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# Phosphatase PTEN Is Inactivated in Bovine Aortic Endothelial Cells Exposed to Cyclic Strain

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**Abstract** Hemodynamic forces, including cyclic strain (CS) and shear stress (SS), have been recognized as important modulators of vascular cell morphology and function. PTEN (also known as MMAC1/TEP1) is a lipid phosphatase that leads to a decrease in intracellular phosphatidylinositol 3,4,5 trisphosphate (PIP3) and therefore can modulate the stimulating effect of phosphatidylinositol 3-kinase (PI3K). In this study, we focused on the upstream regulators of the PI3K-Akt pathway by assessing Akt, PTEN, casein kinase 2 (CK2) (a kinase that catalyzes phosphorylation of PTEN), and PI3K activity in endothelial cells (EC) exposed to CS. The activity of phospho-PTEN ($n = 4$) and phospho-CK2 ($n = 4$) increased in a time-dependent fashion, reaching maximal activity by 10 min of CS stimulation. The peak of phospho-Akt activity ($n = 4$) occurred later, at 60 min. Akt activity was altered by transfection of EC with dominant negative PTEN plasmids. Furthermore, CS increased PIP3 immunoreactivity in a time-dependent manner, reaching maximal activity after 60 min of CS stimulation, and these effects were affected by transfection of EC with dominant negative PTEN plasmids. Inhibition of PTEN activity had no effect on CS-mediated cell proliferation but inhibited CS-mediated suppression of apoptosis. J. Cell. Biochem. 100: 515–526, 2007. 

**Key words:** cyclic strain; endothelial cell; Akt; PTEN; PIP3

The mechanical forces associated with blood flow, shear stress (SS), and cyclic strain (CS), play an important role in the regulation of vascular tone, vascular remodeling, and the focal development of atherosclerotic lesions [DeBakey et al., 1985; Ku et al., 1985; Gimbrone et al., 1997; Frangos et al., 1999]. CS represents the constant, rhythmic deformation of the vascular wall by pulsatile, hydrostatic pressures during systole and diastole. Both endothelial cells (EC) and the underlying vascular smooth muscle cells (SMC) are exposed to this repetitive external force [Sumpio, 1993]. Furthermore, CS is one of many stimuli that regulate protein synthesis, morphology, migration, apoptosis, proliferation, and survival in EC [Iba and Sumpio, 1992; Sumpio et al., 1997, 1998; Li et al., 2003; Li and Sumpio, 2005]. This delicate balance of survival, proliferation, and death is essential for the regulation of angiogenesis, vessel regression and remodeling, maintenance of vascular integrity, and, when disturbed, may become the pathogenesis of vascular disease [Cho et al., 1995; Isner et al., 1995; Stefanec, 2000]. CS and SS are recognized to have important roles in modulating EC function both in vitro [Frangos et al., 1985; Kuchan and Frangos, 1993; Sumpio et al., 1998] and in vivo [Ku et al., 1985; Huynh et al., 1999]. The mechanism by which these forces activate intracellular signal transduction in EC is becoming increasingly understood [Letsou et al., 1990; Awolesi et al., 1995; Tseng et al., 1995; Azuma et al., 2000], as are some of the downstream pathways [Sumpio and Banes, 1988; Iba and Sumpio, 1992; Davies, 1995; Patrick and McIntire, 1995; Sumpio et al., 1997; Lehoux and Tedgui, 1998; Ikeda et al., 1999; Azuma et al., 2001; Frangos et al., 2001]. But very little information is available on the role of phosphatases that counteract kinase activity [Murata et al., 1996; Lee et al., 2003; Lee and Sumpio, 2004].

Phosphatidylinositol 3-kinase (PI3K) catalyzes the phosphorylation of phosphatidylinositol

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at the 3′-OH position to generate phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 3,4-bisphosphate (PI(3,4)P2), and phosphatidylinositol 3,4,5-trisphosphate (PIP3). Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) appears to be the major substrate for PI3K, with PIP3 being the major reaction product [Katso et al., 2001]. Lipid phosphatase PTEN is a tumor suppressor protein that functions, in large part, by dephosphorylating the lipid second messenger PIP3 [Maehama and Dixon, 1998] and by doing so. attenuates PI3K/Akt signaling [Vazquez and Sellers, 2000; Maehama et al., 2001]. Phosphorylation of the PTEN C-terminal domain negatively regulates its function as an antagonist of PI3K signaling [Vazquez et al., 2000]. Inhibition of PTEN activity by phosphorylation of PTEN results in elevated levels of PIP3 and consequent Akt activation [Vazquez et al., 2001]. Dephosphorylation of PTEN will increase its activity leading to downregulation of Akt activity [Vazquez et al., 2000, 2001]. A cluster of serine/threonine residues located at the PTEN C terminus are targets for phosphorylation by protein kinase CK2 [Torres and Pulido, 2001]. CK2-mediated phosphorylation of PTEN has been demonstrated to inhibit PTEN function. [Vazquez et al., 2000; Miller et al., 2002].

We recently reported on the upregulation of AKT activity in response to CS [Li and Sumpio, 2005]. In the present study, we focus on the upstream regulation of the PI3K-Akt pathway. Here we compare the time course of activation of Akt, CK2, and PTEN and the changes in PI3K activity in ECs exposed to CS. The functional role of PTEN in CS-mediated cell proliferation and inhibition of apoptosis was also examined.

## MATERIALS AND METHODS

### Cell Culture and Cyclic Strain Application

Bovine aortic EC were obtained by scraping the intimal surface of calf thoracic aorta as previously described [Gallagher and Sumpio, 1997] and were cultured in Dulbecco modified Eagle Medium F-12 (Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT), 5 μg/ml deoxycytidine/thymidine (Sigma Chemical, St. Louis, MO), and antibiotics (penicillin 100 U/L, streptomycin 100 μg/ ml, and amphotericin B 250 ng/ml) (Gibco-BRL) at 37°C in a humidified incubator with 5% carbon dioxide. Cells of passage 3 to 8 were seeded on flexible-bottomed collagen I-coated culture plates (Flex I plates; Flexcell International, Hillsborough, NC). Prior to exposure to CS, cells were synchronized with serum-free medium for 24 h. Cells were exposed to CS by placing the Flex I plates on FX-4000 Flexcell strain unit (Flexcell International), and deforming the membrane bottoms with 150 mmHg of pressure, resulting in an average strain of 10%, at a frequency 60 cycles/min (0.5 s deformation alternating with 0.5 s in neutral conformation) as previously described [Li et al., 2001, 2003; Chen et al., 2003]. The control group consisted of ECs seeded in Flex 1 collagen plates that were left in the same incubator without exposure to strain.

### Plasmids and Transfection Protocol

The plasmids pSG5L-HA-PTEN; Wild type (WT), pSG5L-HA-PTEN; G129R and pSG5L-HA-PTEN; 380–385A were kindly provided by Dr. William R. Sellers [Nakamura et al., 2000; Vazquez et al., 2000, 2001]. G129R lacks both protein and lipid phosphatase activity [Ramaswamy et al., 1999; Nakamura et al., 2000] while 380–385A has a mutation on the PTEN phosphorylation sites [Vazquez et al., 2000, 2001]. Transfections were conducted according to manufacturers' recommendations (Invitrogen, Carlsbad, CA). DNA (0.5 μg) were diluted to 100 μl in OPTI-MEM (Gibco-BRL) and then combined with 2.5 μl of LIPOFECTAMINE 2000 Reagent (Invitrogen) diluted in 100 μl of MEM. The mixture was incubated for 30 min at room temperature, and then added drop wise onto the cells. The medium was replaced after 4 h and the cells incubated at 37°C for 20 h. The efficiency of transfection was about 80% as determined by immunohistochemical detection using an anti-HA antibody (Cell Signaling, Beverly, MA). Control cells were EC only treated with LIPOFECTAMINE 2000 Reagent.

### PI3 Kinase Inhibition Studies

We compared the effects of specific chemical inhibitors of PI3 kinase, LY294002 and wortmannin with the dominant negative PTEN plasmids. Although the specificity for PI3K is high, other pathways such as myosin light-chain kinase (MLCK) [Yano et al., 1995] or mitogen-activated protein kinase [Suga et al., 1997] may be inhibited depending on the dose of LY294002 or wortmannin. Nevertheless, these two inhibitors are well established and broadly

used by others and have been reported in our previous studies [Chen et al., 2003; Li et al., 2003; Li and Sumpio, 2005]. EC were subjected to CS in the presence and absence of the LY294002 (10 μmol/L, Calbiochem, La Jolla, CA) and wortmannin (25 nmol/L, Calbiochem) and studied as above. EC were preincubated with the inhibitors 1 h before the initiation of cyclic strain [Kraiss et al., 2000]. Since these inhibitors were dissolved in DMSO, control cells in these studies were similarly treated with DMSO to a final concentration of 0.2%.

### Immunohistochemical Analysis of PIP3

After exposure to CS, the cells were washed twice in 25 mM Tris buffered saline (TBS), fixed for 5–10 min in 4% paraformaldehyde, and washed three times in TBS. After blocking with 5% normal goat serum (NGS) and 2% bovine serum albumin (BSA), samples were incubated overnight at 4°C with mouse anti-PIP3 monoclonal antibody (Echelon, Salt Lake City, UT) at 1:200 dilution. Primary antibodies were detected with Alexa488 conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR) at a dilution of 1:100 [Niswender et al., 2003]. The negative immune control was an equivalent concentration of non-immune mouse IgG (DakoCytomation, Carpinteria, CA) substituted for the primary antibody at an equivalent dilution. All antibodies were diluted in TBS containing 2% BSA and 5% NGS.

Samples were counterstained with SYTOX-Orange (Molecular Probes) to determine the number of nuclei per field of view. Images were captured via standard epifluorescence using an Olympus BH-2 microscope (Olympus, Tokyo, Japan) equipped with a Diagnostic Instruments Color digital camera. All images for a single experiment were obtained using identical acquisition parameters in one imaging session. PIP3 immunofluorescence was quantified using Scion Image freeware (Scion, Frederick, MD) and expressed as the simple average pixel intensity for the field of view. SYTOX-Orange fluorescence was evaluated via particle analysis using Scion Image freeware, yielding the total number of cells present per field of view. For PIP3 immunofluorescence study, values are reported as the mean ± SEM of 3–5 fields of view per well and at least 4-wells per measurement. The average pixel intensity was analyzed for the negative control this way from a set of wells in which an equivalent concentration of non-immune mouse IgG was substituted for the PIP3 antibody.

### Immunoblotting Technique

After exposure to CS, cells were washed with ice-cold phosphate-buffered saline (PBS) and scraped in lysis buffer containing 50 mmol/L HEPES, 150 mmol/L sodium chloride, 10% glycerol, 1% Triton X-100, 1.5 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin. Cell lysate were centrifuged to collect supernatant, and equal amounts of protein (30 μg per lane; BioRad protein assay system, BioRad, Hercules, CA) were loaded on 10% sodium dodecyl sulfate–polyacrylamide gels and transferred to nitrocellulose membrane (Amersham, Arlington Heights, IL). The membranes were incubated with primary antibody, either anti-Akt antibody or anti-phospho-specific Akt (Ser473) antibody or anti-PTEN antibody, or anti-phospho-specific PTEN (Ser380/Thr382/383) antibody (Cell Signaling), or anti-CK2 antibody, or anti-phospho CK2 antibody (Calbiochem), and horseradish peroxidase-conjugated secondary antibody (Cell Signaling), prior to detection of immunoreactivity by enhanced chemiluminescence (Amersham). All blots were quantified with densitometry (BioImage, Ann Arbor, MI). Densitometry data from each of at least three similar blots from separate and distinct experiments was pooled prior to statistical analysis after normalization against band intensity for the control lane (static untreated cells).

### Cell Counting

For proliferation studies, EC were seeded at 100,000 cells/cm$^2$, serum-starved for 24 h, and transfected with plasmids, or lipofectamine or vehicle as described above. The cell number was counted before and after CS for 24 h. Control cells were exposed to static conditions for 24 h. Cells were detached with trypsin or ethylenediamine tetraacetic acid, and an aliquot was counted with both an automatic counter (Model ZM, Coulter Electronics, Hialeah, Fla) and metallized hemacytometer (Hausser scientific, Horsham, PA). All proliferation studies were done in two 6-well plates (12 well/group) with cell counts from each of the 12-wells counted independently in triplicate. Triplicate counts were averaged to yield a mean cell count/well, and data from each experiment was thus analyzed with 12 observations in each group.

### Detection of Apoptosis

Transfected and non-transfected EC were synchronized for 24 h with serum-free medium and then exposed to CS for 24 h; control cells were exposed to static conditions. Cells were then fixed with 4% paraformaldehyde and apoptosis was assayed using 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 in five representative high power fields for each sample.

### Statistical Analysis

Data are presented below as mean ± standard error, and analyzed by Student unpaired *t*-test or ANOVA with Bonferroni correction. $P < 0.05$ was considered significant. We performed each study at least three times with similar results.

## RESULTS

### Cyclic Strain Stimulates PIP3 Immunoreactivity in Bovine Aortic ECs

To determine the effect of CS on PIP3 immunofluorescence, ECs were serum starved for 24 h and then treated with vehicle, lipofectamine, PI3K inhibitors, DMSO or transfected with plasmids WT, G129R, or 380–385A, and PIP3 fluorescence quantitated by pixel intensity. PIP3 immunoreactivity was readily visible in ECs stained with anti-PIP3 antibody but was not detected in cells incubated with an equal concentration of non-immune mouse IgG (Data not shown). Figure 1 demonstrates that CS increased PIP3 immunofluorescence in a time-dependent manner. PIP3 immunofluorescence peaked at 60 min, then decreased to basal levels by 240 min after the initiation of strain in EC, vehicle, or EC transfected with lipofectamine or WT. In ECs transfected with G129R, that lacks both protein and lipid phosphatase activities (43), PIP3 immunofluorescence intensity was higher than vehicle, lipofectamine, or WT (Fig. 1D). On the other hand, PIP3 immunofluorescence intensity was generally weaker in EC transfected with 380–385A (Fig. 1E).

Figure 2 demonstrates PIP3 immunofluorescence in EC treated with PI3K inhibitors and subjected to CS. There was abolition of PIP3 immunofluorescence intensity in the presence of these inhibitors. Figure 3 is a schematic of the time course of average PIP3 immunofluorescence intensity from four different experiments. CS increased PIP3 immunofluorescence and peaked at 60 min after CS stimulation by $2.03 \pm 0.18$, $1.75 \pm 0.20$, $1.93 \pm 0.21$, and $1.85 \pm 0.17$-fold in ECs treated with vehicle, lipofectamine, WT, and DMSO, respectively relative to static condition. Figure 3B indicates that PIP3 immunofluorescence in ECs transfected with G129R rapidly increased and was maximum at 60 min after CS stimulation by $2.14 \pm 0.28$ relative to static condition. PIP3 intensity in EC transfected with 380–385A was generally weak but gradually increased to a maximum at 240 min after CS stimulation by $1.48 \pm 0.15$ relative to static condition (Fig. 3B). To rule out a random increase in the number of cells per field of view accounting for higher PIP3 signal, a corresponding SYTOX-Orange nuclear stain image was collected for each PIP3 fluorescence image obtained. There was no significant difference in the average number of cells per field of view for vehicle ($49 \pm 4$), lipofectamine ($51 \pm 4$), WT ($52 \pm 5$), G129R ($48 \pm 4$), 380–385A ($49 \pm 5$), DMSO ($46 \pm 4$), LY294002 ($50 \pm 5$), or wortmannin ($51 \pm 4$).

### Akt, PTEN, and CK2 Are Phosphorylated by Cyclic Strain

To confirm that CS activates Akt, we measured the phosphorylation of Akt in EC exposed to CS with time. Figure 4A shows that strain induced Akt phosphorylation occurred in a time-dependent manner. Similar to our previous results (15), activation peaked at 60 min by $2.23 \pm 0.42$-fold (Fig. 4D), after which Akt activation declined and decreased to the basal levels 240 min after the initiation of strain. To study the upstream regulation of Akt, the phosphorylation of PTEN by CS was assessed. Exposure of EC to CS resulted in a time-dependent PTEN phosphorylation. In contrast to Akt, maximal phosphorylation occurred earlier, at 10 min after the CS stimulation by $2.21 \pm 0.50$-fold (Fig. 4B,D) relative to static condition. In pilot experiments, there was no change in PTEN protein expression in EC exposed to up to 24 h of CS (data not shown). We also examined CK2 phosphorylation in EC exposed to CS. There was a small ($1.48 \pm 0.15$-fold) transient increase in phosphorylation at 10 min after CS stimulation (Fig. 4C,D).

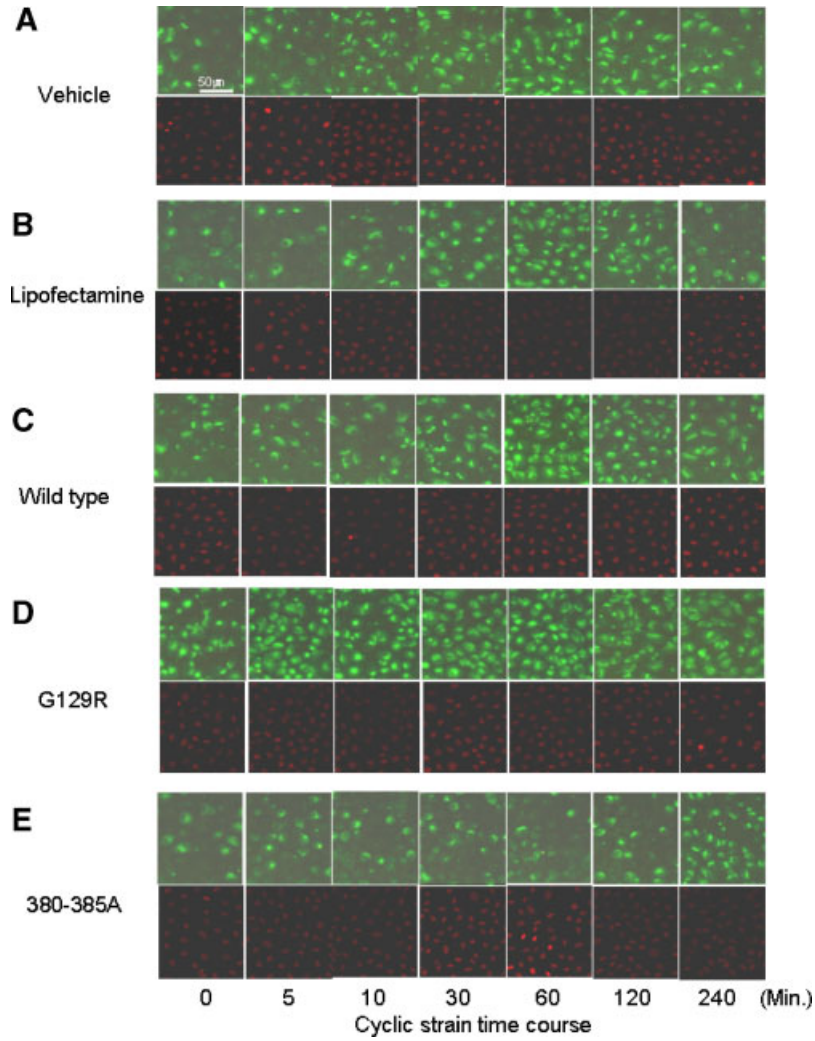


**Fig. 1.** Representative PIP3 immunofluorescence (Green) and its nucleic counter stain (Red) in bovine aortic EC treated with vehicle (**A**), Lipofectamine (**B**), or transfected with WT (**C**), G129R (**D**), and 380–385A (**E**) on CS time course. CS induce PIP3 immunoreactivity (peaks at 60 min) in ECs that were treated with vehicle (A), Lipofectamine (B), and WT (C) in a time-dependent fashion. PIP3 immunoreactivity were higher in ECs transfected with G129R (D). PIP3 immunofluorescence were low and gradually increase in ECs transfected with 380–385A (E). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

### Akt Activation Is Influenced by PTEN

To determine how Akt activity is influenced by PTEN, we assessed phosphorylation of Akt in EC transfected with dominant negative plasmids. Maximal Akt phosphorylation occurred at 60 min by $2.23 \pm 0.42$, $2.86 \pm 0.48$, and $2.60 \pm 0.17$-fold in EC treated with vehicle, lipofectamine, and WT, respectively relative to static condition (Fig. 5A). On the other hand, phosphorylated Akt rapidly increased by 10 min of exposure to CS in ECs that were transfected with G129R(Fig. 5B). In EC that were transfected with 380–385A, there was only a slow gradual increase in Akt activity that only became significant by 240 min (Fig. 5B).

### Proliferation and Apoptosis in EC

Figure 6 shows the 24-h proliferation for EC in the presence or absence of CS. There was no significant difference before CS stimulation for all groups. Bovine aortic EC maintained under CS conditions for 24 h significantly increased their cell number an average $1.23 \pm 0.10$, $1.15 \pm 0.10$, and $1.17 \pm 0.10$-fold in ECs treated with vehicle, lipofectamine, and WT, respectively (Fig. 6). ECs transfected with the plasmids G129R and 380–385A and then subjected

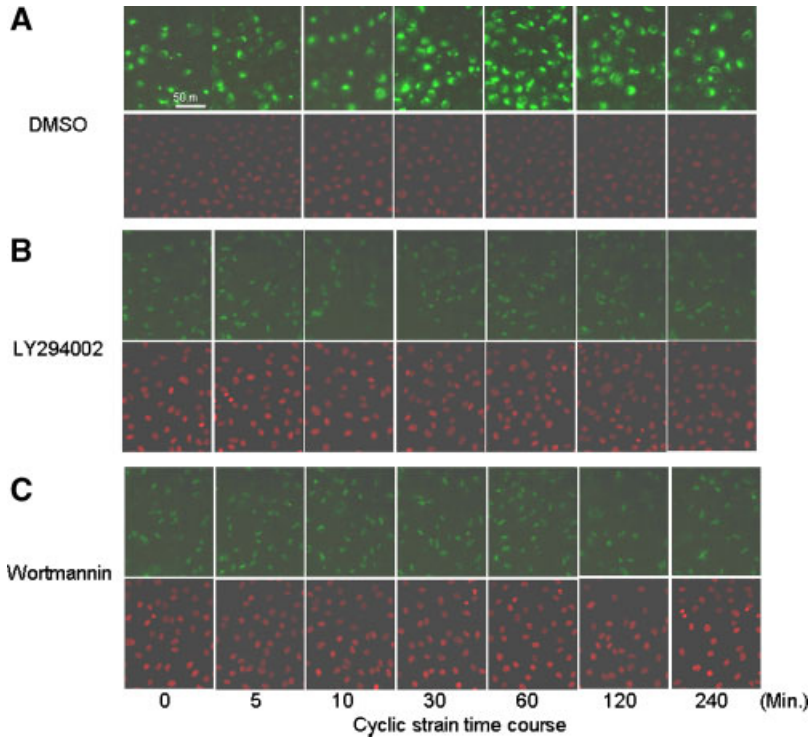


**Fig. 2.** Representative PIP3 immunofluorescence (Green) and its nucleic counter stain (Red) in bovine aortic EC treated with DMSO (**A**), LY294002 (**B**), and wortmannin (**C**) on CS time course. LY294002 and wortmannin were initially solubilized in DMSO to yield a final concentration of 0.2% after the inhibitors were added to the cell culture medium, so an equivalent DMSO control is shown (A). CS induces PIP3 immunoreactivity (peaks at 60 min) in ECs that were treated with DMSO (A) in a time-dependent manner. The PIP3 immunofluorescences are generally weak in ECs that were treated with PI3K inhibitors, LY294002 (B) and wortmannin (C) compared to the control DMSO. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to CS for 24 h also significantly increased cell number an average $1.16 \pm 0.10$ and $1.17 \pm 0.10$-fold relative to static condition in response to strain respectively (Fig. 6). Transfection of EC with dominant negative plasmids to PTEN had no effect on the CS-induced increase in cell number that has been previously reported [Li and Sumpio, 2005].

To determine the effect of CS on EC apoptosis, EC were maintained for 24 h in serum-free medium, with or without CS stimulation. After 24-h in serum-free medium, apoptosis was detected in cells grown in the absence of CS (Fig. 7). In contrast to control vehicle, less apoptotic cells were seen in EC transfected with G129R under both static and CS condition (Fig. 7B). In ECs transfected with 380–385A, more apoptotic cells were detected compared to vehicle in both static and CS conditions (Fig. 7B). However, CS stimulation for 24 h suppressed EC apoptosis an average $0.41 \pm 0.21$, $0.38 \pm 0.43$, $0.46 \pm 0.22$, $0.35 \pm 0.30$, $0.79 \pm 0.23$-fold relative to static condition in EC treated with vehicle, lipofectamine, WT, G129R, and 380–385A, respectively (Fig. 7).

## DISCUSSION

Hemodynamic forces have been recognized as important modulators of vascular cell morphology and function. The mechanism by which vascular cells sense and transduce the extracellular mechanical signals into the cell nucleus is only slowly becoming elucidated. CS is an important hemodynamic force that represents the repetitive deformation which occurs as the vessel wall rhythmically distends and relaxes with the cardiac cycle [Sumpio, 1993].

CS has been previously reported to stimulate EC production of prostacyclin [Sumpio and Banes, 1988], endothelin [Sumpio and Widmann, 1990], tissue plasminogen activator [Sumpio et al., 1997], and nitric oxide [Awolesi et al., 1995]. Our group has also previously reported that CS induces Akt activation in EC in a time-dependent manner [Li and Sumpio,

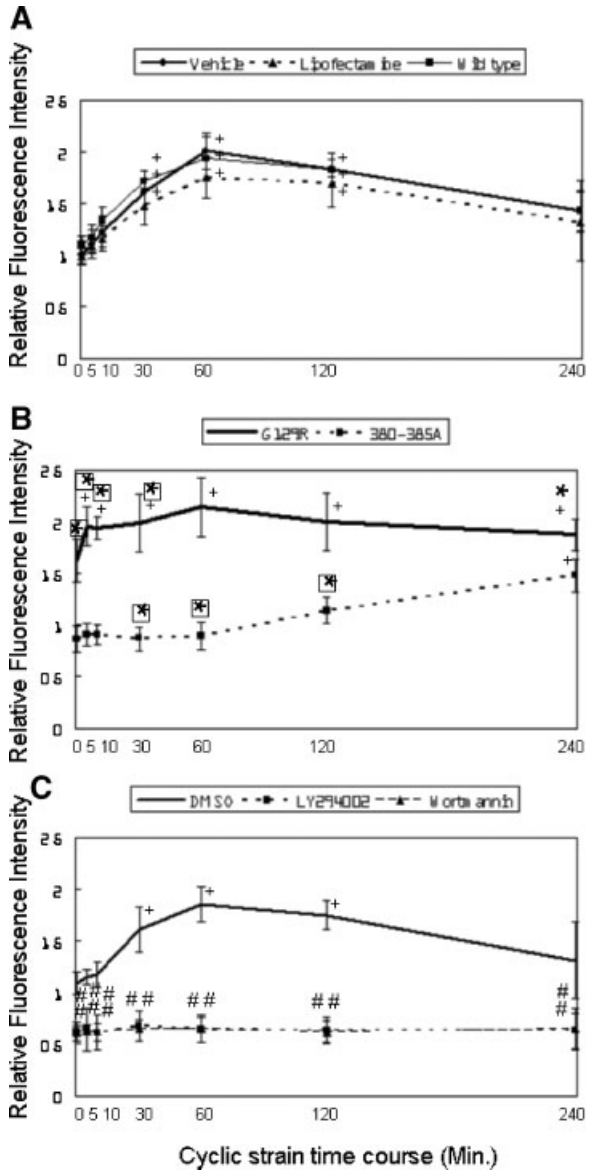


**Fig. 3.** Time course of PIP3 immunoreactivity by CS. Graphs depict the mean increase ratio, from at least four different experiments, of densitometric analyzed PIP3 fluorescence intensity that are divided by the cell number compared to static condition (time 0 min) of vehicle. PIP3 immunofluorescence peaked at 60 min after the initiation of strain in EC treated with vehicle (**A**), lipofectamine (A), WT (A), and DMSO (**C**). In ECs transfected with G129R, PIP3 immunofluorescence intensity rapidly increased and was higher than vehicle at the time point 0, 5, 10, 30, and 240 min (**B**). PIP3 immunofluorescence intensity was generally weak but gradually increased at the maximum 240 min after CS stimulation relative to static condition in EC transfected with 380–385A (B). There was abolition of PIP3 immunofluorescence intensity in the presence of PI3K inhibitors (LY294002 and wortmannin) (C). (+$P < 0.05$ to static, *$P < 0.05$ to vehicle, #$P < 0.05$ to vehicle, n = 4).

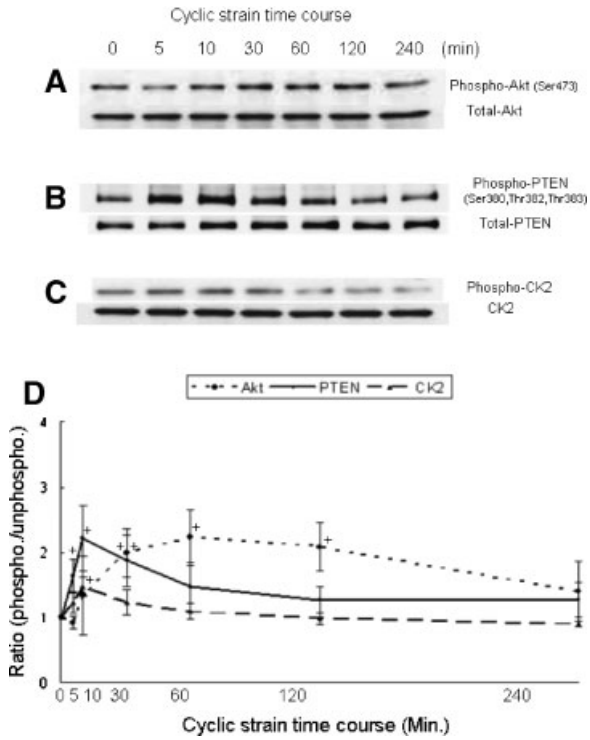


**Fig. 4.** Time course of Akt, PTEN, and CK2 phosphorylation by CS. The three top panels are representative Western Blots for Akt (**A**), PTEN (**B**), and CK2 (**C**), and the bottom panel (**D**) represents densitometric analysis of four similar experiments for them. Graph depicts the mean increase ratio of phosphorylated to total, compared to baseline (time 0 min). CS induced Akt phosphorylation occurred in a time-dependent manner and the activation peaked at 60 min after the initiation of strain (A). CS resulted in a time-dependent PTEN phosphorylation in contrast to Akt, maximal phosphorylation occurred earlier, at 10 min after the CS stimulation (B). There was a small CK2 phosphorylation transient increase in activity at 10 min after the CS stimulation (C). (+$P<0.05$ to static, n = 4).

2005]. In the present study, we focused on the upstream regulators of the PI3K-Akt pathway. We first confirmed that strain induced the phosphorylation of Akt with a peak at 60 min and was consistent with our previous results [Li and Sumpio, 2005]. To assess PI3K activity, we measured PIP3 by an immunocytochemical method. The PIP3 antibody and technique that we utilized has been recently reported as a valid and useful non-radioactive tool for detection of changes in intracellular PI3K signaling [Chen et al., 2002; Niswender et al., 2003]. Our results indicate that CS increased PIP3 immunofluorescence in bovine aortic EC in a time-dependent fashion which peaked at 60 min after CS stimulation (Fig. 1A). This result is consistent with the time course of Akt activation by CS. We

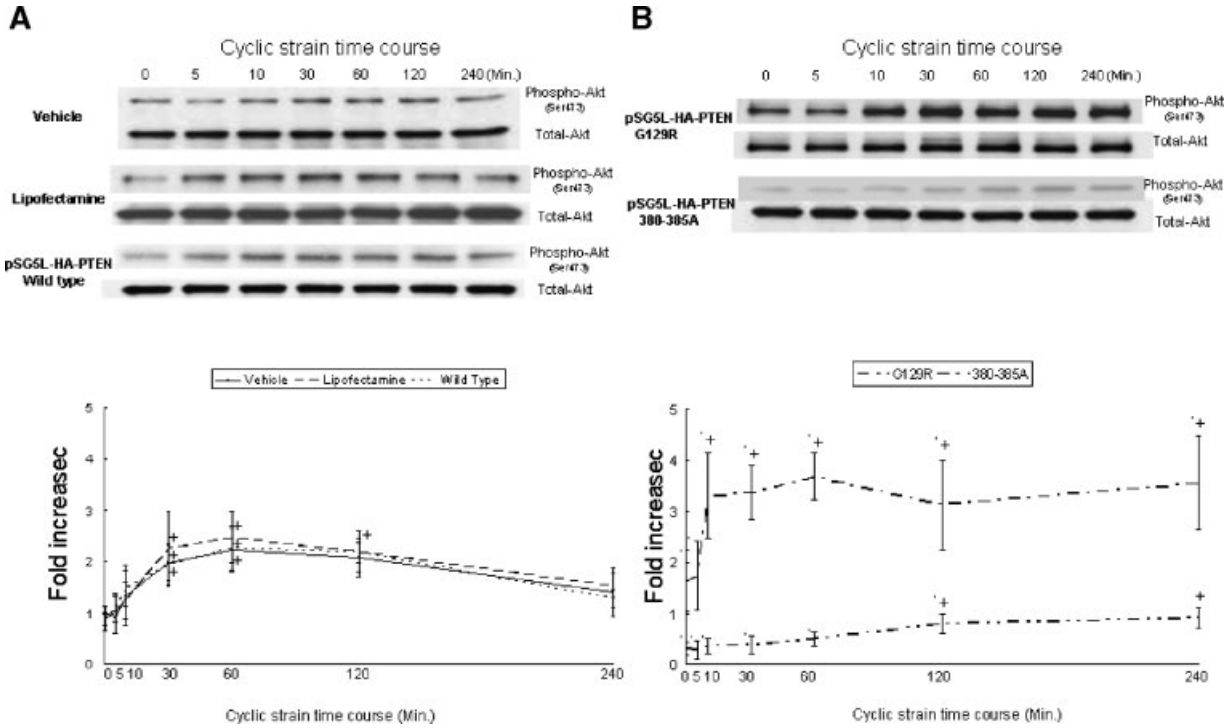


**Fig. 5.** Time course of Akt phosphorylation by CS in ECs treated with vehicle (**A**), lipofectamine (A), WT (A), G129R (**B**), and 380–385A (B). The top panels of (A) and (B) are representative Western Blots for Akt and the bottom panels of (A) and (B) are densitometric analysis of four similar experiments for them. Graphs depict the mean increase ratio of phosphorylated to total, compared to baseline (time 0 min) of vehicle. Akt phosphorylation peaked at 60 min after the initiation of strain in EC treated with vehicle (A), lipofectamine (A), and WT (A). In ECs transfected with G129R, Akt phosphorylation rapidly increased and was higher than vehicle (B). Akt phosphorylation was generally weak but gradually increased at the maximum 240 min after CS stimulation relative to static condition in EC transfected with 380–385A (B). ($+P < 0.05$ to static, $*P < 0.05$ to vehicle, $n = 4$).

also demonstrate that CS induced CK2 and PTEN phosphorylation at an earlier time point (Fig. 4). It has been previously reported that the level of PIP3 can be regulated by the effect of PTEN, PI3K, and SH2-containing inositol 5-phosphatase (SHIP) which converts PIP3 into PI(3,4)P2 [Pesesse et al., 1997; Ishihara et al., 1999]. In our pilot studies, CS induced no

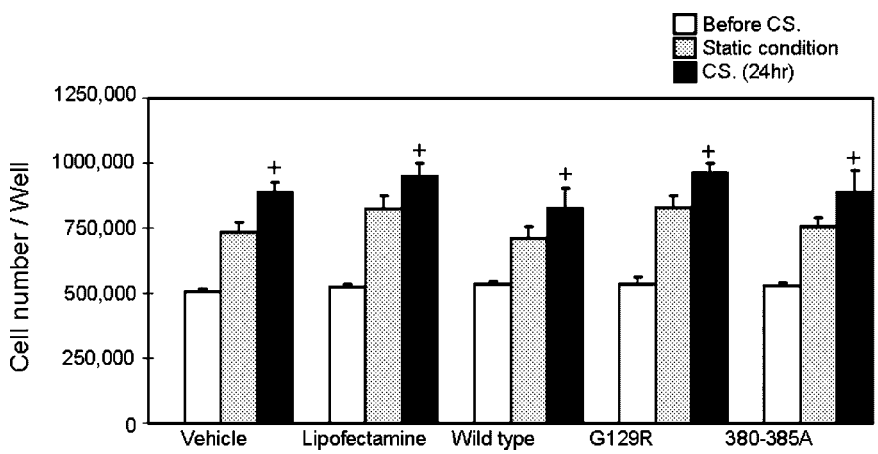


**Fig. 6.** Cell proliferation studies in bovine aortic ECs treated with vehicle, lipofectamine, WT, G129R, and 380–385A. Replicate EC monolayers were serum-starved for 24 h (open bars), and then either maintained under static conditions (dotted bars) or repetitively strained (shaded bars) for a period of 24 h prior to trypsinization and cell counting. Strain increased average cell number in cell monolayers in all groups and there was no significant difference in all groups. ($+P < 0.05$ to static condition, $n = 12$).

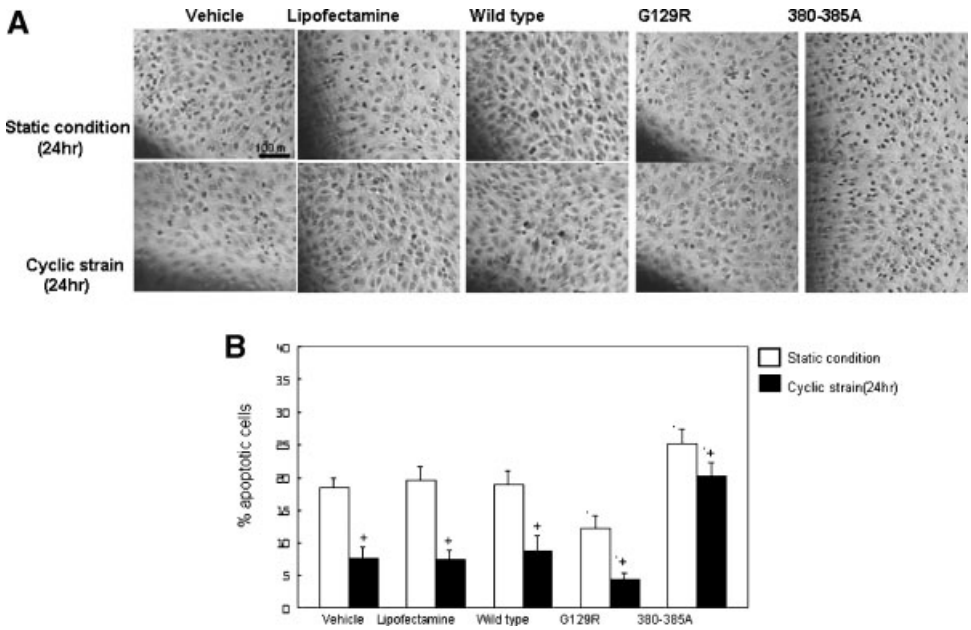


**Fig. 7.** TUNEL studies in bovine aortic ECs treated with vehicle, lipofectamine, WT, G129R, and 380–385A. The top panels (**A**) are representative TUNEL studies under static conditions and 24 h cyclic strained conditions and the bottom panel (**B**) represents the mean percentage of apoptotic cells in each treatment groups. Apoptosis is reduced in the presence of CS in all groups. Compared to control vehicle, the percentage of apoptotic cells are lower in G129R and higher in 380–385A in both static and CS conditions. ($+P < 0.05$ to static, $*P < 0.05$ to vehicle, n = 3).

activation of SHIP in bovine aortic EC (data not shown). Based on the time course of activation that we observed, we hypothesize that the phosphatase PTEN is phosphorylated and inactivated in EC exposed to CS prior to the phosphorylation and activation of Akt. In the early phase (up to 10 min), phospho-CK2 and phospho-PTEN were rapidly increased while the level of dephosphorylated PTEN decreased. In the late phase (about 60 min), the PI3K-Akt pathway is activated and phospho-Akt reaches a peak while phospho-PTEN starts to decrease to basal levels. This regulates the intracellular levels of PIP3 leading to a gradual decrease in Akt activity. It should be noted that a functional role of CK2 in phosphorylating PTEN has not been determined in our studies since we did not have access to PTEN plasmids with mutations at the CK2 consensus motif at position 370 [Vazquez et al., 2000].

In the present study, the Akt activity, PIP3 immunofluorescence, and apoptosis in ECs were strongly influenced by PTEN dominant negative plasmids (Figs. 1D,E, 5B, and 7). Previous studies have showed that PTEN displays weak tyrosine phosphatase activity, which may downregulate signaling pathways that involve focal adhesion kinase (FAK) or Shc [Besson et al., 1999; Yamada and Araki, 2001]. In our pilot experiments, FAK activity and extracellular signal-related kinases 1 and 2 activity, that is downstream of Shc, were not influenced by these dominant negative plasmids (data not shown). However, in ECs transfected with G129R plasmid, that has no lipid phosphatase activity, Akt activity, and PIP3 immunofluorescence levels were dramatically increased (Figs. 1D and 5B) and the increased Akt correlates with suppression of apoptosis (Fig. 7). Interestingly, CS suppressed apoptosis in plasmid G129R tranfected EC to a greater degree compared to the static condition. On the other hand, in ECs transfected with plasmid 380–385A, which has mutated phosphorylation sites, Akt activity, and PIP3 immunofluorescence intensity were decreased (Figs. 1E and 5B), and apoptosis was increased compared to the control vehicle (Fig. 7). Although antisense or siRNA approaches were not utilized, these results are consistent with the hypothesis that the levels of PIP3, Akt activity, and the apoptosis in EC due to serum withdrawal were significantly influenced by the phosphatase PTEN.

It is also interesting that even though both plasmid 380–385A and the PI3K inhibitors downregulated PIP3 levels and Akt activity (Figs. 1E, 2B,C, and 5B), plasmid 380–385A could not prevent the decrease in apoptosis induced by CS (Fig. 7). In plasmid 380–385A transfected EC, proliferation stimulated by CS was unaffected (Fig. 6). In our previous reports, bovine aortic EC proliferation was significantly reduced and CS-induced suppression of apoptosis was inhibited by the PI3K inhibitors [Haga et al., 2003; Li and Sumpio, 2005]. To address this apparent discrepancy between inhibitor of PI3K with dominant negative PTEN and chemical inhibitors, PIP3 immunofluorescence studies in EC treated with chemical PI3K inhibitors and the control DMSO were performed to compare with the dominant negative transfected EC data (Figs. 1E and 2). Figure 2 shows that the PIP3 immunofluorescences were generally weak in PI3K inhibitors compared to those of the control DMSO. Furthermore, the slight and gradual increase of Akt activity and PIP3 immunofluorescence that were seen in 380–385A dominant negative plasmid transfection study (Figs. 1E and 5B) was not seen in EC treated with chemical PI3K inhibitors (Fig. 2).

Based on these findings, we hypothesize that CS induces activation of both PI3K and PTEN independently (Fig. 8). If this hypothesis is correct, the slight and gradual CS-induced increase of Akt activity and PIP3 immunofluorescence in ECs transfected with plasmid 380–385A (Figs. 1E and 5B) results from the CS effect on PI3K. Since dephosphorylation of PIP3 to PI(4,5)P2 is enhanced by plasmid 380–385A, the increase in PIP3 immunofluorescence and Akt activation as a result of direct PI3K activation will be lower compared to WT plasmid. (Figs. 3B and 5B). This slight and gradual increase of Akt activity and PIP3 immunofluorescence in EC transfected with the plasmid 380–385A may still result in CS-induced cell proliferation (Fig. 6) and the CS-induced suppression of apoptosis (Fig. 7). However, in ECs treated with PI3K inhibitors, CS could not increase PIP3 immunofluorescence and Akt activation, because the direct effect of CS on PI3K is completely abolished by inhibitors [Gallis et al., 1999]. This abolition of PIP3 and Akt significantly reduces cell proliferation [Li and Sumpio, 2005] and inhibits CS-induced suppression of apoptosis [Haga et al., 2003]. On the other hand, dephosphorylation of PIP3 to PI(4,5)P2 is attenuated in EC transfected with the G129R plasmid. Therefore, PIP3 immunofluorescence and Akt activation should enhance the CS direct effect on PI3K (Figs. 3B and 5B). Of note, recent studies suggest that PTEN can be oxidized on sensitive cysteines and thereby inactivated [Connor et al., 2005]. Further studies on the role of oxidation of PTEN in EC exposed to CS need to be done to determine the role of this pathway of inactivation.

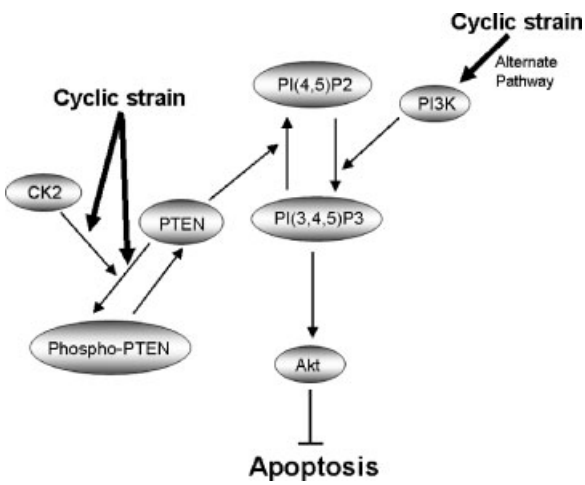


**Fig. 8.** Proposed CS effects on Akt pathway. CS induces the phosphorylation of CK2 and PTEN. CS may have alternate direct effect on PI3K activity.

In conclusion, the present study demonstrated that phosphatase PTEN is phosphorylated and inactivated in EC exposed to CS prior to the phosphorylation and activation of Akt. CS stimulation also resulted in a marked enhancement of immunoreactive PIP3 in a time-dependent manner. The level of PIP3 and Akt activity exposed to CS were influenced by phosphatase PTEN.

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