Physiological cyclic stretch causes cell cycle arrest in cultured vascular smooth muscle cells

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VASCULAR SMOOTH MUSCLE CELLS (SMC) proliferation has been implicated in the pathogenesis of several vascular diseases, including atherosclerosis, hypertension, and restenosis following vascular interventions (9, 20, 30). Therefore, numerous studies have examined the regulation of SMC growth in vitro primarily under static conditions. In vivo, however, SMC are continuously exposed to hemodynamic forces. Blood flow through the systemic blood vessels generates shear stresses on the luminal surface, whereas pulsatile blood flow in arteries and arterioles causes the compliant blood vessel wall to distend and contract. The luminal surfaces of blood vessels are lined with endothelial cells so that shear stress predominately acts on, and is sensed by, endothelial cells. However, both endothelial cells and SMC are subjected to cyclic stretch, or strain, arising from the periodic change in vessel circumference.

Although there are many possible mechanisms by which SMC proliferation is affected by stretch, the final common pathway of proliferative signaling cascades is the cell cycle (4). Regulatory proteins control transitions between phases of the cell cycle. Retinoblastoma protein (Rb) must be hyperphosphorylated in the late G1 phase for cells to enter the S phase. Cyclin-dependent kinase inhibitors, including p21 and p27, have been shown to play a critical role in blocking cell cycle progression (19, 34) by preventing Rb phosphorylation. Presently, little is known about the effect of cyclic stretch on these cell cycle regulators in vascular cells.

A balance between cell proliferation and cell death governs a change in cell number. Apoptosis, or programmed cell death, has increasingly been recognized to be important in the homeostasis of a healthy in vivo environment (31). There is only limited information about the effects of hemodynamic forces on apoptosis in vascular cells. Studies with cultured endothelial cells suggest that shear stress prevents apoptosis (21). However, it has been reported that increased apoptosis occurs in SMC cultured from spontaneously hypertensive rats compared with normotensive control rats (16). In addition, stretch has been shown to induce apoptosis in cardiac myocytes (7, 24).

This study was designed to determine the effects of a physiologically relevant level of cyclic stretch on vascular SMC and to elucidate the mechanism of action of this hemodynamic force on SMC growth. We now demonstrate that cyclic stretch inhibits SMC proliferation and that this inhibition is not due to apoptosis or necrosis, but rather to a stretch-induced inhibition of Rb phosphorylation. We conjecture that inhibition of Rb phosphorylation with physiological levels of cyclic stretch contribute to vascular homeostasis by inhibiting the proliferative pathway of SMC.

METHODS

Materials. Calf serum, penicillin, streptomycin, neomycin, trypsin-EDTA, and MEM were purchased from Gibco BRL (Gaithersburg, MD). SDS, β-mercaptoethanol, BSA, PBS, Triton X-100, Tris, TES, and HEPES were purchased from Sigma Chemical (St. Louis, MO). Platelet-derived growth factor (PDGF)-A/B, propidium iodide (PI), and RNase were purchased from Boehringer-Mannheim (Indianapolis, IN). Collagen was purchased from ICN Biomedicals (Aurora, OH), and the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
and Silastic membranes for uniaxial stretch experiments were purchased from SF Medical (Hudson, MA). Recombinant mouse tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) were purchased from Genzyme (Cambridge, MA); recombinant mouse interleukin-1β (IL-1β) from R&D Systems (Minneapolis, MN); human α-thrombin from Enzyme Research Labs (South Bend, IN).

Cell culture. SMC were isolated by elastase and collagenase digestion of the thoracic aorta obtained from adult 12-wk-old Sprague-Dawley male rats. The cells were cultured serially in MEM containing Earle’s salts, 5.6 mM glucose, 2 mM L-glutamine, 20 mM TES-NaOH, 20 mM HEPES-NaOH, 10% heat-inactivated FCS, and 100 U/ml of penicillin, streptomycin, and neomycin antibiotics. Subcultured strains were used between passages 6 and 29. For the proliferation and cell cycle experiments, cells were seeded subconfluently (5 × 10⁴ cells/well). To obtain sufficient cell number for the uniaxial stretch experiment and sufficient protein for Western blots and caspase-3 assays, confluent cells were also examined. The SMC were quiesced for 48 h in serum-free media (FCS replaced by 0.1% BSA) before exposure to the experimental treatments. The media were aspirated and replaced with fresh media every 48 h.

Cyclic stretch apparatus. SMC were stretched by two different devices that imparted stretch along one axis (uniaxial) or along all axes (equiaxial). The Flexercell Strain Unit (FX-3000, Flexcell, McKeesport, PA) was used to expose SMC to an equiaxial cyclic stretch of 10% magnitude and a frequency of 1 Hz. SMC were cultured on 2-well Bioflex plates coated with type I collagen (Flexcell). A computer-controlled vacuum pump regulated the magnitude, frequency, and duration of stretch. In parallel, static control SMC were also grown on Bioflex plates under identical conditions but were not exposed to stretch.

The effects of uniaxial stretch were examined by passing SMC on a taut Silastic membrane coated with collagen. The stretch apparatus was previously used in the laboratory of Wagner et al. (38) and was similar to that used by Carosi et al. (5). Briefly, the membrane is mechanically deformed by attaching one end to a piston, the stroke and frequency of which can be controlled, while the other end is fixed. The control for this apparatus consists of a similar membrane, the other end of which is not fixed. This provides a control for the effects of fluid motion over the surface of the cells generated by the moving piston. As with the Flexercell experiments, cells were stretched at a 10% magnitude with a frequency of 1 Hz.

Cell proliferation. Cells were washed with PBS and removed from the wells by 0.05% trypsin/0.53 mM EDTA digestion. Cell number was measured by counting SMC with a Coulter Counter (model ZF, Coulter Electronics, Miami, FL) with an orifice size of 70 μm and sample volume of 100 μl. The number of detached cells was measured by colorimetrically determining caspase-3 activity (Enzyme Chem, Rockford, IL) to ensure equal protein loading.

Cell cycle. DNA content, assessed by flow cytometry of PI-labeled cells, was used as an index of cell cycle. SMC were trypsin-EDTA digested from the culture plates, centrifuged, and fixed in 70% ice-cold ethanol for 30 min. The cells were subsequently washed twice with ice-cold PBS. The pellet was resuspended in 800 μl of PBS and 10 μl of RNase (0.5 mg/ml) for 30 min. One hundred microliters of PI were added to the suspension before flow cytometry analysis on a Becton-Dickinson FACScan flow cytometer (Franklin Lakes, NJ). The area versus the width of the fluorescent signal was analyzed to gate out cellular multiplets. Histograms of DNA content were analyzed using Modfit Lt V1.01 (Verity Software House, Topsham, ME) to determine fractions of the population in each phase of the cell cycle (G₀, G₁, S, and G₂/M).

Apoptosis. Two assays of SMC apoptosis were employed. An apoptosis detection kit that measured phosphatidylserine (PS) was purchased from R&D Systems. The assay was conducted as per the manufacturer’s directions. Briefly, cells were washed with PBS and removed from the plate with trypsin-EDTA. The cells were washed twice with ice-cold PBS and resuspended in the supplied binding buffer so that the final cell concentration was 10⁶ cells/ml. Approximately 10⁵ cells were transferred to a flow cytometric vial, and 10 µl each of PI and fluorescein-conjugated annexin V (Ann) were added to the suspension. The suspension was vortexed and incubated in the dark for 15 min. Nine hundred microliters of binding buffer were added to each sample before analysis on a Becton-Dickinson FACScan flow cytometer.

Caspase-3 activity was measured using either the fluorescent peptide Ac-DEVD-AMC (Calbiochem, La Jolla, CA) (28) according to the method of Higuchi et al. (17) or with a colorimetric assay kit from Clontech (Palo Alto, CA). Briefly, cells were trypsinized, washed, and lysed. The caspase-3 activity was measured in the lysate using 20 μM of the peptide in a fluorescent plate reader (PE Biosystems, Foster City, CA) at room temperature every 3 min for 3 h, or colorimetrically from 5 × 10⁶ cells with a μQuant spectrophotometer (Bio-Tek Instruments, Winooski, VT) per the manufacturer’s directions. The rate of fluorescence increase was normalized to protein concentration, and the colorimetrically determined caspase-3 activity was expressed relative to static controls.

Protein analysis. Cellular protein was collected by washing the SMC with ice-cold PBS and then scrape-harvesting with sample buffer (30 mM Tris·HCl, pH 6.8, 0.8% SDS, and 5.6% glycerol), and then processed by a 10-min boil, a 20-min sonicaton in a water bath, and a 20-min 10,000 g spin. The supernatant was collected and the protein concentration was determined by the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL) to ensure equal protein loading.

Fig. 1. Time course of effects of equiaxial cyclic stretch (10% at 1 Hz) and serum (1%) treatment on cultured smooth muscle cell (SMC) proliferation. Results are means ± SE of 4 separate experiments, each consisting of a minimum of 2 wells. *Statistically greater (P < 0.05) cell number under static compared with stretch conditions.
**RESULTS**

A physiologically relevant level of cyclic stretch inhibited growth factor-induced proliferation of SMC. Figure 1 shows a 5-day time course of proliferation of SMC stimulated with 1% serum and demonstrates that proliferation is inhibited after 3 or more days of cyclic stretch. The inhibitory effect of cyclic stretch did not depend on culture conditions containing specific types...
of growth factors, because stretch-induced growth inhibition was observed for thrombin, PDGF, and various serum concentration treatments (Fig. 2). Static cells, prestretched for 2 days, proliferated in response to 1% serum treatment in a similar manner to cells that were not prestretched. Stretch-induced growth inhibition was not due to higher levels of cell detachment with stretch (not shown).

To assess whether stretch induces cell death, either by necrosis or apoptosis, we measured the orientation of PS in the cell membrane of SMC in concert with measurements of cell membrane integrity. Figure 3 shows that SMC remained viable under both static and cyclic stretch conditions, in contrast to the effects of a combination of cytokines (40 ng/ml TNF-α, 10 ng/ml IL-1β, and 400 U/ml IFN-γ) that have been previously demonstrated to induce apoptosis in vascular SMC (15). This assay indicates that under both stretch and static conditions, ~95% of the cells remain viable, and stretch does not increase the fraction of apoptotic or necrotic cells. The fraction of viable (static 95.2 ± 0.4%; stretch 94.6 ± 0.4%), apoptotic (static 1.4 ± 0.2%; stretch 1.9 ± 0.2%), and necrotic (static 2.5 ± 0.3%; stretch 2.6 ± 0.3%) cells was not significantly different between static and stretch treatment and remained constant throughout treatment duration (n = 10, duration between 1 and 5 days). Static cells that were pretreated with cyclic stretch for 2 days responded to cytokine treatment in a similar manner to nonstretched cells. An additional apoptosis assay for caspase-3, an integral enzyme involved in DNA fragmentation, also indicated that stretch does not induce apoptosis. In agreement with PI/Ann labeling experiments, caspase-3 activity did not vary with treatment duration, and there was no significant difference between the caspase-3 activity of stretched (72.3 ± 4.3 fluorescent units·mg⁻¹·s⁻¹; mean ± SE, n = 11) and static (74.7 ± 3.3; n = 11) cells. However, 3 days of cytokine treatment increased caspase-3 activity 1.54 ± 0.06-fold relative to static controls, whereas the activity for 3 days of stretch remained unchanged (1.02 ± 0.02-fold) as measured by the colorimetric assay.

Permeabilized SMC were incubated with PI to determine whether cyclic stretch affected the cell cycle. Figure 4 shows representative histograms of the DNA content of static (Fig. 4A) and stretched (Fig. 4B) cells at 24 h. There was an increase in the fraction of cells in the first peak (G0/G1) and a corresponding decrease in the fraction of cells in S phase with stretch. Analysis of these DNA-content histograms shows cyclic stretch significantly increases the fraction of cells in the G0/G1 phases of the cell cycle (Fig. 5). A similar increase in the fraction of cells in G0/G1 was observed for cells uniaxially stretched (data not shown). The increase in the fraction of cells in G0/G1 is persistent and continuous throughout the 5-day treatment. Figures 4 and 5 suggest 10% cyclic stretch inhibits either entry into the G0/G1 phase
cell cycle or inhibits G₁ to S phase transition. Because there was not a significant increase in the fraction of cells in G₀/G₁ with increasing duration of stretch, we examined proteins involved in the regulation of the cell cycle after 1 day of stretch.

Western blot analysis indicated that cyclic stretch inhibited phosphorylation of Rb protein (Fig. 6). In agreement with our cell proliferation data (Fig. 2), cyclic stretch did not completely block Rb phosphorylation with 5% serum. Rb phosphorylation was examined under both confluent and subconfluent culture conditions. Although cyclic stretch inhibited Rb phosphorylation under both confluent and subconfluent conditions, the effect was more pronounced in subconfluent cultures. In addition, cyclic stretch significantly increased protein levels of the cyclin-dependent kinase inhibitor p21 (Fig. 7A), whereas p27 protein levels remained unchanged (Fig. 7B).

DISCUSSION

This study demonstrates that a physiological level of cyclic stretch inhibits vascular SMC proliferation. This growth inhibition occurred irrespective of the growth factors to which the SMC were exposed (thrombin, PDGF, or various concentrations of serum). Cyclic stretch did not increase cell detachment or cell death, either by necrosis or apoptosis, but rather caused an arrest of cells in the G₁ phase of the cell cycle. Cyclic stretch upregulated the level of p21, a cyclin-dependent kinase inhibitor that has been shown to prevent G₁ to S phase transition in vascular SMC by inhibiting Rb phosphorylation (19). We propose that physiological levels of cyclic stretch increase p21 protein levels, which may inhibit phosphorylation of Rb, thereby arresting SMC in the G₁ phase of the cell cycle.

During the cardiac cycle, the maximum stretch of the human aorta has been reported to be 9–12% under normotensive conditions (10). Other large diameter vessels in humans, such as the femoral and pulmonary arteries, are exposed to similar levels of stretch. Therefore, our stretch conditions (10%, 1 Hz) modeled physiologically relevant levels of stretch in normotensive arteries. SMC in the walls of most arteries are oriented in the circumferential direction (10), whereas the motion of the wall occurs predominately in the radial direction so that SMC experience a type of uniaxial stretch. The Flexcell model exerts an "equiaxial" stretch over the majority of the flexible membrane. Previously used devices impart a uniaxial stretch to the SMC (38). We used both devices to determine whether the type of stretch affects SMC proliferation. Both types of cyclic stretch caused an increase in the fraction of cells in the G₀/G₁ phase of the cell cycle, suggesting that stretch-induced cell cycle arrest does not depend on a specific type of stretch.

There are conflicting data about whether stretch inhibits (9, 36) or promotes (26, 39, 41) SMC proliferation. Previous studies have found that stretch potenti-
ates proliferation in SMC obtained from venular but not arterial blood vessels. In addition, Nakamura et al. (27) found that the proliferative response due to cyclic stretch of SMC obtained from small resistance arteries was significantly greater than that of SMC obtained from large conduit arteries. There are phenotypically distinct SMC within a given blood vessel and also between blood vessels of the same vascular bed (3, 13, 27). Therefore, the conflicting results regarding the effects of cyclic stretch on SMC growth can be attributed to differences in SMC phenotype. Furthermore, opposite results have been noted in arterial SMC cultured from newborn and adult animals (35, 39). In support of our study, it has been found that mechanical strain alters SMC myosin isoform expression comparable to that found in a more differentiated, nonproliferating contractile phenotype (32).

Hemodynamic forces have been shown to be a factor in regulating apoptosis in vascular cells under certain conditions (21). DeBlais et al. (8) found that SMC isolated from hypertensive rats had an increased number of apoptotic cells compared with normotensive control rats. Under hypertensive conditions, the amount of strain has been estimated to be 15% higher than under normotensive conditions (33). In addition, SMC isolated from atherosclerotic plaques have been shown to be more susceptible to apoptosis (1). There are no previous studies on the effects of cyclic stretch on apoptosis in cultured vascular SMC. However, in myocytes apoptosis increases with stretch (7, 24). Thus we considered the possibility that a decrease in the number of stretched cells (Figs. 1 and 2) could reflect an increase in cell death, rather than stretch-induced inhibition of proliferation. Therefore, we examined whether stretch induces cell death relative to static controls.

We used two different assays to examine the effects of cyclic stretch on apoptosis in SMC. The first assay measured the enzymatic activity of caspase-3. Caspase-3 is a protease that, when activated by apoptotic signals, degrades a protein that inhibits chromosomal DNA degradation (11). There is evidence that caspase-3 may be involved in apoptosis of SMC (14). However, we did not detect an increase in caspase-3 activity with stretch versus static controls. A second, more sensitive assay used flow cytometry to measure the fraction of cells that were viable, necrotic, or undergoing apoptosis. An early event in apoptosis is the loss of membrane asymmetry, with the movement of PS from the internal to external surface of the cellular membrane (12). PS flip has been previously measured in SMC undergoing apoptosis (2). Apoptosis can be measured using fluorescently conjugated Ann, which has a high specificity for PS (37). During necrosis, the cell membrane loses its integrity so that Ann can enter the cell and bind PS, making it impossible to distinguish between apoptosis and necrosis by Ann alone. To help us distinguish between necrotic and apoptotic cells, membrane integrity was simultaneously assessed by measuring the fluorescence of PI, a dye that binds to DNA and cannot penetrate an intact cell membrane. We found that ~95% of the SMC were viable under both static and stretch conditions throughout the treatment (up to 5 days). In contrast, a combination of cytokines that has been previously shown to induce apoptosis in SMC (15) increased the fraction of apoptotic cells from less than 5% to 40%. Thus we found that physiological levels of cyclic stretch did not increase cell death.

Physiological cyclic stretch inhibited SMC proliferation via G1 cell cycle arrest. Cyclins and their associated cyclin-dependent kinases phosphorylate Rb in late G_1 phase to drive the cell into S phase. The cyclin-dependent kinase inhibitors, p27 (34) and p21 (19), prevent Rb phosphorylation and are expressed in vascular SMC (6, 22). In the present study we found that cyclic stretch inhibits Rb phosphorylation and specifically increases p21, but not p27, protein expression. A previous study (19) found a specific increase in p21, but not p27, protein levels in vascular SMC. This suggests that physiological levels of cyclic stretch cause an increase in p21, which may prevent Rb phosphorylation and therefore, SMC entry into S phase.

Cyclic stretch may exert its SMC antiproliferative effects through the release of mediators, such as ANG II and transforming growth factor–β (25), which can regulate vascular remodeling processes such as SMC proliferation, hypertrophy, and matrix deposition. Another possible mechanism for regulation of SMC proliferation may involve the ability of cyclic stretch to transduce its cellular responses via integrins (23). It has been suggested that the effects of cyclic stretch on SMC proliferation is matrix dependent and mediated by integrin binding to specific matrix proteins (40). In addition, Hu et al. (18) demonstrated that mechanical stresses may directly alter receptor conformation to initiate signaling pathways normally used by growth factors.

In hypertension, arterial vessel walls are exposed to chronically elevated levels of cyclic stretch, resulting in SMC proliferation, hypertrophy, and apoptosis (16). However, under normal physiological conditions, these remodeling events do not occur. In fact, endothelial cells overlying SMC normally exert antiproliferative effects on vascular SMC (29). This study demonstrates that a physiological hemodynamic force (cyclic stretch) can also exert a direct effect on SMC to induce G_1 cell cycle arrest and thereby maintain their nonproliferative state.

We thank Dr. Thomas Chow for assistance with flow cytometry and Dr. Genevieve Sparagna for help with the caspase-3 assay. We also thank Lan Liao for SMC culture, Jimmy Wu for help with the uniaxial stretch experiments, and Kelly Peyton for excellent advice and assistance.

This work was supported by the National Institutes of Health under Grants HL-59976, HL-18584, and HL-36045; a Grant-in-Aid from the American Heart Association; and a grant from the Veterans Affairs Merit Review Board.

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Received 16 April 1999; accepted in final form 11 October 1999.

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