

# Leukotrienes and Tyrosine Phosphorylation Mediate Stretching-Induced Actin Cytoskeletal Remodeling in Endothelial Cells

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We studied actin cytoskeletal remodeling and the role of leukotrienes and tyrosine phosphorylation in the response of endothelial cells to different types of cyclic mechanical stretching. Human aortic endothelial cells were grown on deformable silicone membranes subjected to either cyclic one-directional (strip) stretching (10%, 0.5 Hz), or biaxial stretching. After 1 min of either type of stretching, actin cytoskeletons of the stretched cells were already disrupted. After stretching for 10 and 30 min, the percentage of the stretched cells that had disrupted actin cytoskeletons were significantly increased, compared with control cells without stretching. Also, at these two time points, biaxial stretching consistently produced higher frequencies of actin cytoskeleton disruption. At 3 h, strip stretching caused the formation of stress fiber bundles, which were oriented nearly perpendicular to the stretching direction. With biaxial stretching, however, actin cytoskeletons in many stretched cells were remodeled into three-dimensional actin structures protruding outside the substrate plane, within which cyclic stretching was applied. In both stretching conditions, actin filaments were formed in the direction without substrate deformation. Moreover, substantially inhibiting either leukotriene production with nordihydroguaiaretic acid or tyrosine phosphorylation with tyrphostin A25 did not block the actin cytoskeletal remodeling. However, inhibiting both leukotriene production and tyrosine phosphorylation completely blocked the actin cytoskeletal remodeling. Thus, the study showed that the remodeling of actin cytoskeletons of the stretched endothelial cells include rapid disruption first and then re-formation. The resulting pattern of the actin cytoskeleton after remodeling depends on the type of cyclic stretching applied, but under either type of cyclic stretching, the actin filaments are formed in the direction without substrate deformation. Finally, leukotrienes and tyrosine phosphorylation are necessary for actin cytoskeletal remodeling of the endothelial cells in response to mechanical stretching. *Cell Motil. Cytoskeleton* 46:137–145, 2000.

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**Key words:** endothelial cells; stretching; cytoskeleton; leukotriene; tyrosine phosphorylation

## INTRODUCTION

Mechanical forces are known to alter cell form and regulate cell function [Ingber, 1991; Davies and Tripathi, 1993]. In culture, for example, when endothelial cells are cyclically stretched, the actin cytoskeleton undergoes a dramatic reorganization: It changes from a collection of randomly distributed actin filaments to uniformly distributed bundles of actin filaments (i.e., stress fibers) [Dartsch et al., 1989; Iba and Sumpio, 1991]. Concomitant with this change in actin cytoskeleton, extracellular matrix proteins, cytokines, and a multitude of genes are

expressed [Sadoshima and Izumo, 1993; Davies and Tripathi, 1993; Banes et al., 1995]. These changes in cell

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function may result from the remodeling of the actin cytoskeleton, since many elements of cell metabolic machinery are associated with actin cytoskeleton [Ingber, 1993]. However, the process of the actin cytoskeletal remodeling in response to different types of mechanical stretching remains unclear.

Primarily from studies of Swiss 3T3 fibroblasts in static cultures, growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), are shown to induce rapid actin polymerization at the plasma membrane and lamellipodia and then lead to formation of stress fibers [Ridley and Hall, 1992; Ridley et al., 1992]. Metabolites of arachidonic acid (AA) are implicated in the growth factor-induced actin remodeling [Peppelenbosch et al., 1993]. This signaling pathway requires 5-lipoxygenase (a key enzyme that converts AA to leukotrienes), since treatment of the cells with nordihydroguaiaretic acid (NDGA), which inhibits 5-lipoxygenase, abolished the EGF-induced formation of stress fibers [Peppelenbosch et al., 1995]. Besides the growth factors, lysophosphatidic acid (LPA) can also induce stress fiber formation through a second pathway involving protein tyrosine kinases [Craig and Johnson, 1996]. The involvement of the protein tyrosine kinases (PTK) in stress fiber formation has been shown in many previous studies. For example, when treated with vanadate, a potent inhibitor of tyrosine phosphatases, serum-starved Swiss 3T3 cells form stress fibers, and this effect by vanadate is similar to that of LPA [Chrzanowska-Wodnicka and Burrige, 1994]. Moreover, the LPA-induced formation of stress fibers is blocked by tyrphostin A25, a specific tyrosine kinase inhibitor [Nobes et al., 1994]. However, it is unclear whether the same pathways are also responsible for the actin cytoskeletal remodeling of the endothelial cells under dynamic culture conditions.

This study has two objectives: (1) to examine the process of the actin cytoskeletal remodeling under different types of mechanical stretching; and (2) to test the hypothesis that the stretching-induced actin cytoskeletal remodeling requires leukotrienes and tyrosine phosphorylation. We found that actin cytoskeletons of the stretched endothelial cells were rapidly disrupted and subsequently re-formed. The actin structures after the remodeling depend on the type of stretching applied. Moreover, leukotrienes and PTK together control the actin cytoskeletal remodeling of the endothelial cells in response to cyclic stretching.

## MATERIALS AND METHODS

### Materials

Human aortic endothelial cells (HAECs), essential culture medium (EBM) and supplements were supplied

from Clonetics (Walkersville, MD). Deformable, 0.5-mm-thick silicone membranes were obtained from Specialty Manufacturing (Saginaw, MI). ProNectin-F, a bio-engineered polymer for promoting cell attachment to silicone surfaces, was purchased from Protein Polymer Technologies (San Diego, CA). NDGA, phorbol myristate acetate (PMA), anti-phosphotyrosine, H<sub>2</sub>O<sub>2</sub>, orthovanadate, anti-leukotriene B<sub>4</sub>, and leukotriene E<sub>4</sub> were obtained from Sigma Chemical Co. (St. Louis, MO). Tyrphostin A25 was purchased from Sigma and Life Technologies (Grand Island, NY). Rhodamine phalloidin and fluorescein phalloidin were obtained from Molecular Probes (Eugene, OR).

### Methods

**Cell culture.** HAECs were cultured in EBM, containing 2% fetal bovine serum (FBS) and other supplements (supplied by the manufacturer) and grown to subconfluence on uncoated plastic dishes at 37°C in a humidified 5% carbon dioxide atmosphere. For stretching experiments, the cells were transferred to the central region (see below) of the silicone membranes coated with 10 µg/ml ProNectin-F, and incubated overnight (12–16 h). For all experiments, cells from 9th to 16th passages were used.

**Stretching apparatus.** A custom-made stretching apparatus was used to stretch the silicone membranes. Briefly, the apparatus consists of four metal arms and four driving motors arranged tandemly in pairs along two orthogonal axes. Each of the metal arms attaches to one edge of a square (55 × 55 mm) pre-punched membrane via four metal posts. The motors move the pairs of arms independently to cyclically stretch the membrane at an extent and frequency set by a control unit. One significant advantage of this stretching apparatus is that it can apply both biaxial stretching and strip stretching, with which the membrane is controlled, so it deforms in one direction without lateral deformation (Fig. 1). In all stretching experiments, the cells were plated in the central region (2 cm × 2 cm) of the silicone membrane. In this region, the strains were measured to be  $10.2 \pm 0.6\%$  (mean ± SD) for a 10% biaxial stretching, and  $10.8 \pm 0.8\%$  for a 10% strip stretching. Therefore, under the two types of stretching, the strains were uniformly distributed in the central region, within which cells were subjected to the same membrane deformation, regardless of their location.

**Staining actin filaments.** To examine change in actin cytoskeleton with and without stretching, actin filaments were stained with the following procedures. Briefly, HAECs were quickly washed once with cold PBS, and fixed in 3.7% formaldehyde for 5 min. After fixation, the cells were permeabilized in 0.25% Triton X-100 for 5 min, followed by washing twice with PBS

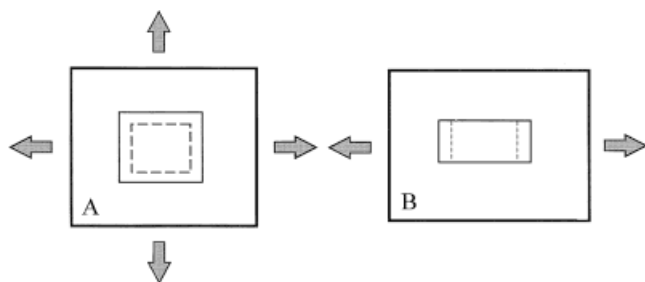


Fig. 1. Schematic of two types of stretching on a silicone membrane: (A) biaxial stretching, and (B) strip stretching. For biaxial stretching, the membrane is deformed in two perpendicular directions; and for the strip stretching, the membrane is deformed in the stretching direction, but not its perpendicular direction. Under the two types of stretching conditions, the strain in the central region of the membrane (about 2 cm  $\times$  2 cm) was  $10.2 \pm 0.6\%$  (mean  $\pm$  SD) and  $10.8 \pm 0.8\%$  for a 10% biaxial stretching and strip stretching, respectively. The endothelial cells were grown in the central region, and therefore were subjected to the same membrane deformation, regardless of their location.

and then incubating with rhodamine phalloidin or fluorescein phalloidin ( $0.165 \mu\text{M}$ ) in phosphate-buffered saline (PBS). The stained cells were viewed on a Zeiss microscope and photographed with Kodak 400 color slide film.

**Examining actin cytoskeleton integrity.** To assess the effect of cyclic stretching on the integrity of actin cytoskeleton, a semiquantitative approach was used. This involved staining the actin filaments, and then on a fluorescence microscope with a  $100\times$  objective, examining actin cytoskeletons of individual cells carefully. Based on gross appearance, the actin cytoskeleton of each observed cell was categorized as intact, partially disrupted, and completely disrupted (i.e., almost no actin filaments were present in the cell). A total of 234–396 cells were randomly selected to avoid possible biases of the cell selection. These cells were pooled together from at least two experiments with no stretching for control, or with 1, 10, and 30 min of both cyclic strip and biaxial stretching.

**Examining the effect of leukotrienes and tyrosine phosphorylation.** To probe the role of leukotrienes and tyrosine phosphorylation in actin cytoskeletal remodeling of the endothelial cells subjected to cyclic stretching, two proven inhibitors, NDGA and tyrphostin A25, were used. First, cells were treated with either tyrphostin A25 ( $200 \mu\text{M}$ ) for 2 h, or with NDGA ( $50 \mu\text{M}$ ) for 0.5 h, or both. Then, cells were cyclically stretched (10% strip stretching, 0.5 Hz) for 3 h.

The concentrations of these two inhibitors used in the stretching experiments represent optimal ones, determined as follows. HAECs were incubated with 50–400  $\mu\text{M}$  tyrphostin A25 in EBM for 2 h, then stimulated for 15 min with  $\text{H}_2\text{O}_2$  ( $100 \mu\text{M}$ ) in the presence of or-

thovanadate ( $0.25 \text{ mM}$ ), a potent inhibitor of tyrosine phosphatase. After fixation and permeabilization, the cells were incubated with a 1:75 dilution of mouse FITC-conjugated anti-phosphotyrosine antibody. Control cells not stimulated with  $\text{H}_2\text{O}_2$ , and unblocked cells not exposed to the tyrphostin but treated with  $\text{H}_2\text{O}_2$  and orthovanadate were similarly stained for phosphotyrosine. The stained cells were washed thoroughly and then scanned for measurement of fluorescence intensity (Millipore CytoFluor 2300). The optimal concentration of tyrphostin A25 was found to be  $200 \mu\text{M}$ , which substantially inhibited tyrosine phosphorylation induced by  $\text{H}_2\text{O}_2$  but did not cause cells to detach, as higher concentration of the tyrphostin did. In addition, Western blot analysis was performed to confirm that  $200 \mu\text{M}$  of tyrphostin A25 inhibited tyrosine phosphorylation substantially (see Fig. 3).

Using methods similar to those above, we found that the optimal concentration of NDGA that inhibited PMA ( $16.2 \text{ nM}$ ) stimulated leukotriene activity was  $50 \mu\text{M}$ . To verify the effect of leukotrienes on the formation of stress fibers, the following experiment was performed. Unstretched cells were first treated with tyrphostin A25 ( $200 \mu\text{M}$ ) and NDGA ( $50 \mu\text{M}$ ) for 3 h and then incubated for another 10 min with leukotriene  $\text{E}_4$  ( $5 \mu\text{M}$ ). Another group of cells was treated similarly but without the addition of the leukotriene. Both groups of cells were then stained with rhodamine phalloidin to examine whether stress fibers were present.

**Statistical analysis.** To compare the effects of various concentrations of the inhibitors (i.e., tyrphostin and NDGA) on leukotriene production and tyrosine phosphorylation, analysis of variance (ANOVA) was used with the Neuman-Keuls test for multiple comparisons. To compare the degrees of actin cytoskeletal disruption between two stretching conditions, the chi-square test was applied. The difference was considered to be significant if  $P < 0.05$ .

## RESULTS

### Disruption of Actin Cytoskeletons in Response to Cyclic Stretching

Fluorescence microscopy was used to examine the response of actin cytoskeleton to cyclic mechanical stretching. After only 1 min of cyclic strip stretching (10%, 0.5 Hz), actin cytoskeletons of the endothelial cells were already disrupted. Further increasing stretching time to 10 min, and then 30 min, led to more severe disruption of the actin cytoskeletons (Fig. 2A–C). After 3 h of stretching, however, the disrupted actin cytoskeleton of the stretched cell was re-formed into dense stress fibers, oriented in the direction perpendicular to the stretching direction (Fig. 2D).

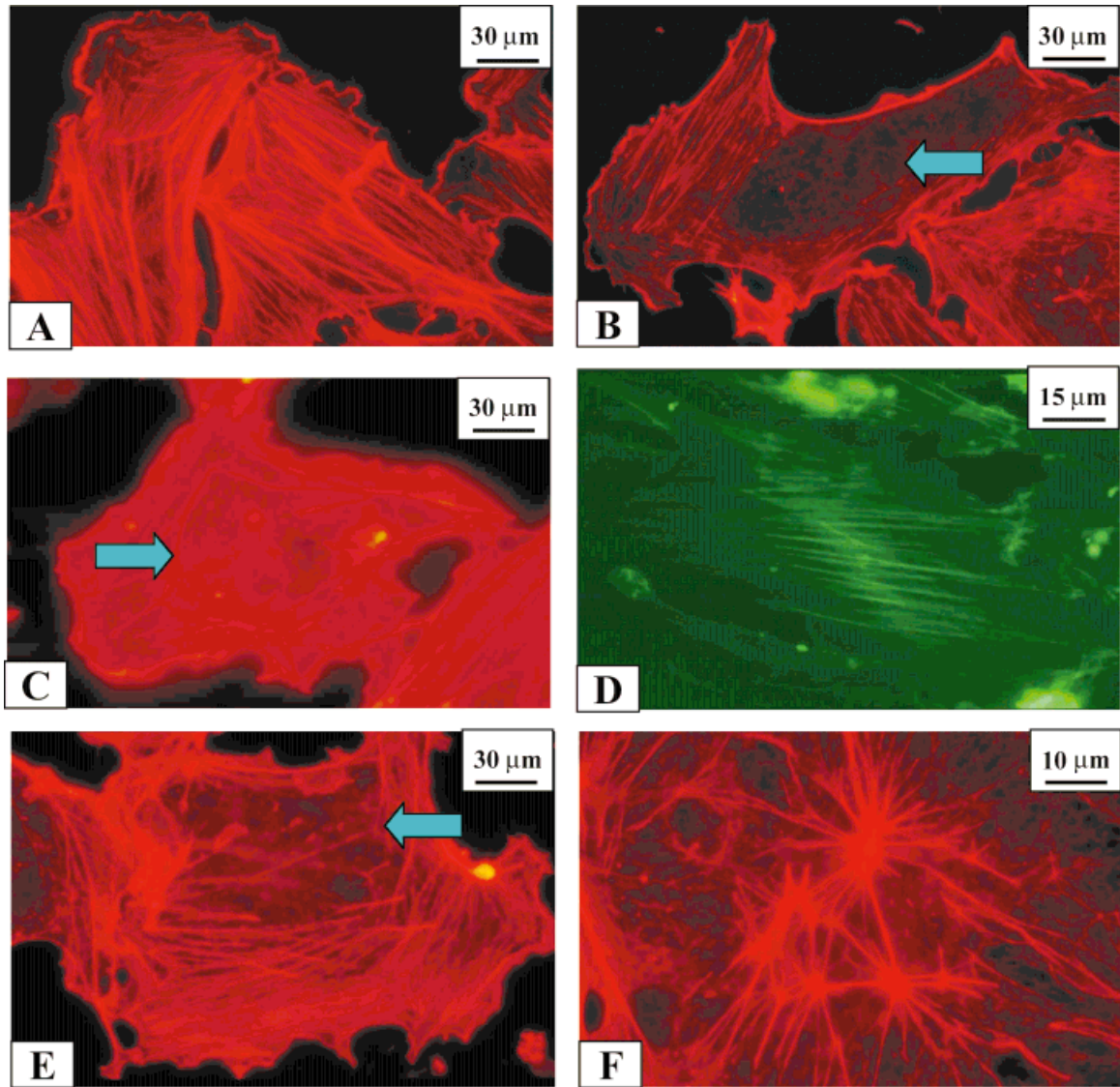


Fig. 2. Representative fluorescent microphotographs of the actin cytoskeletons of the endothelial cells without stretching (A), after 1 min (B) and 10 min (C) of cyclic strip stretching (10%, 0.5 Hz). It can be seen that these short periods of stretching led to disruption of the actin cytoskeleton. However, after 3 h of cyclic stretching, the actin cytoskeleton was remodeled such that bundles of stress fibers were formed in the direction perpendicular to the stretching direction—the

vertical direction (D). Under the condition of cyclic biaxial stretching (10%, 0.5 Hz), 1 min of stretching led to the disruption of the actin cytoskeletons (E). In particular, after 1 h of cyclic biaxial stretching (F), the actin cytoskeletons were remodeled into three-dimensional, “tent-like” actin structures protruding outside the substrate plane, in which the stretching was applied. Arrows indicate areas where actin filaments were disrupted.

Similarly, under cyclic biaxial stretching (10%, 0.5 Hz), actin cytoskeletons of the cells were also disrupted, but the degree of the actin cytoskeleton disruption (ACD) appeared to be more severe than under the strip stretching at the 10-min and 30-min time points. Moreover, after cyclic stretching for a certain time (e.g., 1 h), “tent-like,” three-dimensional actin structures in stretched endothelial cells were observed. Confocal microscopy confirmed that these actin structures protruded outside the substrate plane, within which cyclic biaxial stretching was applied.

To quantify the degree of ACD in response to cyclic stretching, ACD frequencies under both strip and biaxial stretching were determined, and the results are summarized in Table I. Compared with the condition of no stretching, cyclic stretching, both strip and biaxial, caused significant disruption of the actin cytoskeleton at all time points (1, 10, and 30 min;  $P < 0.002$ ). But at 1 min, the two types of stretching did not cause significant difference in the ACD frequency ( $P = 0.21$ ). At 10 min and 30 min, however, biaxial stretching caused signifi-

**TABLE I. Frequency (%) of Actin Cytoskeleton Disruption of the Endothelial Cells in Response to Cyclic Stretching (10%, 0.5 Hz)\***

|                    | Intact (%) | Partially disrupted (%) | Completely disrupted (%) |
|--------------------|------------|-------------------------|--------------------------|
| Without stretching | 73         | 24                      | 3                        |
| Strip (1 min)      | 42         | 57                      | 1                        |
| Biaxial (1 min)    | 35         | 62                      | 3                        |
| Strip (10 min)     | 29         | 67                      | 4                        |
| Biaxial (10 min)   | 5          | 65                      | 30                       |
| Strip (30 min)     | 31         | 66                      | 3                        |
| Biaxial (30 min)   | 1          | 41                      | 58                       |

\*After only 1 min of strip or biaxial stretching, actin cytoskeletons of the stretched cells were already disrupted compared with those of the cells without stretching. With the increase of stretching time from 1 to 10 min, more cells had actin cytoskeletons that were disrupted. Especially, biaxial stretching caused severer actin cytoskeleton disruption than strip stretching at 10 min. When stretching time was increased to 30 min under strip stretching, the extent of actin cytoskeleton disruption, both partial and complete disruption, appeared unchanged, but the extent of actin cytoskeleton disruption was further increased. Statistical analysis indicated that frequencies of the actin cytoskeleton disruption under strip and biaxial stretching from 1 to 10 and 30 min were significantly different from that of the cells without stretching ( $P < 0.001$ ). Furthermore, at 10 and 30 min, frequencies of the actin cytoskeleton disruption under the biaxial stretching were significantly higher than those under the strip stretching. However, at 1 min, there was no significant difference between the two stretching conditions ( $P = 0.21$ ). Note that a total of 234–396 cells from at least two experiments were used to determine the frequencies shown in the table.

cantly higher ACD frequencies than the strip stretching ( $P < 0.0001$ ).

### Effects of Inhibiting Leukotriene Production and Tyrosine Phosphorylation

In the presence of NDGA (50  $\mu\text{M}$ ) or tyrphostin A25 (200  $\mu\text{M}$ ) alone, actin cytoskeletons of the endothelial cells under cyclic strip stretching were still remodeled. Specifically, after stretching for 3 h, dense stress fibers were formed in the direction perpendicular to the stretching direction (Fig. 3A,B). This was true, in spite of the fact that NDGA and tyrphostin A25, at the doses used, substantially inhibited leukotriene production and tyrosine phosphorylation, respectively (Fig. 3C,D).

By contrast, when both NDGA (50  $\mu\text{M}$ ) and tyrphostin A25 (200  $\mu\text{M}$ ) were present during cyclic strip stretching, the remodeling of the actin cytoskeleton was completely blocked. As a result, few, if any, stress fibers were formed in the stretched cells (Fig. 4A). The inhibiting effect by NDGA and tyrphostin A25 was not due to carriers for NDGA and tyrphostin A25 (100% EtOH and DMSO, respectively), as in the presence of these carriers alone, stress fibers were able to form after 3 h of stretch-

ing (data not shown). Furthermore, after addition of leukotriene  $\text{E}_4$  (5  $\mu\text{M}$ ) for 10 min, 54% of 254 cells, which were randomly selected in two experiments, regained dense stress fibers (Fig. 4B), 41% regained many stress fibers, and only 5% were still absent of the stress fibers.

## DISCUSSION

This study has several major findings: (1) actin cytoskeletons of the endothelial cells were rapidly disrupted in response to cyclic stretching; (2) the degree of the disruption in the actin cytoskeleton depended on the type of cyclic stretching applied (i.e., biaxial stretching caused more extensive disruption in the actin cytoskeleton than occurred with strip stretching); (3) under strip stretching, the disrupted actin cytoskeletons were remodeled into stress fibers, with the direction nearly perpendicular to the stretching direction; (4) under biaxial stretching, the disrupted actin cytoskeleton was unable to form the stress fibers (instead, it formed a “tent-like” structure protruding outside the plane within which cyclic stretching was applied); and (5) inhibiting leukotriene production or tyrosine phosphorylation alone did not block the remodeling of the actin cytoskeletons of the endothelial cells subjected to cyclic stretching. Inhibiting both the leukotriene production and tyrosine phosphorylation, however, completely abolished the actin cytoskeletal remodeling.

Although this study showed that cyclic stretching caused disruption of the actin cytoskeleton, the precise mechanism of the disruption is unclear. One possibility is the direct “breaking” of the actin filaments by the applied cyclic stretching. It is now established that actin filaments are linked with focal adhesions via integrins on a cell surface [Ingber, 1991]. Therefore, through the linkage, substrate strains can be transmitted to actin filaments and consequently, the transmitted strains may “break” the filaments. However, there is little evidence for this mechanism. A more likely mechanism is that stretching the cell activates some factors in the cell membrane or cytoplasm, which then lead to breakdown of stress fibers. Reactive oxygen species could play such a role, as there is evidence that cyclic stretching causes release of  $\text{H}_2\text{O}_2$  in the endothelial cells [Howard et al., 1997], and low doses of  $\text{H}_2\text{O}_2$  can remodel actin cytoskeleton rapidly [Liu and Sundqvist, 1995; Zhao and Davis, 1998]. This possible mechanism responsible for the stretching-induced disruption of actin cytoskeleton awaits further studies.

However, whatever the mechanism, the substrate deformation due to applied stretching seems to dictate the re-formation of the actin cytoskeleton. Under strip stretching, in which cells are stretched in the stretching

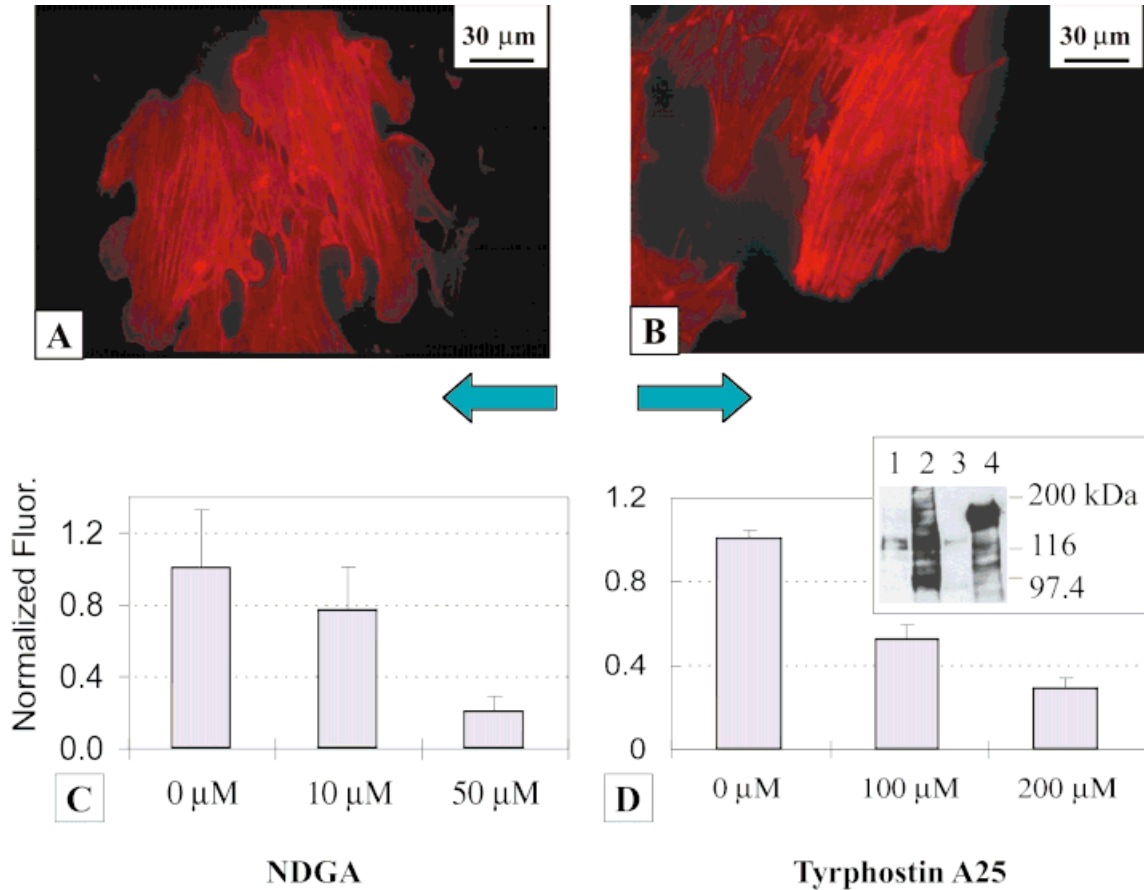


Fig. 3. Representative fluorescent microphotographs of actin cytoskeletons of the endothelial cells. The cells were cyclically stretched (10% strip stretching, 0.5 Hz) in the presence of either 50  $\mu\text{M}$  of nordihydroguaiaretic acid (NDGA) (A), or 200  $\mu\text{M}$  of tyrphostin A25 (B) for 3 h. It is evident that the actin cytoskeletons of the stretched cells were remodeled into stress fibers oriented nearly perpendicular to the stretching direction, despite the fact that NDGA (50  $\mu\text{M}$ ) led to a substantial decrease in leukotriene production (C). Similarly, tyrphostin A25 (200  $\mu\text{M}$ ) markedly decreased tyrosine phosphorylation, as shown by both semiquantitative immunocytochemistry and Western blot analysis (D). Note that for the Western blot, three groups of cells in 6-well plates were used. One group was untreated, and others were

treated with 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  and 0.25  $\mu\text{M}$  sodium orthovanadate in the presence or absence of tyrphostin A25 (200  $\mu\text{M}$ ). The cells were extracted with ice-cold lysis buffer (1% SDS, 1.0 mM sodium orthovanadate, 10  $\mu\text{M}$  Tris, pH 7.4). Equal amounts of protein samples were loaded and run on a 7.5% SDS-PAGE and transferred to nitrocellulose paper (BioRad). Blots were probed with a 1:2,500 dilution of PY20 anti-phosphotyrosine antibody conjugated with horseradish peroxidase (Transduction Laboratories). The blots were visualized with an ECL kit (Amersham) according to the manufacturer's instructions. Note that lane 1 was the treatment with  $\text{H}_2\text{O}_2$  and tyrphostin A25; lane 2 was treated with  $\text{H}_2\text{O}_2$  only; lane 3 lacked  $\text{H}_2\text{O}_2$  treatment; and lane 4 was a positive control.

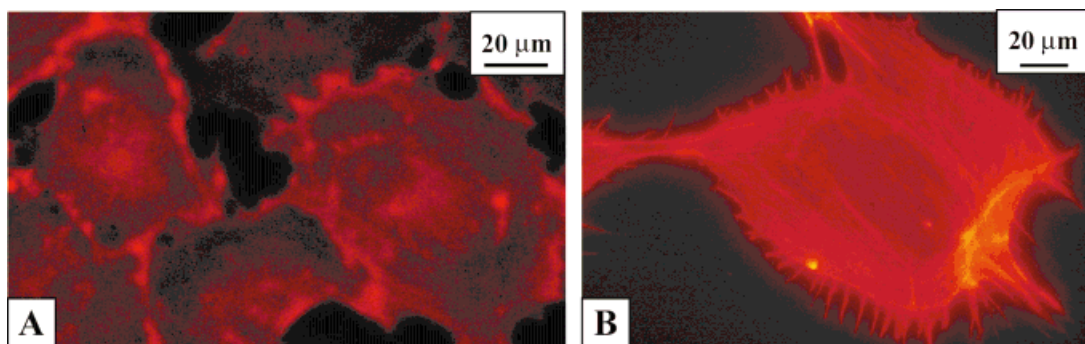


Figure 4

direction, but not in its perpendicular direction (Fig. 1), the actin cytoskeleton was re-formed into stress fibers perpendicular to the stretching direction. Under biaxial stretching, the actin cytoskeleton was re-formed into three-dimensional, "tent-like" structures, which protrude outside the substrate plane. Note that in both these directions, i.e., the perpendicular to the stretching direction and the direction perpendicular to the substrate plane, there are no substrate deformations. Thus, the results suggest that the formation of the actin filaments is directly controlled by substrate deformations: Actin filaments are only able to form in the direction without substrate deformation. In other words, substrate deformation is an unfavorable signal for the cells to form actin filaments [Wang, 2000]. The reason why is unclear from this study, but it does explain why biaxial stretching produced a higher frequency of ACD. This is because under biaxial stretching, cells were equally stretched in every direction in the substrate plane. Under strip stretching, however, cells were not stretched in all directions in the substrate plane. Specifically, they were not stretched in the direction perpendicular to the stretching direction. In addition, with the same magnitude of stretching, say 10%, as in the present study, strip stretching causes smaller total deformation than biaxial stretching [Wang et al., 1995].

It is now established that the actin cytoskeleton plays a crucial role in various cellular functions such as proliferation and differentiation [Ingber, 1991, 1993]. Hence, the pattern of the actin cytoskeleton of a cell may determine the expression of cellular phenotype in response to mechanical stretching. Thus, the stretched induced phenotype expression of cells may depend on the type of mechanical stretching applied, because strip stretching and biaxial stretching produce a different pattern of actin cytoskeleton, as shown in this study.

The formation of stress fibers involves AA metabolism to leukotrienes and the tyrosine phosphorylation of a number of proteins [Peppelenbosch et al., 1995]. Our finding that inhibiting leukotriene production or tyrosine phosphorylation alone did not block the actin cytoskeletal remodeling of the stretched endothelial cells, suggests that like Swiss 3T3 fibroblasts in static cultures, the AA metabolism to leukotriene production and tyrosine

phosphorylation are two independent signaling pathways for formation of stress fibers in the endothelial cells. The fact that inhibiting both leukotriene production and tyrosine phosphorylation blocks the stress fiber response suggests that the leukotrienes and tyrosine phosphorylation are necessary in the actin cytoskeletal remodeling of endothelial cells subjected to cyclic stretching.

Leukotrienes have been found to play an important role in many cellular functions. For example, treatment of leukotriene B<sub>4</sub> enhances the adhesion of the endothelial cells to neutrophils and also increases the adhesion of the neutrophils to culture surfaces [Heimburger and Palmblad, 1996]. In addition, when the production of leukotrienes is enhanced with PMA stimulation, the endothelial cells increase tyrosine phosphorylation of two adhesive proteins, paxillin and pp125FAK [Yuan et al., 1998], which can lead to formation of stress fibers [Craig and Johnson, 1996]. Furthermore, leukotrienes mediate PMA-induced extensive rearrangement of actin filaments and formation of stress fibers in endothelial cells [Zhao and Davis, 1999; Dantew et al., 1993; Palmblad et al., 1994]. On the other hand, when endothelial cells were incubated with NDGA (50  $\mu$ M), an inhibitor of 5-lipoxygenase, which converts AA to leukotrienes through oxidative metabolism [Dantew et al., 1993; Peppelenbosch et al., 1995], the PMA induced adhesion of endothelial cells was significantly inhibited [Dantew et al., 1993]. Taken together, these previous studies indicate that arachidonic acid metabolites, such as leukotrienes, regulate adhesion and actin cytoskeleton of the endothelial cells. Thus, leukotrienes are likely involved in the remodeling of actin cytoskeletons of the stretched endothelial cells, as shown in this study.

A previous study showed that treatment with NDGA abolished the EGF-induced formation of stress fibers [Peppelenbosch et al., 1995]. However, this study showed that incubating cells with NDGA alone decreased leukotriene production substantially but did not affect the formation of stress fibers. The discrepancy between the two studies may be attributable to the use of serum-free medium in the previous study, whereas this study used serum-containing medium. It has been shown that the serum contains LPA, which stimulates cells to form stress fibers through phosphorylation of tyrosine kinases [Craig and Johnson, 1996]. In addition, the present study shows that the addition of the leukotriene to cells whose stress fibers had been disrupted by the presence of NDGA and tyrphostin A25 induced the re-formation of stress fibers. This finding confirmed that leukotrienes are necessary for the formation of stress fibers.

In Swiss 3T3 fibroblasts, the EGF and LPA pathways appear to converge at the level of the small GTP-

Fig. 4. Effect of nordihydroguaiaretic acid (NDGA) and tyrphostin A25 on the stress fiber remodeling of the endothelial cells. The cells were cyclically stretched (10% strip stretching, 0.5 Hz) in the presence of the two inhibitors for 3 h. Few stress fibers were present in these cells (A). Note that in some cells F-actins formed clusters (arrows), which gave strong signals. Furthermore, after leukotriene E<sub>4</sub> (5  $\mu$ M) was added for 10 min, the cells, which had been treated with NDGA (50  $\mu$ M) and tyrphostin A25 (200  $\mu$ M), regained numerous stress fibers (B).

binding protein Rho, which alone can induce the formation of stress fibers and focal adhesions as well [Craig and Johnson, 1996]. So it may be possible that inactivating Rho may sufficiently block the stress fiber remodeling of endothelial cells in response to cyclic stretching. Indeed, a previous study showed that ADP-ribosylation of Rho p21 by C3 transferase decreased the formation of actin filaments in bovine aortic endothelial cells subjected to cyclic stretching [Yano et al., 1996]. In addition,  $Ca^{2+}$  has been shown to be involved in the stretching-induced responses of endothelial cells [Naruse and Sokabe, 1993]. Our results also showed that blocking  $Ca^{2+}$  entry to stretched endothelial cells with gadolinium abolished formation of stress fibers completely (unpublished data).

Tyrphostins are a family of inhibitors that bind competitively to the phosphor acceptor site in tyrosine kinases [Yaish et al., 1988; Gazit et al., 1989]. The tyrphostins are tyrosine kinase specific and do not inhibit PKA, PKC, or other serine/threonine kinases [Levitzki, 1990]. For example, it has been shown that tyrphostin A25 inhibits phosphorylation of the focal adhesion kinase (FAK) and assembly of actin cytoskeleton in Swiss 3T3 cells [Chrzanowska-Wodnicka and Burridge, 1994]. Furthermore, in bovine endothelial cells, tyrphostins have been shown to inhibit the tyrosine phosphorylation of the FAK and paxillin induced by cyclic stretching [Yano et al., 1996]. Our results showed that 200  $\mu$ M of tyrphostin A25 substantially inhibited phosphotyrosine, consistent with the previous studies in that tyrphostins inhibit tyrosine phosphorylation.

In summary, this study showed that the actin cytoskeletal remodeling of human aortic endothelial cells in response to cyclic stretching includes the two steps: (1) the rapid disruption, and (2) the re-formation of actin cytoskeleton. Strip stretching and biaxial stretching produced markedly different patterns in the remodeled actin cytoskeleton. For both stretching conditions, however, the actin filaments were formed in the direction without substrate deformation. Furthermore, the actin cytoskeletal remodeling of the cyclically stretched endothelial cells appeared to involve the AA metabolism to leukotrienes and tyrosine phosphorylation.

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