loading control. After washing with ice-cold PBS, the membranes were incubated with secondary antibody: anti-rabbit IgG antibody (Cell Signaling Technology) or anti-mouse IgG antibody (Cell Signaling Technology). Blots were developed using the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to film. The films were scanned, and the intensity of the bands was quantified using ImageJ software (Wayne Rasband, NIH, Bethesda, MD) to calculate densitometry.

**Transfection.** The plasmids encoding the hemagglutinin (HA)-tagged forms of AKT, a point mutation in a PH domain AKT (RC25), and a kinase-dead AKT (KA179) were kind gifts from Anke Klippel (Atugen, Berlin, Germany; Refs. 30, 42, 43). According to the manufacturer’s protocol, 0.5 μg of dominant-negative AKT plasmid diluted in 100 μl of Opti-MEM (Invitrogen, Carlsbad, CA) and 2.5 μl of Lipofectamine 2000 (Invitrogen) diluted in 100 μl of Opti-MEM were gently mixed and incubated for 20 min at room temperature. One milliliter of medium in the Flex 1 plate was mixed with two hundred microliters of DNA-Lipofectamine 2000 and incubated at 37°C in a CO₂ incubator for 4 h. After the incubation, culture medium was replaced and cells were incubated for 24 h to allow protein expression. To confirm the transfection efficiency, the cells were assessed by staining with anti-HA-tag antibody (Cell Signaling Technology, Beverly, MA) and a staining kit (R&D Systems, Minneapolis, MN). Cells stained with anti-HA-tag antibody were considered transfection positive. The percentage of HA-tag-positive cells was counted using phase-contrast microscopy (Olympus IMT-2; Olympus Optical, Tokyo, Japan) at ×400 magnification. Figure 1 demonstrates that the efficiency of RC25 and KA179 transfection was 70–80%. On the other hand, no transfection-positive cells were observed using control or Lipofectamine 2000 transfection reagent.

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anti-GAPDH (V-18) antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The membranes were incubated with secondary antibody, anti-rabbit IgG, horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology), or donkey anti-goat IgG horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) before detection of immunoreactivity using enhanced chemiluminescence (Amersham). All blots were quantified using densitometry (BioImage, Ann Arbor, MI).

**AKT immunocytochemical staining.** To determine the effect of CS on AKT expression at both the center and the periphery of the membrane, 50,000 ECs/well were seeded. After the cells were synchronized in serum-free medium for 24 h before exposure to CS, cells were exposed to either static conditions or 60 cpm-10% for up to 24 h. Cells were then fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS, and nonspecific immunostaining was blocked with peroxidase blocking reagent (R&D Systems) before cells were incubated with anti-phospho-AKT antibody (Ser473) (IHC Specific; Cell Signaling Technology) and stained using an R&D Systems staining kit. The AKT-positive cells were visualized by phase-contrast microscopy (Olympus) at ×300 magnification.

**5′-Bromo-2′-deoxyuridine incorporation.** Measurement of cell proliferation was analyzed by DNA incorporation of the thymidine analog 5′-bromo-2′-deoxyuridine (BrdU) as described by the manufacturer (Sigma-Aldrich). Fifty thousand ECs per well were seeded onto flexible six-well plates. After cells were synchronized in serum-free medium for 24 h before exposure to CS, the medium was changed to 10% serum medium including a final concentration of 10 μM BrdU immediately before cells were exposed to static condition or 60 cpm-10% for up to 24 h. Cells were then fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS, and nonspecific immunostaining was blocked with peroxidase blocking reagent (R&D Systems) before incubation with anti-BrdU antibody (Sigma-Aldrich) and the R&D Systems staining kit. The percentage of BrdU-positive nuclei was counted by phase-contrast microscopy (Olympus) at ×200 magnification.

**Detection of apoptosis.** To determine the effect of CS on EC apoptosis, 50,000 ECs/well were seeded onto flexible six-well plates. After cells were synchronized in serum-free medium for 24 h before exposure to CS, cells were exposed to either the static condition or 60 cpm-10% for up to 24 h. Cells were then fixed with 4% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 containing 0.1% sodium citrate in distilled water, and nonspecific immunostaining was blocked with 3% hydrogen peroxide in methanol before use of the In Situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN), which detects terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). The percentage of apoptotic nuclei was counted by phase-contrast microscopy (Olympus) at ×300 magnification.

**Statistical analysis.** Data are presented as means ± SE and were analyzed by ANOVA as appropriate with StatView 5.0 software (SAS Institute, Cary, NC). *P* < 0.05 was considered significant.

**RESULTS**

**Time course of AKT.** To confirm the effect of the dominant-negative AKT plasmids (RC25 and KA179), the phosphorylation of AKT in ECs exposed to 60 cpm-10% CS was assessed. As shown in Fig. 2A, phosphorylation of Ser473 on AKT peaked between 30 and 60 min in control (nontransfected) and Lipofectamine 2000-transfected ECs. On the other hand, there was no significant change in AKT (Ser473) phosphorylation in ECs transfected with RC25 or KA179. The densitometric data from three independent experiments show that the peak activation of AKT (Ser473) in control was 1.64 ± 0.09-fold (*P* < 0.05) at 60 min and returned to baseline at 4 h (1.05 ± 0.13-fold compared with static condition). On the other hand, peak activation of AKT (Ser473) in Lipofectamine 2000-, RC25- and KA179-transfected EC groups was 1.63 ± 0.39-fold (*P* < 0.05) at 30 min, 1.43 ± 0.24-fold [not significant (NS)] at 30 min, and 0.98 ± 0.11-fold (NS) at 60 min, respectively, compared with each static condition.

As shown in Fig. 2B, phosphorylation of Thr308 on AKT peaked at 30 min in the control and Lipofectamine 2000 groups but was not significantly changed in the RC25- or KA179-transfected cells. The densitometric data from three independent experiments showed that the peak activation of AKT (Thr308) was 1.54 ± 0.18-fold (*P* < 0.05) at 30 min in control, 1.46 ± 0.26-fold (*P* < 0.05) at 30 min in Lipofectamine 2000-transfected cells, 1.47 ± 0.40-fold (*P* = 0.09) at 30 min in RC25-transfected cells, and 1.10 ± 0.11-fold at 240 min in KA179-transfected cells compared with each static condition.

**AKT immunocytochemical staining.** The Flexcell unit used in these experiments resulted in a heterogeneous strain pattern (6, 33). When cells were exposed to the 60-cpm-10% strain regimen, cells seeded in the center of the membrane demon-
strated insignificant strain, whereas cells seeded close to the periphery showed ~4% strain. To confirm the effect of CS on AKT activation, the phosphorylation of AKT (Ser473) was assessed using immunocytochemistry. Figure 3 demonstrates that CS phosphorylated AKT in nontransfected ECs and ECs transfected with Lipofectamine or RC25 in the high-strain region of the membrane (periphery) at 60 min. At the periphery of ECs transfected with KA179 and in the center of the membrane, where there was 0–1% strain, there were no positive-staining cells.

**Effect of different CS regimens on AKT activation.** To determine the effect of change in amplitude or frequency of CS on AKT activation, a downstream target of AKT, the phosphorylation of GSK-3β was assessed. As shown in Fig. 4A, at 60 cpm-10%, GSK-3β phosphorylation in the control was maximal at 60 min. At both 60 cpm-15.6% (Fig. 4B) and 100 cpm-10% (Fig. 4C), GSK-3β phosphorylation in the control peaked at 10 and 5 min, respectively. The densitometric data (Fig. 4D) from at least three independent experiments in the control indicate that at 60 cpm-10%, the peak activation of GSK-3β in the control was 2.14-fold that at 60 min. The peak in the control at both 60 cpm-15.6% (1.85-fold) and 100 cpm-10% (1.90-fold) was earlier than that at 60 cpm-10%. Moreover, the peak activation of GSK-3β in Lipofectamine 2000-transfected ECs in all three regimens studied demonstrated a pattern similar to that in control. On the other hand, GSK-3β phosphorylation was unchanged in ECs transfected with RC25 and KA179 in all three regimens studied.

![Fig. 3. Immunocytochemistry of AKT (Ser473) phosphorylation in response to CS.](http://ajpcell.physiology.org/)

After cells were synchronized in serum-free medium for 24 h, nontransfected ECs and ECs transfected with Lipofectamine 2000, RC25, or KA179 were exposed to static condition (represented by time = 0 min) (A) or to 60 cpm-10% CS (B) for 60 min. A and B, top, center of membrane; bottom, periphery of membrane. Representative phase-contrast photomicrographs showing results of immunocytochemistry experiments in which anti-phospho-AKT (Ser473) antibody was used. Magnification, ×300. AKT phosphorylation in nontransfected ECs and ECs transfected with Lipofectamine 2000 or RC25 occurred only in the high-strain region of the membranes exposed to CS.
Importance of AKT on CS-induced cell number. As shown in Fig. 5A, after 24 h of exposure to 60 cpm-10% CS, the number of nontransfected ECs and ECs transfected with Lipofectamine 2000, RC25, and KA179 were exposed to static condition (represented by time = 0 min) or to CS for 5, 10, 30, 60, 120, and 240 min. A: representative immunoblot with anti-phospho-GSK-3β (Ser9) antibody and anti-GSK-3β antibody exposed to 60 cpm-10% CS (n = 4). B: representative immunoblot with anti-phospho-GSK-3β (Ser9) antibody and anti-GSK-3β antibody exposed to 15.6% CS at a frequency of 60 cycles/min (60 cpm-15.6%) (n = 4). C: representative immunoblot with anti-phospho-GSK-3β (Ser9) antibody and anti-GSK-3β antibody exposed to 10% CS at a frequency of 100 cycles/min (100 cpm-10%) (n = 4). D: densitometric data of GSK-3β bands from 4 separate experiments. Data are expressed as the density relative to each static condition; values are means ± SE. *P < 0.05 compared with static condition at 100 cpm-10%; **P < 0.05 compared with static condition at 60 cpm-15.6%.

Effect of AKT on DNA synthesis. The Flexcell unit used in these experiments results in a heterogeneous strain pattern (6, 33). When cells were exposed to 60-cpm-10% CS, cells seeded in the center of the membrane demonstrated insignificant strain, whereas cells seeded close to the periphery showed ~24% strain. The ratio of BrdU-positive cells was increased at the periphery of ECs transfected with RC25 or KA179 as well as nontransfected ECs and ECs transfected with Lipofectamine 2000 exposed to CS for 24 h compared with static conditions for 24 h (Fig. 6).

TUNEL staining of ECs exposed to CS. Figure 7 demonstrates that TUNEL staining of ECs subjected to 60 cpm-10% CS revealed no significant difference in pattern in either the center or the periphery of control cells or in cells transfected with Lipofectamine 2000 under static or CS conditions. However, ECs transfected with RC25 had positive-staining cells that were slightly increased at the periphery of stretched wells, and ECs transfected with KA179 were much more increased at the periphery after CS for 24 h compared with the static condition or the center of the stretched membrane for 24 h.

BAD and caspase-3 activation with CS. To assess whether apoptotic signaling pathways were activated, the phosphoryla-
Figure 9 demonstrates that cleaved caspase-3 was increased in a time-dependent manner in all groups. However, there was no significant difference between the experimental groups. In all groups, cleaved caspase-3 was detected by 1 h and slowly increased with exposure of ECs to CS.

**DISCUSSION**

The goal of this study was to determine the role of AKT in EC proliferation and survival when ECs were exposed to CS. We used plasmids containing either a point mutation in a PH domain of AKT (RC25) or a kinase-dead AKT (KA179) to determine the importance of these domains in the AKT signaling cascade. In our earlier paper (26), we reported that 60 cpm-10% CS stimulated AKT in bovine aortic ECs by 5 min. However, because the purpose of that paper was to compare the effects of shear and strain on ECs, we coated the Flex 1 membrane with 60 μg/ml collagen I for 24 h. This difference in surface concentration of collagen I probably accounts for the different temporal pattern compared with our present study. We also conducted immunocytochemical experiments that indicated that AKT activation occurred primarily in the high-strain region of the membrane (i.e., the periphery). The present study further demonstrates the role of the PH domain and kinase domain. In ECs transfected with RC25 both sites were only slightly phosphorylated, and, as expected, those sites were not phosphorylated in ECs transfected with KA179. Although there are reports that suggest that AKT with a mutation in the PH domain could not be activated by PI3-kinase in vivo or phosphatidylinositol-3,4-bisphosphate in vitro because of lower-affinity binding of phosphoinositides to the domain (24, 42), our results are consistent with reports that constructs lacking a PH domain but having the membrane attachment myristoylation motif may be activated, but to a lesser extent than the native protein (2, 30). The PH domain functions primarily to anchor the protein at the membrane, and AKT activity depends primarily on the myristoylation site at the NH2 terminus and the consequent constitutive kinase activity. Our results also seem to be in conflict with previous reports that the kinase-inactive mutant (KA179) could still be phosphorylated on both Thr308 and Ser473 after vanadate, IGF-I, or insulin stimulation (1, 2, 32). However, in those studies, the phosphorylation level was very low and similar to the results shown in Fig. 1, which may still be consistent with our findings. It should also be noted that other investigators use a different kinase-dead AKT construct (Lys179-to-alanine mutant) which is refractory to the consequent constitutive kinase activity. Our results also seem to be in conflict with previous reports that the kinase-inactive mutant (KA179) could still be phosphorylated on both Thr308 and Ser473 after vanadate, IGF-I, or insulin stimulation (1, 2, 32). However, in those studies, the phosphorylation level was very low and similar to the results shown in Fig. 1, which may still be consistent with our findings. It should also be noted that other investigators use a different kinase-dead AKT construct (Lys179-to-alanine mutant) which is refractory to Thr308 and Ser473 phosphorylation (53, 54). Together, our results are consistent with the hypothesis that the kinase domain of AKT, and to a lesser extent the PH domain of AKT, is necessary for the AKT phosphorylation induced by CS.

Because GSK-3 is a critical downstream element of the PI3-kinase/AKT pathway and GSK-3 reacts similar to AKT (14, 57), we studied GSK-3 when we determined the effect of dominant-negative AKT plasmids. The present study also confirms that both RC25 and KA179 can affect downstream AKT substrate activation using the various force regimens that we used. Increasing the amplitude or frequency of strain resulted in an earlier sustained activation of GSK-3β, although the magnitude of phosphorylation was similar in all of the various force regimens used. Other investigators have reported that the activation of c-JNK was regulated by a magnitude-dependent...
but not a frequency-dependent response (4). Although the CS regimens were different in our study, our findings are consistent with the hypothesis that the amplitude or frequency of CS affects the profile of signal activation.

In all the CS regimens tested, the number of control and nontransfected ECs at 24 h was increased, which is consistent with our previous results (45, 61). The amplitude or frequency of strain did not affect the magnitude of CS-induced EC number at 24 h, although other investigators who studied human osteoblast-like cells have demonstrated a frequency- and cycle number-dependent proliferative response (36). The seminal findings of our study are that AKT is necessary for CS-induced increase in EC number and inhibition of apoptosis. ECs transfected with RC25 and especially with KA179 demonstrated no CS-induced increase in cell number.

Cooper (13) reported that if a cell in exponential growth had a measured doubling time of 20 h, it might be expected that in a well-synchronized culture started with a pure collection of newborn cells, a large majority of cells would divide between 18 and 22 h. Even a lower-resolution synchrony in which most of the cells divided between 17 and 23 h might be acceptable (13). The present study demonstrated that the ratios of BrdU-positive cells, after an assessment of DNA synthesis and consequently of cellular proliferation, were increased in nontransfected ECs or dominant-negative transfected ECs exposed to CS for 24 h. This finding strongly suggests that AKT does...
not play a critical role in DNA synthesis in the absence or presence of CS.

AKT has been shown to promote cell survival via its ability to phosphorylate BAD at Ser136 (16, 17). BAD is a proapoptotic member of the Bcl-2 family that regulates apoptosis by controlling mitochondrial permeability and the release of cytochrome c (25). Moreover, the mitochondrial pathway of programmed death involves the release of not only cytochrome c but also pro-caspase-9 and Apaf-1 from the mitochondrial intermembrane space and a series of subsequent biochemical interactions that include the activation of caspase-9. This leads to the activation of caspase-3 (53), which is thought to play a crucial role in apoptosis (52).

Apoptosis is generally characterized in the early stages by cleavage and subsequent activation of caspases and at later stages by internucleosomal DNA fragmentation (12, 39). We previously reported (26) that CS could prevent apoptosis in ECs. In the present study, when the center region of the well (where the strain was <1%) was analyzed, the ratios of TUNEL-positive cells after 24 h were similar under both static and CS conditions. At the periphery of the Flex 1 membrane (where the strain was 10–24%), the TUNEL-positive cell number of control ECs and ECs transfected with Lipofectamine 2000 after CS for 24 h was slightly decreased. However, TUNEL staining showed a slight increase in positive cells at the periphery after 24 h in ECs transfected with RC25 and a significant increase in positive cells in ECs transfected with KA179. Consistent with the TUNEL studies, we also demonstrated BAD phosphorylation and the generation of cleaved caspase-3 in ECs exposed to CS. BAD phosphorylation was attenuated in the AKT dominant-negative transfected ECs, especially in the KA179 group. However, our results also demonstrated that cleaved caspase-3 was activated in a time-
dependent manner in nontransfected control ECs as well as in dominant-negative transfected ECs. This finding is in conflict with the data regarding TUNEL staining and BAD phosphorylation. In this regard, Osaki et al. (49) showed that Bcl-2 and Bcl-XL, which are members of the Bcl-2 family associated with the Fas-mediated apoptotic signal pathway and consequently related to caspase-induced apoptosis, did not change after treatment with LY-294002, a PI3-kinase inhibitor in the human gastric carcinoma cell line MKN-45. Thus our results indicate not only that AKT is necessary for CS-induced apoptosis and BAD phosphorylation but also that the caspase-induced apoptosis pathway may be different from the CS-induced apoptosis pathway. AKT does not seem to be involved in the direct activation of cleaved caspase-3, but further studies are needed to resolve this question.

In conclusion, the magnitude of AKT activity based on GSK-3β phosphorylation was independent of the CS amplitude and frequency regimes tested, whereas the time until peak response was shortened at the highest CS amplitude and the highest frequency tested. The increase in EC number induced by CS appears to be due to an increase in cell survival and an increase in DNA synthesis, whereas the decrease in the number of ECs transfected with KA179 is due to a decrease in cell survival and not a decrease in DNA synthesis. Our results also suggest that the kinase domain of AKT, and to a lesser extent the PH domain of AKT, is necessary for the AKT phosphorylation induced by CS. AKT is important in preventing apoptosis but is not involved in EC proliferation.

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