Cyclic stretch induces vascular smooth muscle cell alignment via NO signaling

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VASCULAR WALL THICKENING is a hallmark of several disease states, such as hypertension and postinjury restenosis (25, 27). Such thickening appears to be due to a triad of pathophysiological phenomena, namely vascular smooth muscle cell (VSMC) hyperplasia, VSMC hypertrophy, and enhanced extracellular matrix protein deposition. In many cases, the ability of VSMCs to hypertrophy and undergo proliferation is linked to their abilities to migrate from their normal intimal location toward the vascular lumen (9). Thus vascular intimal thickening appears to be dependent both on VSMC growth and migration, both of which necessitate VSMC to transform from a quiescent secretory phenotype to a growing mobile phenotype.

Vascular cells are continuously subjected to biophysical stimuli such as shear stress, oscillatory strain, and pulse pressure waveforms. In response to such stimuli during fetal development, angiogenesis, and vascular remodeling, cells align to form the most efficient functional configuration while adapting to a dynamic milieu (15). Two examples of such cellular alignment are endothelial cell orientation in the direction of their longitudinal axis and VSMC arrangement in the form of a fibrous helix. In the case of VSMC, there is evidence that such orientation may be a result of the cyclic nature of vessel stretch resulting from arterial pressure waveforms generated by the left ventricle (15, 28). Despite observations noting VSMC alignment in this manner, the mechanisms by which this is accomplished remain poorly understood.

Another notable effect of vascular cell cyclic stretching is cell proliferation. Our studies (24, 26) and others (13, 17, 28) have shown that VSMC subjected to cyclic stretch undergo a hyperplastic response mediated by IGF-1, PDGF, EGF, and other autocrine growth factors. The degree of hyperplasia correlates with the magnitude of the stretch stimulus (15, 24), suggesting that a stretch-response element, perhaps part of the extracellular matrix, underlies such growth. Interestingly, others (6, 3, 10) report that equivalent biophysical perturbations of these cells results in VSMC production of apoptotic molecules, such as nitric oxide (NO), several eicosanoids, and various superoxide anions. Therefore, it seems clear that the overall growth response to cyclic stretch results from a combination of mitogenic and antimitogenic/apoptotic signaling modalities.

In vivo, VSMC appear as circumferentially arranged layers of contractile cells within the vascular intima. This arrangement appears to fully exploit the intracellular contractile protein arrangement such that maximal vessel contraction or dilation occurs over a comparatively small range of VSMC shortening or lengthening, respectively (7). Thus control of vascular lumen caliber (and consequently blood flow and pressure) is extremely dependent on the anatomic alignment of VSMC relative to the biophysical stretching exerted by pulsatile blood flow and their parallel orientation with each other. These two phenomena, vascular growth and anatomic alignment/orientation with respect to biophysical...
cal perturbation and with respect to neighboring VSMC, have not been investigated in an integrated fashion to provide a complementary view of pathophysiological establishment of altered pressure/flow profiles. In this study, we investigated the effects of in vitro cyclic stretch (designed to mimic in vivo pulse pressure) on VSMC alignment in an attempt to identify potential cell-signaling mediators common to both stretch-mediated cellular alignment and hyperplasia.

MATERIALS AND METHODS

Culturing of aortic VSMC. Studies were carried out using a7r5 aortic smooth muscle cells from DB1X rats that were purchased from American Type Culture Collection (Rockville, MD). These cells have been used extensively by our group and by other researchers (24–26) in the study of VSMC growth, ion transport mechanisms, and contractility. VSMC were cultured in DMEM supplemented with 9% FBS and a 1% solution of penicillin-streptomycin at 37°C, 5% CO2, and 100% humidity. The cells were fed with serum-free medium and passaged on confluent (usually 3–7 days). For each experiment, the cell passage number was matched such that all experimental groups originated from a single VSMC plate of passages 4–15.

Stretch apparatus. The Flexercell apparatus (Flexcell; McKeeport, PA) is a computer-controlled device designed to deliver preprogrammed stretch regimens to elastomeric wells attached to a vacuum-assisted gasketed baseplate. The baseplate remains in a tissue culture incubator throughout the duration of the stretch paradigm. The rate and duration of both stretch and relaxation phases are programmed for an infinite number of stretches. Valves located in the base plate allow selected culture plates to be stretched, whereas control wells remain unstretched. Many investigators (17, 25, 26) have utilized this apparatus to study a variety of vascular cell functions. It has been determined that force on the attached cells is uniaxial (17, 18). An important aspect of the stretch paradigm imparted by this device is that stretch is nonuniform across the well (2). Data from the Flexercell apparatus have been principally analyzed as integrated responses of the entire population of cells subjected to a continuum of stretch magnitudes. In selected studies, we took advantage of this stretch nonuniformity by assessing the physiological variables in cells obtained by “punch biopsies” from various regions of the elastomeric culture support. Specifically, 5-mm punches were obtained (using a leather punch and mallet) from selected, carefully measured coordinates on the well that represent percent strains from 0 to 25%. We then correlated our physiological end point [i.e., orientation angle (Φ)] with the calculated strain magnitude for each punch.

Stretch paradigms. VSMC were seeded (50,000 cells/ml) onto 6-well collagen-I-coated FlexI plates in the DMEM/9%/FBS solution. These plates have an elastomeric surface that allows the cells grown on them to stretch under vacuum. Once cells were ~50–60% confluent (a density at which the cells have recovered from the seeding process, but before the time they are confluent and differences in growth could no longer be assessed; usually 24–48 h postseeding), the growth medium was replaced with serum-free medium for 24 h, and the cell viability and adherence to the collagen plate remained in a tissue culture incubator throughout the duration of the stretch paradigm. The rate and duration of the stretch paradigm imparted by this device is that stretch is nonuniform across the well (2). Data from the Flexercell apparatus have been principally analyzed as integrated responses of the entire population of cells subjected to a continuum of stretch magnitudes. In selected studies, we took advantage of this stretch nonuniformity by assessing the physiological variables in cells obtained by “punch biopsies” from various regions of the elastomeric culture support. Specifically, 5-mm punches were obtained (using a leather punch and mallet) from selected, carefully measured coordinates on the well that represent percent strains from 0 to 25%. We then correlated our physiological end point [i.e., orientation angle (Φ)] with the calculated strain magnitude for each punch.

Measurement of orientation angle. Forty-eight hours poststretch, selected areas (i.e., periphery, middle, and central zones) of the silicone elastomers were viewed with phase-contrast optics (magnification ×400). Digital images were captured with a zoom camera (model MDS120 DC, Eastman Kodak; Rochester, NY) and saved as tagged image files. Each image from the cyclically stretched or corresponding static cultures was analyzed by a blinded observer with the use of Scion Image software. Briefly, each image file was bisected vertically and horizontally to yield four equal quadrants. Four random cells within each quadrant (total of 16 cells per image) were randomly chosen for angular measurements. Orientation angle was then measured by the method of Kanda et al. (15). Briefly, the long axis of each of the 16 cells was drawn on a digitizing tablet (Scion Image). A second line (reference line) was then drawn on the tablet parallel to the direction of stretch, or, in the case of static cultures, this line was drawn horizontally. The orientation angle values originally ranged from 0° to 180°. A 0° or 180° reading indicated an orientation parallel to the stretch vector, whereas a 90° reading indicated a perpendicular orientation. By convention (15), these values were then corrected to average orientation angles, which is the average of minimal deviation values of the cellular long axis from the reference line. Therefore, when the orientation angle is <90°, the orientation angle was taken, and when the orientation angle is >90°, its complementary value is recorded. Therefore, a 90° average orientation angle represents a perfectly perpendicular orientation of cells to the direction of stretch, whereas 45° and 0° represent completely random and parallel orientations, respectively.

Western blotting. After the appropriate treatment with Cytomix (containing 10 μg/ml lipopolysaccharide, 400 U/ml interferon, 1,000 U/ml tumor necrosis factor, and 100 U/ml interleukin) or vehicle, cell lysates were immediately obtained by incubating cells in ice-cold lysis buffer (30 μl/well) containing 10% protease inhibitor cocktail (Sigma; St. Louis, MO) and gently scraping cells with a cell scraper. The protein content of the lysates was determined with the Pierce Bicinchoninic Acid Protein Assay (Pierce; Rockford, IL), and aliquots of lysates (1–10 μg/well) were prepared for Western blotting, as we have previously described (26). Volumes of lysates were adjusted for each gel so that total protein content was equalized among all wells. The gel (7.5% acrylamide) was run at 30 mA for 40 min. The proteins were transferred (Bio-Rad Mini-Protein II Electrophoresis Cell and Mini Trans-Blot Electrophoretic Transfer Cell) to nitrocellulose (Nitrobind 0.45 μm; Fisher Scientific) for 1 h at 185 mA. The nitrocellulose was incubated with goat anti-rabbit inducible NO synthase (iNOS) polyclonal antibody (1:15,000) for 45 min at 37°C, washed repeatedly, and then incubated with anti-rabbit IgG horseradish peroxidase (HRPO) conjugate (1:80,000) at 37°C for 45 min. Protein bands were visualized...
with the Pierce Super Signal West Femto Kit, and band densities were quantified on a pixel-by-pixel basis with Un-ScanIt software (San Diego, CA).

Replicates and statistical analysis. Each experiment was performed a minimum of three times. For each experimental trial, triplicate wells from each group (i.e., control, stretch, and stretch + inhibitor) were assayed. All data are expressed as means ± SE. Replicate experiments all showed equivalent changes in the assayed variable compared with the representative graphs illustrated. Outliers, when present, were identified and removed via Dixon’s gap test. Student’s t-test or analysis of variance with post hoc Bonferroni correction assessed differences in population means. Population means were considered significantly different if \( P < 0.05 \). All data were analyzed using the InStat software suite (GraphPad Software; San Diego, CA).

Chemicals, kits, and antibodies. Human recombinant IGF-1 was a kind gift from Genentech (San Francisco, CA). Recombinant mouse interferon-γ was purchased from PBL Laboratories (New Brunswick, NJ); lipopolysaccharide, mouse recombinant tumor necrosis factor-α, \( \text{N}^\text{G}-\text{monomethyl-} \text{L-arginine (l-NMMA)}, \text{PD}-98059, \text{l-citrulline, wortman-} \text{nin, GdCl}_3, \text{tyrphostin-1, sodium nitroprusside, and rat re-} \text{combinant interleukin-1β were purchased from Sigma. Goat anti-iNOS polyclonal antibody, anti-rabbit IgG HRPO conjugate, and iNOS-positive control lysate were from Transduc-}\text{tion Laboratories (Lexington, KY). The Cell Titer 96 Cell Proliferation Assay kits were from Promega (Madison, WI) and the fluorometric nitrate/nitrite assay kit was from Cay-}\text{man (Ann Arbor, MI).}

RESULTS

Cyclic stretch does not affect cell viability. Before stretch was commenced, and after 24 and 48 h of cyclic stretch, cell viability was assessed. Microscopic evaluation revealed no gross areas of cell detachment from the elastomeric substrate in nonstretched control (i.e., static) cells or stretched groups. Furthermore, cell viability was ≥95% in stretched as well as static wells as determined by the CellTiter 96 kit, which tests the ability of the cells to bioreduce MTS into formazan. Similar viability indexes were obtained from cells treated with all reported concentrations of inhibitors (see below).

Cyclic stretch causes VSMC to align nearly perpen-\text{dicular to stretch vector. Figure 1 illustrates that cyclic stretch at 1 Hz and 120% } L_o \text{ for 48 h results in VSMC alignment in a manner almost perpendicular to the stretch vector. Figure 1A depicts VSMC grown in static conditions for 48 h. These cells display a typical spindle-shaped appearance, with the long axis of the cells}
arranged in a random orientation angle configuration (see histograms, Fig. 1). Stretched cells (Fig. 1B) also appear as spindle-shaped cells; however, the long axes of the cells are aligned nearly perpendicular to the stretch vector (double-headed arrow signifies stretch vector direction) as illustrated by the high incidence of cells measured at 80–90° to the stretch vector. As we (24) have previously reported, cells in the middle of the elastomeric well are stretched the least, whereas cells at the periphery undergo the greatest transient increase in length during a typical waveform. Figure 2 illustrates that cells stretched the least (center) respond by partially aligning, whereas cells stretched to a maximum (periphery) respond by maximally aligning. If cyclically stretched cells are then returned to a static environment, they revert to a completely random orientation within 48 h (data not shown).

**Inhibition of IGF-1 signaling decreases stretch-induced proliferation, but not stretch-induced alignment.** We (24) reported previously that cyclic stretch induces a dramatic increase in VSMC proliferation, a response that is completely blocked by pretreatment of cells with antibodies to IGF-1. Therefore, we tested whether stretch-induced cellular alignment was similarly dependent on stretch-induced IGF-1. Figure 3A shows that stretch-induced alignment was not affected by identical anti-IGF-1 pretreatment. To validate the lack of effect of IGF-1 on cellular alignment, we treated static VSMC with 10−7 M IGF-1 for 48 h. This maneuver resulted in a 70% increase in proliferation versus nontreated cells (data not shown; see similar results in Ref. 24), but did not affect cellular orientation (Fig. 3B).

We then tested the effects of three IGF-1 signaling inhibitors: tyrphostin-1 (a tyrosine kinase inhibitor), PD-98059 (a MAPK inhibitor), and wortmannin [a phosphatidylinositol 3-kinase (PI3-kinase) inhibitor]. None of these inhibitors, at concentrations known to dramatically inhibit IGF-1 growth responses (24), affected stretch-induced alignment compared with similarly stretched control cells. Similarly, none of the inhibitors affected cellular orientation in static VSMC (Table 1).

**Inhibition of stretch-activated calcium channels inhibits proliferation but not cellular alignment.** We (24) have previously shown that blockade of stretch-activated calcium channels (SACCs) inhibits stretch-induced VSMC proliferation. Therefore, we tested whether identical pretreatment of VSMC altered stretch-induced alignment. Table 1 shows that SACC blockade with a Gd3+ concentration, known to dramatically inhibit stretch-induced VSMC proliferation (24), had no effect on stretch-induced cellular alignment compared with similarly stretched control cells. In addition, blockade of SACCs in static VSMC had no effect on cellular orientation.

**Inhibition of NOS attenuates NO secretion and blocks cyclic stretch-induced alignment in VSMC.** In addition to enhancing VSMC IGF-1 secretion and proliferation, cyclic stretch also increases iNOS expression and consequent NO secretion (26). We tested whether this secreted NO affected cellular alignment in response to stretch. Blockade of NOS by treatment with...
L-NMMA (various concentrations from 1 to 100 μM) resulted in the reversal of stretch-induced alignment (Fig. 4A). Figure 4B confirms that pretreatment with 100 μM L-NMMA results in an 85% reduction in VSMC NO secretion, suggesting that NO is normally poised to induce or maintain alignment of VSMC in response to stretch. To this end, we tested whether a suprabasal concentration of NO (sodium nitroprusside, 100 nM) was capable of inducing alignment in static VSMC. This maneuver was chosen because we have shown that, although VSMC produce NO under static basal conditions, they do not align. Figure 5A shows that exogenous NO without stretch is not a sufficient stimulus to induce cellular alignment. Citrulline, the other product of NOS-catalyzed arginine metabolism, has been shown to affect cellular proliferation (21). Therefore, we tested whether citrulline (10 nM; a concentration known to relax rabbit aortic rings and significantly attenuate rat aortic vascular smooth muscle proliferation, Ref. 21) was capable of inducing cellular alignment in static cells or capable of affecting stretch-induced cellular alignment. Figure 5B illustrates that citrulline (not present in our growth medium) does not affect cellular alignment in static or stretched VSMC.

### Table 1. Effects of various cell signaling inhibitors on orientation angle in static and cyclically stretched vascular smooth muscle cells

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Purpose</th>
<th>Static</th>
<th>Static + Inhibitor</th>
<th>Stretch</th>
<th>Stretch + Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tyrphostin-1 (10 μM)</td>
<td>Block tyrosine kinase activity</td>
<td>44.0 ± 6.0</td>
<td>50.1 ± 6.1</td>
<td>88.2 ± 2.1*</td>
<td>81.5 ± 3.2*</td>
</tr>
<tr>
<td>PD-98059 (100 μM)</td>
<td>Block MAPK activity</td>
<td>48.6 ± 4.3</td>
<td>42.8 ± 6.2</td>
<td>81.6 ± 1.2*</td>
<td>83.2 ± 1.1*</td>
</tr>
<tr>
<td>Wortmarin (100 nM)</td>
<td>Block PI3 kinase activity</td>
<td>41.3 ± 7.2</td>
<td>45.5 ± 4.4</td>
<td>90.2 ± 3.1*</td>
<td>79.5 ± 3.1*</td>
</tr>
<tr>
<td>GdCl3 (30 μM)</td>
<td>Block SACCs</td>
<td>45.1 ± 8.5</td>
<td>47.5 ± 8.1</td>
<td>88.2 ± 1.6*</td>
<td>88.2 ± 1.6*</td>
</tr>
</tbody>
</table>

Values are means ± SE for a minimum of 48 cells in peripheral fields for each data set. PI3, phosphatidylinositol-3; SACCs, stretch-activated calcium channels. All inhibitors were added at the commencement of the 48-h cyclic stretch (see MATERIALS AND METHODS) or static regimen. *P < 0.05 vs. nonmarked groups in the same inhibitor row.
Cytokine induction of NOS does not affect VSMC alignment. Cytomix has been shown to dramatically stimulate iNOS expression, stimulate NO secretion, inhibit VSMC proliferation, and reduce blood pressure (4, 26). Therefore, we tested whether induction of iNOS in this manner affected cellular alignment in static and stretched VSMC. Figure 6A shows that treatment of static cells with Cytomix had no effect on cellular orientation. Furthermore, superinduction of NOS with Cytomix failed to uncouple alignment in cyclically stretched VSMC. We confirmed, however, that identical Cytomix treatment indeed resulted in induction of iNOS expression as shown by Western blotting (Fig. 6B) and also results in a two- to fourfold enhancement VSMC NO secretion (26).

DISCUSSION

Our studies show that cyclic stretch causes reversible cellular alignment in a manner nearly perpendicular to the stretch vector. The mechanism of alignment is independent from the several signaling modalities that are involved in cyclic stretch-induced hyperplasia. Specifically, IGF-1, MAPK, PI3-kinase, tyrosine kinase, and SACCs appear not to be involved in the alignment process. Conversely, NO, which is known to inhibit cyclic stretch-induced hyperplasia, is involved in cyclic stretch-induced alignment, as blockade of NOS uncouples this alignment process. Attempts at stimulating alignment in static cells by 1) upregulating NOS, 2) adding NO donors, or 3) adding citrulline (another product of NO catalysis of arginine) proved to be unsuccessful. These data suggest that both cyclic stretch and VSMC autocrine NO are required to induce cellular alignment.

Our data showing that VSMC align in response to cyclic stretch confirms the findings of previous studies. For example, others have shown using the Flexercell apparatus that cyclic stretch causes bovine aortic VSMC to align nearly perpendicular to the stretch vector (18). However, the stretch stimulus imparted to VSMC at various locations throughout the Flexercell well is nonuniform (2). In fact, cells in the center of such wells are stretched 0–4% above resting length, those in the middle regions are stretched 4–10% above resting length, and those in the peripheral regions are stretched 10–25% above resting length (24). We (24) have shown that as stretch magnitude increases from the center to peripheral regions, the hyperplastic response also increases. Similarly, our current study illustrates that the equivalent increases in stretch magnitude (i.e., 0–25%) result in greater degrees of alignment (Fig. 2). Because secretions from all cells exposed to such a continuum of stretch magnitudes are ultimately homogenized within the conditioned media of the wells, it is unlikely that such secretions (i.e., IGF-1 and NO) are alone responsible for various degrees of alignment. Rather, we speculate that the greater stretch magnitudes seen in the peripheral regions are associated with greater stretch of extracellular/intercellular adhesion proteins, which in turn result in a greater alignment stimulus as suggested by the tensegrity model posed by others (12). Because alignment is reversible within 48 h, it appears that potential stretch activation of such adhesion proteins/receptors must be maintained to continue to support the alignment phenomena. This represents an area we are currently investigating.

Despite attempts to explain the underlying mechanism by which NO mediates cyclic stretch-induced alignment, the precise signaling cascade remains elusive. For example, despite cyclic stretch induction of cAMP and protein kinase C in bovine aortic VSMC, neither signaling molecule appears to be related to the proliferation response or to the alignment phenomenon (18). Others have shown that NO selectively attenuates PDGF-induced increases in protein kinase B (PKB)-α, a process that appears to diminish VSMC proliferation by an apoptotic-independent mechanism (22). If autocrine PDGF is present in our system in adequate concentrations, then it is plausible that NO blockade (via l-NMMA) increases PKB-α, which in turn may mediate cellular alignment. This notion requires further investigation. We (24) have shown that stretch-induced hyperplasia is indeed dependent on autocrine actions of IGF-1, activation of tyrosine kinase, enhancement of MAPK, induction of PI3-kinase, and opening of SACCs. The current findings show that these five signaling modalities are not involved in stretch-induced alignment (Fig. 3 and Table 1). This lack of effect of IGF-1 blockade on stretch-induced alignment was somewhat surprising because others (11, 19) have shown that IGF-1 induces rat aortic VSMC migration in a wound model by a mechanism
requiring PI3-kinase activation. Although conditions are not identical between wounding-induced and stretch-induced migration, one might expect a degree of overlap in their signaling cascades. To date, our studies do not support a role for IGF-1 in this respect.

We and others (13, 24) have shown that blockade of SACC (with GdCl3) attenuates cyclic stretch-induced hyperplasia as well as stretch-induced phosphorylation of ERK1/2 and EGF receptor. Because our current studies show that similar blockade of SACC does not affect stretch-induced alignment, we may reasonably presume that this alignment response is independent from ERK1/2 activation. Despite reports showing that cyclic stretch induces MAPK, and that enhancement of MAPK is required for stretch-induced hyperplasia (20), MAPK blockade in the current study was without effect on the cyclic stretch-induced alignment response.

In all, these data suggest that the alignment response likely occurs independently of ERK1/2/MAPK activation.

We have also shown that cyclic stretch increases VSMC expression of the iNOS gene and NO secretion (26). NO, an antimitogenic molecule, in turn limits the overall stretch-induced proliferative response. The current study suggests that NO is also involved in the stretch-induced alignment response, as a NOS inhibitor (L-NMMA) abolishes stretch-induced alignment. Interestingly, alignment cannot be induced in static cells treated with a NO donor (sodium nitroprusside), with L-citrulline (another product of NOS-mediated catabolism of L-arginine with known antiproliferative effects; see Ref. 21), nor with cytokine mix (known to induce iNOS expression and NO secretion in a stretch-independent fashion, Ref. 26). Because we have shown that a7r5 VSMCs secrete basal levels of NO (26) we can conclude that VSMCs require both cyclic stretch and minimal basal NO levels to align.

Of the signaling pathways investigated so far, NO appears to be a lone point of overlap that connects cyclic stretch-induced hyperplasia and alignment. The following question remains, however: what is the mechanism by which NO is involved in alignment? Some investigators (8, 23) have shown that NO donors inhibit endothelial cells as well as VSMC migration (1, 5), whereas others (14) suggest that NO stimulates, or at least maintains, hormone-induced VSMC migration. However, given that there are arguable differences between such a migration in response to wounding and alignment in response to biophysical perturbation (see above), such comparisons and conclusions are problematic. Because alignment cannot simply be induced in static cells by the addition of the NO donor L-citrulline or by the induction of expression of the iNOS enzyme with cytokines (Figs. 4–6), suggests that a portion of this response is indeed dependent on biophysical perturbation of the cells and/or their extracellular/intercellular matrix proteins (e.g., integrins, collagen, etc.). These represent areas we are currently investigating.

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