

Contractility Affects Stress Fiber Remodeling and Reorientation of Endothelial Cells Subjected to Cyclic Mechanical Stretching

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Abstract—We studied the effect of contractility on stress fiber remodeling and orientation response of human aortic endothelial cells subjected to cyclic mechanical stretching. The cells were grown on silicone membranes subjected to 10% cyclic pure uniaxial stretching in the presence or absence of 2,3 butanedione monoxime (BDM), a proven inhibitor of cellular contractility. It was found that treatment of the cells with BDM (40 mM) abolished stress fibers and blocked cell reorientation in response to cyclic stretching, indicating that contractility is required for these two cellular responses. When cells were stretched in the presence of N-acetylcysteine (NAC, 20 mM), a hydrogen peroxide (H₂O₂) scavenger, stress fibers were still formed and the cells reoriented—but more slowly. Specifically, compared with untreated cells, NAC treated cells after 0.5, 1, and 3 h of 10% stretching had significantly ($p < 0.005$) less skewed orientation distributions than those of untreated cells. After the cells were treated with both NAC (20 mM) and nordihydroguaiaretic acid (NDGA, 50 μ M), another antioxidant, however, stress fibers were abolished and cell reorientation was completely blocked. These results indicate that reactive oxygen species (ROS), including H₂O₂, affect stress fiber remodeling and reorientation of endothelial cells in response to cyclic stretching. We suggest that the effect of ROS on stress fiber remodeling and cell reorientation is due to the ability of ROS to regulate cellular contractility, which is crucial for these cellular responses. © 2000 Biomedical Engineering Society. [S0090-6964(00)00110-7]

Keywords—Stretching, Contractility, Reactive oxygen species, Hydrogen peroxide.

INTRODUCTION

It is well recognized that mechanical stretching induces wide-ranging cellular responses. These include change in cell orientation, synthesis of various extracellular matrix proteins, and expression of a multitude of genes.^{2,14,27,36} Among these, cell reorientation is one of the most obvious cellular responses—when grown on a deformable substrate and subjected to cyclic uniaxial

stretching, cells orient away from the stretching direction.^{13,20,36} Together with the cell reorientation, actin cytoskeleton of the stretched cell is remodeled, resulting in the formation of parallel bundles of actin filaments, i.e., stress fibers.²⁰

Stress fibers, which attach to the cytoplasmic side of focal adhesions,^{4,21} are associated with cellular contractility.^{6,41} For example, endothelial cells and fibroblasts have been shown to wrinkle underlying thin silicone sheet.¹² This contractile force is measurable by sensitive transducers.¹⁵ Contractility in these nonmuscle cells has been shown to be mediated by myosins.⁷ On the one hand, inhibiting the myosin activity by diverse agents, such as 2,3 butanedione monoxime (BDM), blocks contractility in fibroblasts, resulting in the disassembly of stress fibers.⁶ On the other hand, when contractility was induced, increased assembly of stress fibers was observed.¹² However, most of these studies were conducted with static cultures. It is not clear whether contractility also influences stress fiber remodeling and reorientation of endothelial cells when subjected to cyclic mechanical stretching.

Besides contractility, it has been established that reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), can affect actin polymerization and actin cytoskeletons of cells.^{11,16,17} For example, a low concentration (10 μ M) of exogenous H₂O₂ causes change in actin filaments of bovine endothelial cells from a diffuse pattern to a clear stress-fiber pattern.²⁸ Furthermore, levels of H₂O₂ in endothelial cells can be increased by cyclic stretching.¹⁹ These studies suggest that stress fiber remodeling of the endothelial cells under cyclic stretching conditions may be mediated by ROS.

The purpose of this study was twofold. First, we wanted to test the hypothesis that contractility is required for stress fiber remodeling and reorientation of endothelial cells subjected to cyclic stretching. Second, we wanted to examine whether ROS affect stress fiber remodeling and cell reorientation. The results of this study suggest that during cyclic stretching contractility is re-

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quired for stress fiber remodeling and cell reorientation, and that ROS affect these two cellular responses. These effects are most likely due to the ability of ROS to regulate cellular contractility.

MATERIALS AND METHODS

Materials

Deformable, transparent silicone membranes were purchased from Specialty Manufacturing (Saginaw, MI). ProNectin-F, a bioengineered polymer for promoting cell attachment to silicone surfaces, was obtained from Protein Polymer Technologies (San Diego, CA). BDM, N-acetylcysteine (NAC), and nordihydroguaiaretic acid (NDGA) were obtained from Sigma (St. Louis, MO). Rhodamine phalloidin was purchased from Molecular Probes (Eugene, OR). Human aortic endothelial cells (HAEC) from different donors, essential basal medium (EBM) and supplements were supplied by Clonetics (Walkersville, MD).

Method

Cell Culture. HAECs were cultured in EBM containing 2% fetal bovine serum and other supplements according to the manufacturer's instructions. Briefly, the cells were grown in plastic dishes and incubated at 37°C in a humidified 5% carbon dioxide atmosphere. The medium was changed every two days. Subculturing was done with a trypsin (0.25%)/EDTA (0.02%) solution. Cells, passaged between 5 and 16, were transferred to silicone membranes coated with 10 µg/ml ProNectin-F and incubated for 12–16 h before application of cyclic stretching.

Stretching Apparatus. Cells on the silicone membrane were cyclically stretched using a custom-made stretching apparatus. Briefly, the apparatus consists of four metal arms and four driving motors, arranged in tandem along two orthogonal axes. Each of the metal arms attaches to one edge of a square (55×55 mm) membrane with four metal posts. The motors move the pairs of arms so that the membrane is stretched cyclically. The stretching magnitude and frequency can be set by a control unit. A significant advantage of this apparatus is that it can apply pure uniaxial stretching to a silicone membrane. Pure uniaxial stretching deforms the membrane in such a way that the cells in the central portion are deformed in the stretching but not the perpendicular direction (Fig. 1).

Surface strains on the silicone membrane under pure uniaxial stretching were measured. It was found that the strains are uniformly distributed in the central region (20×20 mm) of the 50×50 mm membrane. Therefore, cells were plated only in this region. Hence, these cells were exposed to the same membrane deformation, regardless of their location within the region.

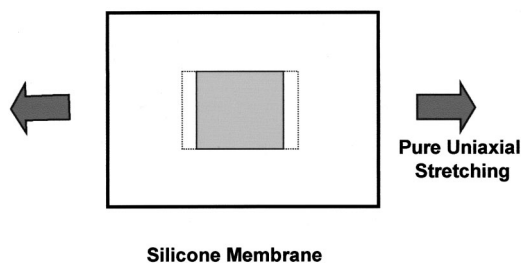


FIGURE 1. Schematic of the deformation of the silicone membrane used in the present stretching experiments. Pure uniaxial stretching deforms the cells on the membrane in the horizontal but not the perpendicular direction in the central region (shaded area) of the membrane. This central area of about 20×20 mm had a uniform strain distribution. Within this area, human endothelial cells were grown and cyclically stretched.

Stretching Experiments. Cyclic stretching, with a magnitude of 10% and a frequency of 0.5 Hz, was applied both in the presence and absence of BDM (40 mM) for 3 h. In separate experiments, cells were stretched in the presence of NAC (20 mM), a ROS scavenger, for 0.5, 1, and 3 h. Higher doses of NAC could not be used because of apparent toxicity to cells. Control cells without NAC treatment were stretched for the same durations. Still in separate experiments, cells were cyclically stretched in the presence of both NAC (20 mM) and NDGA (50 µM), another antioxidant.

Fluorescence Microscopy of Actin Filaments. To determine integrity of the actin cytoskeleton, actin filaments of the endothelial cells were stained with rhodamine phalloidin. Briefly, cells were washed twice with cold PBS, fixed in 3.7% formaldehyde in PBS for 30 min, permeabilized in 0.25% Triton X-100 for 10 min, and incubated with 0.165 µM rhodamine phalloidin at room temperature for 1 h. After washing three times (10 min each) with PBS, the stained cells were viewed on a Nikon fluorescence microscope and photographed with Kodak 400-color slide film.

Determining Cell Orientation Distribution. To evaluate whether NAC affected cell reorientation, cell orientations were measured by digitizing phase contrast photographs of the cells using NIH Image 1.6. The cell orientation was defined to be the angle of a cell's long axis with respect to the stretching direction. Most cells had a bipolar shape, so that their long axes could be readily defined, and their orientations were measured with ease. Those few cells that did not have a bipolar shape were excluded from the measurement.

The distribution of cell orientations was depicted by counting the number of cells that fell into 18 orientation intervals, 5° each, from 0 (the stretching direction) to 90° (perpendicular to the stretching direction).

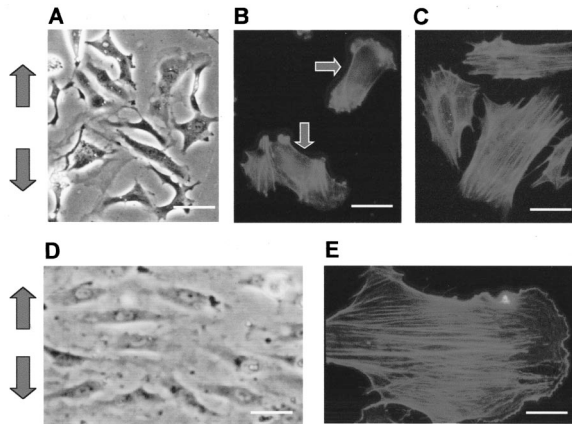


FIGURE 2. Effect of BDM on actin cytoskeleton and cell reorientation. After 10% cyclic uniaxial stretching (the stretching direction is indicated by arrows) for 3 h, reorientation of BDM treated cells was blocked—the cells remained randomly oriented after the stretching [(A) bar: 100 μm]. Also, most stress fibers in the stretched cells were abolished [(B) bar: 100 μm]. Unstretched cells showed abundant stress fibers [(C) bar: 100 μm]. When these cells were stretched, they reoriented perpendicular to the stretching direction [(D) bar: 100 μm], and bundles of stress fibers were formed in the same perpendicular direction [(E) bar: 30 μm]. Notice that the cells after stretching in BDM appeared smaller, and their shape also changed, presumably because stress fibers were abolished [see also Figs. 3(A) and 3(C)].

Statistical Analysis. The Kolmogorov–Smirnov (KS) test was used to compare cell orientation distributions between cell groups. Two distributions were considered to be significantly different if $p < 0.05$.

RESULTS

Effect of BDM Treatment on Endothelial Cells

After 10% cyclic stretching for 3 h in the presence of BDM, reorientation of the endothelial cells was completely blocked [Fig. 2(A)]. Also, only a few stress fibers remained in the stretched cells [Fig. 2(B)], compared with the abundant stress fibers in the unstretched cells [Fig. 2(C)]. The cells stretched without BDM reoriented perpendicular to the stretching direction [Fig. 2(D)], and had dense stress fibers formed in the same perpendicular direction [Fig. 2(E)].

It should be noted that cells stretched in the presence of BDM changed their shape greatly [compare Figs. 3(A) and 3(D)]. However, these cells were viable, as evidenced by their lack of uptake of trypan blue [Fig. 3(B)], a reagent for testing cell viability²² and by the fact that the cells spread quickly after the BDM-containing medium was replaced with the regular growth medium [Fig. 3(C)].

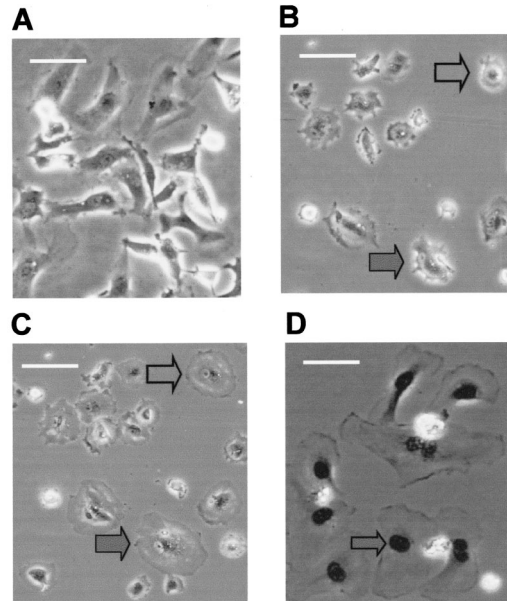


FIGURE 3. Viability of endothelial cells. After the cells were stretched in the presence of BDM for 3 h, they were incubated with trypan blue for 45 min (A). The cells became rounded, but they did not uptake the trypan blue (B). After the medium containing the trypan blue was replaced with the regular growth medium, the cells rapidly spread [compare (B) with (C)]. Paired arrows point to the *same* cells. In contrast, endothelial cells killed by treating with 75% EtOH showed strong trypan blue staining after only 10 min of incubation [(D) arrow]. These results indicated that the BDM treated cells during and after stretching were viable (bar: 100 μm).

Effect of NAC alone on Endothelial Cells

In response to 10% cyclic stretching, at each duration the NAC treated cells showed less skewed orientation distributions than stretched, untreated cells, indicating that the treated cells reoriented less (Fig. 4). In fact, for each of the three stretching periods examined, the median orientations of the NAC treated cells were consistently smaller than those of the untreated cells (Fig. 5).

Effect of NAC and NDGA Together on Endothelial Cells

When cells were stretched in the presence of both NAC (20 mM) and NDGA (50 μM), the stress fibers were abolished and cell reorientation was completely blocked (Fig. 6). However, 50 μM of NDGA alone blocked neither the formation of stress fibers nor the cell reorientation in response to stretching.

To further confirm that the combination of NAC and NDGA blocked stress fiber formation and cell reorientation, we performed additional experiments as follows. The cells were first stretched without NAC or NDGA for 3 h. They reoriented towards the perpendicular to the stretching direction. Then, the stretched cells were treated with the combination of NAC (20 mM) and NDGA (50 μM) for 30 min, and in the presence of the

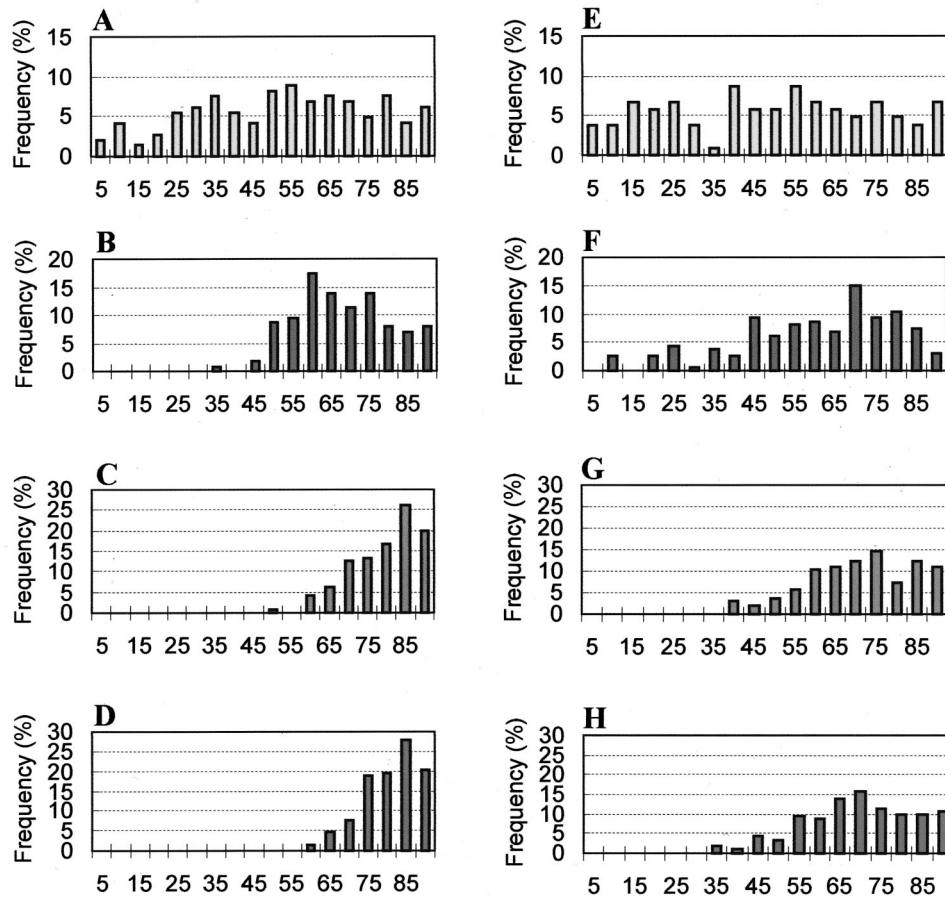


FIGURE 4. Effect of NAC treatment on cell reorientation. The left-hand side column shows the orientation distributions between 0° (the stretching direction) and 90° (perpendicular to the stretching direction) in cells stretched without NAC treatment (A, B, C, D), whereas the right-hand column shows results in cells stretched in the presence of 20 mM NAC (E, F, G, H). The cells were subjected to cyclic pure uniaxial stretching (10%, 0.5 Hz) for 0 (A and E), 0.5 (B and F), 1 (C and G) and 3 h (D and H). Significant differences were found for all three paired distributions between NAC untreated and treated cells at 0.5, 1, and 3 h ($p \leq 0.0001$), but no significant difference was found at time zero (A vs. E) between the untreated and NAC treated cell groups ($p = 0.39$). The total number of cells for the distributions ranged from 115 to 162. The cell orientations for each distribution were pooled together from at least two experiments.

two inhibitors, 10% cyclic stretching was applied in the direction of the cell alignment for 3 h. We found that the cells were devoid of stress fibers and remained aligned in the stretching direction. However, after washing away the NAC and NDGA and then stretching the cells in a regular growth medium, we found that the cells regained stress fibers and reoriented (Fig. 7). This result confirmed that NAC and NDGA together inhibited stress fiber remodeling and cell reorientation, and that this effect was reversible, and not due to nonspecific toxicity of these two inhibitors.

DISCUSSION

Previous studies have shown that BDM, which readily enters cells, inhibits nonmuscle myosin ATPase¹⁰ and decreases myosin light chain (MLC) phosphorylation in

both smooth muscle cells³¹ and fibroblasts.⁶ These effects inhibit actin–myosin interaction thereby affecting shape and locomotion in nonmuscle cells.³⁰ The present study showed that BDM inhibited stress fiber remodeling and blocked reorientation of endothelial cells subjected to cyclic stretching, indicating that contractility is required for these two cellular responses.

Our results are similar to the finding that disrupting the actin cytoskeleton with cytochalasin B, an inhibitor of actin polymerization, completely abolishes cell reorientation in response to cyclic mechanical stretching.²⁰ However, the action of BDM differs from that of cytochalasin B, since BDM does not directly affect actin polymerization.¹⁰ Rather, the disruption of the actin cytoskeleton and subsequent inhibition of stress fiber remodeling during cyclic stretching are likely due to the

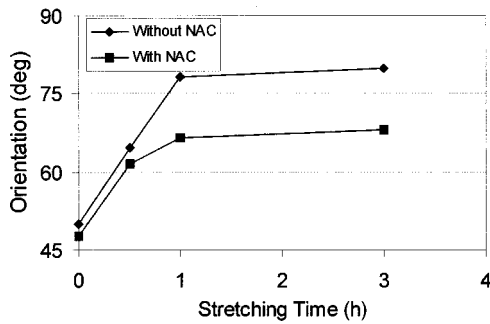


FIGURE 5. Median orientations of the endothelial cells with and without NAC treatment. The cells were cyclically stretched at 10%, 0.5 Hz, for 0.5, 1, and 3 h. Cells treated with NAC had consistently smaller median orientations at all time points.

fact that BDM blocked cellular contractility, which led to the inhibition of stress fiber formation.

This study also showed that NAC slowed reorientation of the stretched endothelial cells. Although the exact mechanism for this is not completely clear, it may also be related to the effect of NAC on cellular contractility. The reasoning is as follows. First, NAC scavenges H_2O_2 .^{25,33} Second, endothelial cells generate significant amounts of H_2O_2 and other ROS.²³ In particular, cyclic stretching of the cells increases levels of H_2O_2 .¹⁹ Third, H_2O_2 induces contraction in bovine aortic endothelial cells²⁹ by increasing MLC phosphorylation,⁴⁰ and possibly calcium and protein kinase C.²⁹ H_2O_2 may also modulate the activity of rho, a small GTP-binding protein, which regulates cellular contractility via MLC kinase⁶ and thereby mediates assembly and disassembly of actin cytoskeletons in fibroblasts.³² High levels of H_2O_2 may activate rho, whereas low levels may inactivate rho. Thus, it is likely that the slowed cell reorientation by NAC was due to reduced levels of H_2O_2 and consequently decreased contractility. Further studies are needed to show that NAC actually scavenges H_2O_2 in

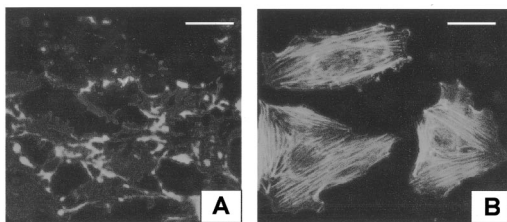


FIGURE 6. Effect of the combination of NAC and NDGA on stress fibers of endothelial cells. After the cells were cyclically stretched (10%, 0.5 Hz) for 3 h in the presence of both NAC (20 mM) and NDGA (50 μ M), stress fibers were completely abolished (A). In contrast, the cells without the treatment of NAC and NDGA had abundant stress fibers (B) (bar: 75 μ m).

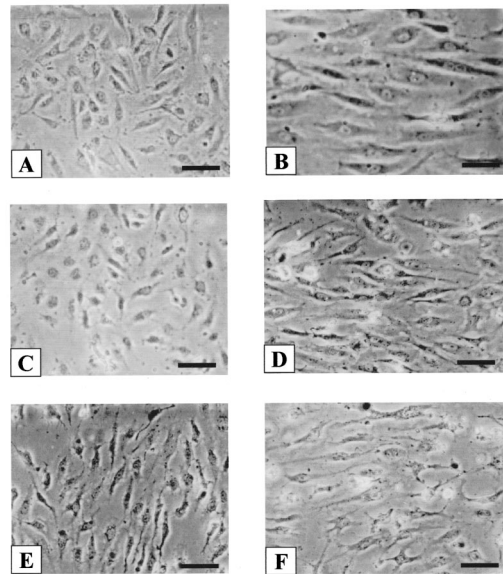


FIGURE 7. NAC and NDGA together blocked reorientation of the endothelial cells in response to cyclic stretching. Unstretched cells were oriented randomly [(A) bar: 175 μ m]. After 10% cyclic stretching for 3 h, they reoriented perpendicular to the stretching (vertical) direction [(B) bar: 75 μ m]. However, after stretching in the presence of NAC (20 mM) and NDGA (50 μ M) [(C) bar: 175 μ m], the cells did not reorient. In fact, they did not move under the same stretching [compare the cells in (A) with those in (C), which were the same group of cells before and after stretching]. The effect of blocking cell reorientation was not due to carriers for NAC and NDGA, since cells reoriented in the presence of carriers alone [(D) 75 μ m]. To further confirm that the combination of NAC and NDGA prevented cell reorientation, cells were first reoriented by stretching without NAC and NDGA for 3 h. Then, the same cells were treated with NAC and NDGA, followed by the same cyclic stretching but now in the direction of the aligned cells (perpendicular to the first stretching direction). The cells were found to remain oriented in the new stretching direction [(E) bar: 175 μ m]. After the NAC and NDGA containing medium was replaced with regular medium and then the cells were cyclically stretched, they reoriented a second time [(F) bar: 75 μ m].

endothelial cells, although this has been shown to be true in smooth muscle cells.³⁷

Many studies have shown that endothelial cells can generate significant amounts of hydrogen peroxide, superoxide, and other free radicals.^{5,24} The exact mechanism of ROS production in endothelial cells, however, is not completely clear. The NADH/NADPH oxidase that resides in the plasma membranes of the endothelial cells may be a possible source for generation of H_2O_2 .⁴² Enzymes like xanthine oxidase and cyclo-oxygenase, which are responsible for generation of superoxide, may also be involved.¹⁹ For example, the latter enzyme is thought to be responsible for the release of superoxide induced by bradykinin.¹⁸ In addition, it has been shown that cyclic stretching depolarizes membranes in smooth muscle cells,⁸ and membrane depolarization initiates the

generation of oxidants in endothelial cells.¹ Hence, it is possible that cyclic stretching of the endothelial cells in this study caused the membrane depolarization, which then led to formation of H₂O₂ and other oxidants.

The finding that NAC and NDGA together completely blocked stress fiber remodeling and cell reorientation may be due to contractility being decreased sufficiently by the combined effects of these two antioxidants. Whereas NAC enters cells to decrease cytoplasmic levels of ROS, NDGA is a lipid soluble antioxidant, which traps ROS in membranes.³⁵ So NAC and NDGA together were able to scavenge sufficient ROS to disable the contractility, leading to the blocking of stress fiber formation and cell reorientation.

It should be noted that NDGA not only scavenges oxidants, but also inhibits 5-lipoxygenase—at least in Swiss 3T3 cells.³⁴ The 5-lipoxygenase is a key enzyme that converts arachidonic acid to leukotrienes, which are also involved in the formation of stress fibers.⁹ Therefore, this effect of NDGA may have contributed to the combined effect of NDGA and NAC to block stress fiber formation and cell reorientation. Nevertheless, this effect of NDGA is likely minor because NDGA alone had no apparent effect on the formation of stress fibers and cell reorientation.³⁸ Furthermore, the leuko-triene pathway that leads to the formation of stress fibers is upstream of the rho pathway. There is, in addition, at least one independent signaling pathway for stress fiber assembly.⁹ In other words, the possible inhibition of leukotriene production by NDGA cannot be fully responsible for the abolishment of stress fibers and cell reorientation observed in this study.

It is the current view that cellular contractility, which requires myosin activation, is necessary for the formation of stress fibers. Myosin activation involves myosin phosphorylation, but whether cyclic stretching of endothelial cells activates MLCK or myosin phosphorylation has not been shown. However, in arterial muscle which includes a layer of endothelial cells, it has been shown that cyclic stretching activates MLC kinase (MLCK).³²⁶ Shear stress on endothelial cells also enhances MLC phosphorylation.³⁹ Furthermore, cyclic stretching of endothelial cells increases the levels of H₂O₂, which induces MLC phosphorylation in the cells, and MLC phosphorylation is inhibited by MLCK inhibitors.⁴⁰ Taken together, the data of these previous studies suggest that cyclic stretching may alter MLCK activity, resulting in the altered contractility in endothelial cells.

In summary, we studied the role of contractility and ROS in stress fiber remodeling and reorientation of human aortic endothelial cells subjected to cyclic stretching. We found that inhibiting cellular contractility abolished stress fiber remodeling and cell reorientation. Furthermore, scavenging ROS affected stress fiber remodeling and reorientation of endothelial cells. This may

be due to the ability of ROS, such as H₂O₂, to regulate cellular contractility.

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