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Prolonged cyclic strain inhibits human endothelial cell growth

Kelly J. Peyton[1], Xiao-ming Liu[1], William Durante[1]

[1]Department of Medical Pharmacology and Physiology, University of Missouri School of Medicine, University of Missouri, One Hospital Drive, Columbia, MO, USA

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1. ABSTRACT

The vascular endothelium is continuously exposed to cyclic mechanical strain due to the periodic change in vessel diameter as a result of pulsatile blood flow. Since emerging evidence indicates the cyclic strain plays an integral role in regulating endothelial cell function, the present study determined whether application of a physiologic regimen of cyclic strain (6% at 1 hertz) influences the proliferation of human arterial endothelial cells. Prolonged exposure of human dermal microvascular or human aortic endothelial cells to cyclic strain for up to 7 days resulted in a marked decrease in cell growth. The strain-mediated anti-proliferative effect was associated with the arrest of endothelial cells in the G_2/M phase of the cell cycle, did not involve cell detachment or cytotoxicity, and was due to the induction of p21. Interestingly, the inhibition in endothelial cell growth was independent of the strain regimen since prolonged application of constant or intermittent 6% strain was also able to block endothelial cell proliferation. The ability of chronic physiologic cyclic strain to inhibit endothelial cell growth represents a previously unrecognized mechanism by which hemodynamic forces maintain these cells in a quiescent, non-proliferative state.

2. INTRODUCTION

The vascular endothelium forms a dynamic cellular interface between the vessel wall and bloodstream that is continuously subjected to hemodynamic forces, principally shear stress and cyclic strain. Emerging evidence suggests that these hemodynamic forces play an important role in regulating vascular tone, vascular remodeling, and vascular disease by influencing endothelial cell metabolism and function (1,2). Laminar fluid shear stress exerts an atheroprotective effect on endothelial cells by inhibiting endothelial cell activation, proliferation, apoptosis, and monocyte adhesion (3). This anti-atherogenic effect involves the activation of distinct signaling pathways that triggers the expression of numerous cytoprotective genes. More recently, cyclic strain, which arises from the periodic change in vessel diameter as a result of pulsatile blood flow, has also been demonstrated to mediate significant effects on the vascular endothelium. Cyclic mechanical strain alters endothelial cell morphology and orientation, and plays a fundamental role in modulating blood flow by stimulating the release of humoral factors from the endothelium (2,4). In addition, cyclic strain enhances endothelial cell motility by stimulating the release and/or activation of various proteases (1-2,5). Interestingly, we previously reported that sustained imposition of cyclic strain inhibits endothelial cell apoptosis (6,7), raising the possibility that similar to shear stress, cyclic strain may mediate anti-atherogenic effects in the circulation. Since the proliferation of endothelial cells also promotes the

development of atherosclerotic lesions (8), the present study investigated whether prolonged administration of a physiologically relevant level of cyclic strain regulates endothelial cell growth.

3. MATERIALS AND METHODS

3.1. Materials

M199 medium, streptomycin, penicillin, heparin, trypan blue, sodium dodecyl sulfate (SDS), hydrocortisone, trypsin, and epidermal growth factor were from Sigma-Aldrich (St. Louis, MO); antibodies against cyclin D1, cyclin E, cyclin A, cyclin B1, p21, p27, cyclin-dependent kinase-1 (cdk1), phospho-cdk1 (cdk1-P, thr^{161}), and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA); antibody against phospho-retinoblastoma protein (pRb-P, $ser^{87/811}$) was from Cell Signaling (Beverley, MA); (^{32}P)dCTP (3000 Ci/mmol) was from GE Healthcare (Boston, MA).

3.2. Endothelial cell culture

Human dermal microvascular endothelial cells (HMECs) and human aortic endothelial cells (HAECs) were purchased from Lonza Corporation (Allendale, NY). HMECs were grown in MCDB 131 medium (Life Technologies, Carlsbad, CA) containing 10% serum, 5 ng/ml epidermal growth factor, 1 mg/ml hydrocortisone acetate, 100 U/ml penicillin, and 100 U/ml streptomycin. HAECs were propagated in M199 medium supplemented with 20% serum, 50μg/ml endothelial cell growth supplement, 50 μg/ml heparin, 100 U/ml penicillin, and 100 U/ml streptomycin (9-11).

3.3. Application of cyclic strain

Cells were seeded onto collagen I-coated 6-well Bioflex plates and subjected to mechanical deformation using the Flexcell Strain Unit (FX 3000 and 4000, Flexcell International Corporation, Hillsborough, NC), as we previously described (6,7,12). Endothelial cells were subjected to three different strain regimens; a physiologically relevant level of 6% strain at 1 hertz (Hz), an intermittent strain of 5 minutes of 6% strain followed by 2 minutes of no strain, or a constant 6% strain. The control group consisted of endothelial cells that were maintained in the incubator and never exposed to strain.

3.4. Endothelial cell viability

Cell viability was determined by measuring the uptake of the membrane impermeable stain, trypan blue. Cells were treated with trypsin (0.2.5%), collected, and diluted (1:4) with trypan blue. Viable cells that exclude trypan blue were counted with a hemocytometer, as we have previously described (6,7,11).

3.5. Endothelial cell proliferation and cell cycle analysis

Endothelial cells were plated at a density of $1x10^5$ cells/well onto collagen I-coated 6-well Bioflex plates and subjected to strain or non-strain conditions for various times. Media were replenished every second day. Cell number determinations were performed by dissociating cells from the plate with trypsin and counting cells in a Coulter Counter (Model ZF, Coulter Electronics, Hialeah, FL). Cell cycle progression and cell viability were assessed by flow activated cell sorting. Cells were permeabilized, stained with propidium iodide and DNA fluorescence measured in a Becton Dickinson FACScan flow cytometer (Franklin Lakes, NY). Histograms of DNA content were analyzed using Modfit (Verity Software House, Topsham, ME) to determine the fractions of the population in each phase of the cell cycle (13,14).

3.6. Protein and mRNA expression

Protein and mRNA expression was determined by western and northern blotting, respectively (7,9,15). Endothelial cells were lysed in electrophoresis buffer (125 mM Tris (pH 6.8.), 12.5.% glycerol, 2% SDS, and trace bromophenol blue) and proteins separated by SDS-polyacrylamide gel electrophoresis. Following transfer to nitrocellulose membranes, blots were blocked with phosphate buffered saline (PBS) and non-fat milk (5%) and then incubated with antibodies against cyclin D1 (1:500), cyclin E (1:500), cyclin A (1:500), phospho-Rb (1:100), p27(1:300), p21 (1:500), cyclin B1 (1:500), cdk1, phospho-cdk1 (1:100), or β-actin (1:200). Membranes were washed in PBS, incubated with horseradish peroxidase-conjugated secondary antibodies and developed with commercial chemoluminescence reagents (Amersham, Arlington Heights, IL). For p21 mRNA expression, total RNA was loaded onto 1.2.% agarose gels and fractionated by electrophoresis. RNA was blot transferred to Gene Screen Plus membranes and then incubated overnight at 68°C in hybridization buffer (Amersham, Arlington Heights, IL) containing (^{32}P)DNA probes ($1x10^8$ cpm) for p21 or GAPDH. After hybridization, membranes were washed, and exposed to X-ray film. Protein and mRNA expression was quantified by scanning densitometry and normalized with respect to β-actin or GAPDH, respectively.

3.7. Small interference RNA (siRNA) protocol

Gene expression was silenced by using siRNA targeting p21, as previously described (14). The experimental and control non-targeting siRNA siRNAs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and delivered to endothelial cells using a commercial transfection reagent (Invitrogen, Carlsbad, CA).

3.8. Statistics

Results are expressed as the means ± S.E.M. Statistical analyses were performed with a Student's two-tailed t-test and P values <0.0.5 were considered to be statistically significant.

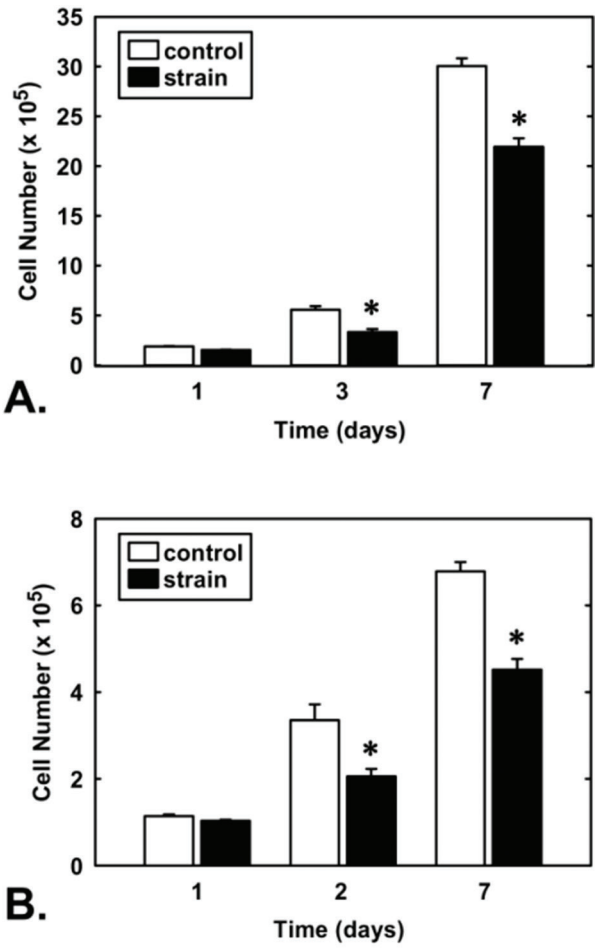

Figure 1. Physiologic cyclic strain inhibits endothelial cell proliferation. HMECs (A) or HAECs (B) were treated with serum (5%) and subjected to strain-free control or cyclic strain (6% at 1 Hz) conditions for up to 7 days. Results are means±SEM (n=3-5). *Statistically significant effect of cyclic strain.

4. RESULTS

4.1. Prolonged cyclic strain inhibits endothelial cell proliferation

Treatment of HMECs with serum (5%) stimulated a time-dependent increase in cell number that was markedly reduced by the application of a physiologic level and frequency of cyclic strain (6% at 1 Hz) (Figure 1A). A significant inhibition in cell growth was observed after 3 days and this became more pronounced following 7 days of cyclic strain. Similarly, the administration of physiologic cyclic strain inhibited the proliferation of HAECs (Figure 1B). Interestingly, the anti-proliferative action of cyclic strain was also observed when endothelial cells were subjected to alternative strain regimens. In particular, application of intermittent strain consisting of 5 minutes of 6% strain followed by a 2 minute strain-free interval resulted in a progressive time-dependent inhibition of HMEC proliferation (Figure 2A). Furthermore, the application of a constant 6% strain also blocked the growth of HMECs (Figure 2B).

4.2. Prolonged cyclic strain arrests endothelial cells in the G_2/M phase of the cell cycle

In subsequent experiments, the effect of physiologic cyclic strain (6% at 1 Hz) on cell cycle progression was determined by flow cytometry. Proliferating HMECs were distributed throughout the cell cycle with a majority of cells in G_0/G_1 (Figure 3). However, application of physiologic cyclic strain for 3 days significantly increased the number of cells in G_2/M and this was accompanied by a significant decrease in the percentage of cells in G_0/G_1 (Figure 3). No cell detachment or toxicity was noted with the application of cyclic strain, as reflected by the lack of a sub-G_0/G_1 fraction. Moreover, trypan blue staining indicated that cell viability was similar between control, unstrained cells (96.4.±2.4.%, n=4) and cells exposed to cyclic strain (95.8.±1.6.%, n=4).

4.3. Prolonged cyclic strain inhibits endothelial cell proliferation via the induction of p21

To elucidate the mechanism by which cyclic strain disrupts cell cycle progression, we examined the effect of physiologic cyclic strain (6% at 1 Hz) on cell cycle regulatory proteins. Application of cyclic strain for 3 days had no effect on the expression of the G_1 cyclins, cyclin D1, E, or A, the cdk inhibitor, p27, the G_2 cyclin, cyclin B1, or the phosphorylation of pRb (Figure 4A). In addition, the levels of the G1 cyclins, cyclin D1, E and A, were unaffected 1 day after the imposition of cyclic strain (Figure 4B). In contrast, physiologic cyclic strain for 3 days decreased the phosphorylation of cdk1 and increased the expression of p21 protein and mRNA (Figure 4A and C). Notably, transfecting HMEC with p21 siRNA inhibited the induction of p21 and reversed the anti-proliferative action of cyclic strain (Figure 4D and E). In contrast, the non-targeting (NT) siRNA had no effect on the ability of cyclic strain to regulate p21 expression or HMEC growth.

5. DISCUSSION

The present study demonstrates that prolonged application of a physiologically relevant regimen of cyclic strain (6% at 1 Hz) inhibits the proliferation of human endothelial cells. The anti-proliferative effect of cyclic strain was associated with the arrest of endothelial cells in the G_2/M phase of the cell cycle, did not involve cell detachment or cytotoxicity, and was due to the induction of p21. Furthermore, the growth inhibitory effect of 6% cyclic strain is also observed with different strain regimes. These findings establish chronic cyclic strain as a novel regulator of arterial endothelial cell growth.

This study is the first to show that sustained administration of a physiologic significant regime of

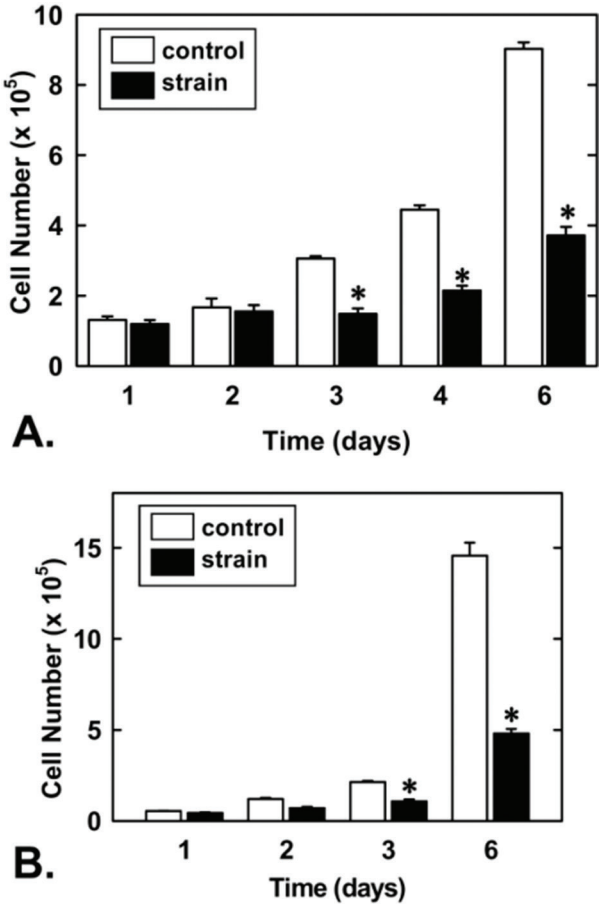

Figure 2. Intermittent or constant strain inhibits endothelial cell proliferation. HMECs were treated with serum (5%) and subjected to strain-free control or intermittent strain (6% strain for 5 minutes followed by a two minute strain-free interval) (A), or constant strain (6%) (B) for up to 6 days. Results are means±SEM (n=3-4). *Statistically significant effect of strain.

cyclic strain (6% at 1 Hz) inhibits endothelial cell growth. This anti-proliferative effect is observed in endothelial cells derived from both the arterial micro- and macro-circulation, and occurs in the absence of cell death indicating that cyclic strain acts via cytostatic rather than cytotoxic mechanisms. Analysis of cell cycle distribution indicates that cyclic strain inhibits cell cycle progression by specifically arresting endothelial cells in the G_2/M phase of the cell cycle. Previous work has determined that cdk1 functions as a master mitotic regulator and that phosphorylation of Thr^{161} is required for cdk1 activation and mitotic entry (16). Interestingly, we found that cyclic strain-induced G_2M arrest is associated with a marked decrease in cdk1 activity, as reflected by a decrease in cdk1-Thr^{161} phosphorylation. This decrease in cdk1 activity occurs independently of any change in the level of cdk1 protein or it's activating cyclin, cyclin B1. However, cyclic strain selectively increases the expression of p21, which has been shown to block cdk1 activity and promote G_2M arrest (17). Moreover, we found that silencing p21 expression prevents the anti-proliferative action of cyclic strain, demonstrating a fundamental role for p21 in mediating the growth-inhibitory action of mechanical strain.

Our finding that chronic application of physiologic cyclic strain inhibits endothelial cell growth contrasts with reports showing that acute imposition of cyclic strain stimulates endothelial cell proliferation (18-20). While cyclic strain transiently triggers the release of endothelial mitogens and activates growth receptors and signaling pathways that acutely promote mitogenesis (18-20), growth-suppression predominates with longer periods of cyclic strain. Significantly, the ability of cyclic strain to retard endothelial cell growth complements studies showing that fluid shear stress likewise inhibits endothelial cell proliferation (21-23). However, these two hemodynamic forces appear to differentially target cell cycle progression: cyclic strain arrests endothelial cells in G_2/M while shear stress blocks entry into S phase (22,23). This raises the possibility that cyclic strain and shear stress may interact in an additive or synergistic manner to prevent cell cycle progression and endothelial cell growth *in vivo*. Consistent with this notion, the fraction of mitotic endothelial cells is extremely low in the arterial tree except near branch arteries where both the shear and strain field are perturbed (24,25). Interestingly, these regions of higher mitotic activity are prone to develop atherosclerotic lesions. This link between endothelial cell turnover and atherosclerosis likely reflects increases in permeability that occurs in areas of endothelial cell growth that permits the infiltration of pro-atherogenic lipoproteins into the vessel wall (25). Thus, the ability of cyclic strain to block endothelial cell proliferation may exert a vasculoprotective, anti-atherogenic effect in the circulation.

Aside from a physiologically relevant frequency of cyclic strain, we found that a currently employed therapeutic schedule of intermittent cyclic strain consisting of 5 minutes of cyclic strain followed by 2 minutes of a strain-free interval blocks endothelial cell growth. This pattern of cyclic strain mimics the strain profile used in vacuum-assisted closure therapy where negative pressure is applied to wounds through the use of an open cell, reticulated foam dressing. Finite element modeling indicates that at typical clinical settings this therapy induces average cellular strains in the range of 5-20%, depending on the stage of wound healing (26). Interestingly, vacuum-assisted closure therapy promotes wound healing and this is associated with increases in microvessel density in wounds (27). The capacity of vacuum-assisted closure to stimulate angiogenesis in wounds may be related to the ability of cyclic strain to induce endothelial cell migration and capillary tube formation (5). Moreover, our present finding demonstrating that cyclic strain restrains endothelial cell proliferation may serve to stabilize the integrity and

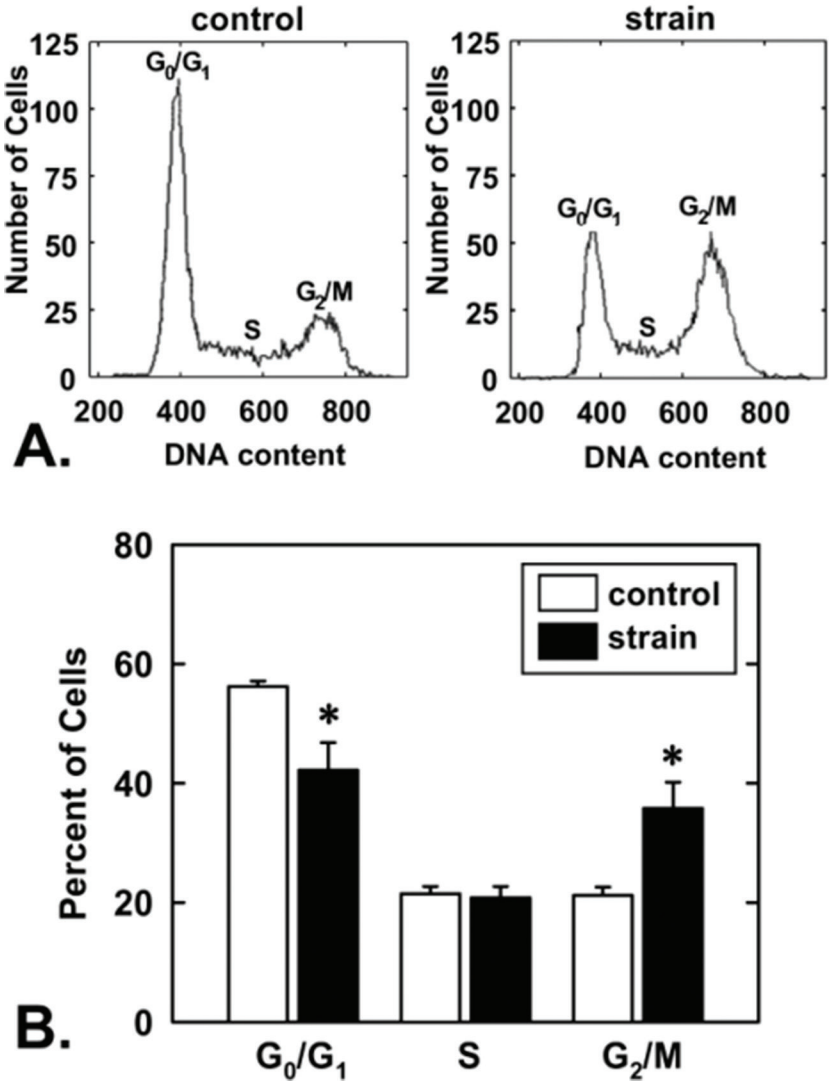

Figure 3. Physiologic cyclic strain inhibits cell cycle progression in endothelial cells. Representative histograms of DNA content (in arbitrary units) in HMECs treated with serum (5%) and subjected to strain-free control or cyclic strain (6% at 1 Hz) conditions for 3 days (A). Distribution of cells in the cell cycle in HMECs treated with serum (5%) and subjected to strain-free control or cyclic strain (6% at 1 Hz) conditions for 3 days (B). Results are means±SEM (n=4). *Statistically significant effect of cyclic strain.

function of newly formed vessels in healing wounds. Significantly, cyclic strain may also directly increase endothelial barrier function of these new vessels by up-regulating the expression of tight junction protein complexes (28). Finally, the continuous application of 6% strain also inhibits endothelial cell growth indicating that mechanical strain is able to block endothelial cell growth irrespective of the cycling regimen. This latter finding may have important implications for the growth of other cells that are subjected to clinically-applied mechanical forces.

In conclusion, the present study identifies prolonged physiological cyclic strain as a novel, potent inhibitor of human endothelial cell growth. In addition, it showed that cyclic strain arrests endothelial cells in the G_2/M phase of the cell cycle and this is due to an increase in p21 expression. The ability of chronic cyclic strain to inhibit endothelial cell growth represents a previously unrecognized mechanism by which hemodynamic forces maintain these cells in a quiescent, non-proliferative state and protect against the development of atherosclerosis.

6. ACKNOWLEDGEMENTS

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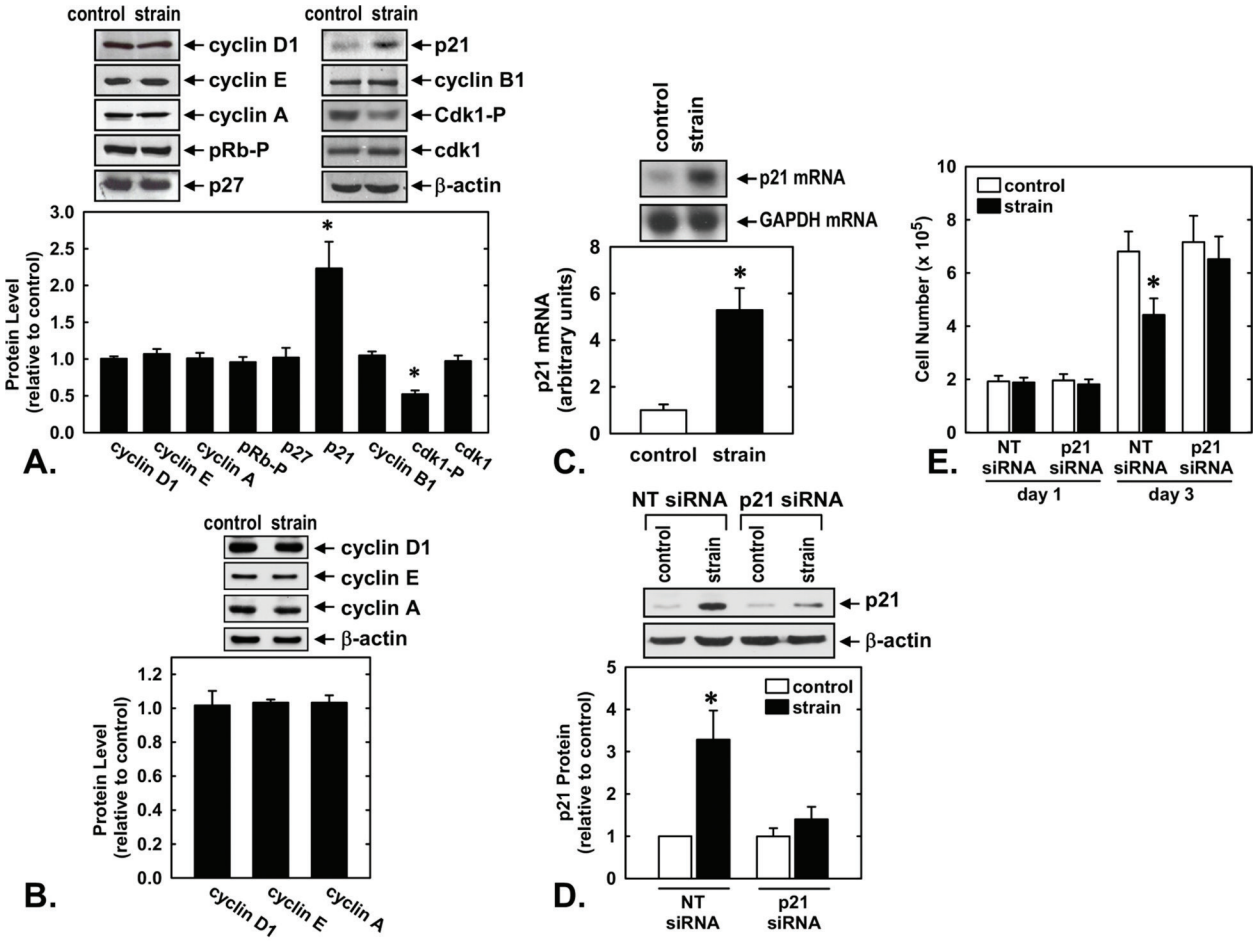

Figure 4. Physiologic cyclic strain inhibits endothelial cell proliferation via the induction of p21. Western blotting demonstrating the expression of cell cycle regulatory proteins in HMECs treated with serum (5%) and subjected to strain-free control or cyclic strain (6% at 1 Hz) conditions for 3 days (A) or 1 day (B). Northern blotting demonstrating the expression of p21 mRNA in HMECs treated with serum (5%) and subjected to strain-free control or cyclic strain (6% at 1 Hz) conditions for 3 days (C). Expression of p21 protein in cells transfected with p21 siRNA (0.1.μM) or non-targeting (NT) siRNA (0.1.μM) and subjected to strain-free control or cyclic strain (6% at 1 Hz) conditions for 3 days (D). HMEC proliferation in cells transfected with p21 siRNA (0.1.μM) or NT siRNA (0.1.μM) and subjected to strain-free control or cyclic strain (6% at 1 Hz) conditions for up to 3 days (E). Protein and mRNA expression was quantified by scanning densitometry, normalized with respect to β-actin or GAPDH, respectively, and expressed relative to that of control cells or in arbitrary units. Results are means±SEM (n=3-5). *Statistically significant effect of cyclic strain.

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Abbreviations: HMECs, human microvascular endothelial cells; HAEC, human aortic endothelial cells; Hz, hertz; SDS, sodium dodecyl sulfate; cdk1, cyclin-dependent kinase-1; cdk1-P, phospho-cyclin-dependent kinase-1; pRB, retinoblastoma protein

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Send correspondence to: William Durante, Department of Medical Pharmacology and Physiology, M409 Medical Sciences Building, University of Missouri, One Hospital Drive, Columbia MO 65212, USA, Tel: 573-882-3886, Fax: 573-884-4276, E-mail: durantew@health.missouri.edu