Mechanical Stretching Stimulates Smooth Muscle Cell Growth, Nuclear Protein Import, and Nuclear Pore Expression through Mitogen-activated Protein Kinase Activation*

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Although it is known that mechanical stretching of cells can induce significant increases in cell growth and shape, the intracellular signaling pathways that induce this response at the level of the cell nucleus is unknown. The transport of molecules from the cell cytoplasm to the nucleoplasm through the nuclear pore is a key pathway through which gene expression can be controlled in some conditions. It is presently unknown if mechanical stimuli can induce changes in nuclear pore expression and/or function. The purpose of the present investigation was to determine if mechanical stretching of a cell will alter nuclear protein import and the mechanisms that may be responsible. Vascular smooth muscle cells that were mechanically stretched exhibited an increase in proliferating cell nuclear antigen expression, cell number, and cell size within 24–48 h. Cells were microinjected with marker proteins for nuclear import. Nuclear protein import was significantly stimulated in stretched cells when compared with control. This was associated with an increase in the expression of nuclear pore proteins as detected by Western blots. Inhibition of the MAPK pathway blocked the stretch-induced stimulation of both cell proliferation and nuclear protein import. We conclude that nuclear protein import and nuclear pore density can adapt to mechanical stimuli during the process of cell growth through a MAPK-mediated mechanism.

Vascular smooth muscle cells (VSMCs)3 within the vessel wall are continuously exposed to mechanical cyclic strain due to changes in blood flow and pressure. Under normal conditions, the vessel wall is subjected to hemodynamic forces that can stretch the large arteries up to 10% (1–3). During conditions, the vessel wall is subjected to hemodynamic forces that can stretch the large arteries up to 10% (1–3). During conditions that can increase to 30% (2, 4–6). This stretch directly leads to the growth of VSMCs (2, 7–10). Cell growth in the form of hyperplasia and/or cell hypertrophy is an active process that requires cells to enter the cell cycle through the induction of cell cycle regulatory proteins (11–14). The mechanism through which mechanical stretch induces cell growth is important to understand the development of diseases such as hypertension, atherosclerosis, and cancer (2, 4, 15). Many cell surface factors have been identified to be involved, including platelet-derived growth factor receptors (4, 5, 16) and endothelin receptors (16). However, this represents only a portion of the cellular pathway to induce growth. It is also possible that the nucleus itself may be a target of mechanical stimuli. The nuclear pore complex (NPC) present on the surface of the nuclear membrane mediates the transport of transcription factors, cell cycle proteins, and signaling proteins into the nucleus to induce cell differentiation, transformation, and proliferation (17–19). Clearly, gene expression and transcription in any cell changes during a growth phase. However, it is still unknown if these changes occur through adaptive changes in nuclear transport in response to physiological and pathological stimuli. Any change in nuclear protein import may take place in concert with or independently of a change in NPC density. It is known that general changes in cell metabolism may influence NPC density (20), but beyond that, little is known. There are no data available to determine if mechanical stretch can induce alterations in nuclear protein import or NPC expression and what specific factors, if any, are involved in the changes. The purpose of this study, therefore, was to examine if the effects of mechanical strain can cause alterations in nuclear pore density and nuclear protein import in vascular smooth muscle cells and the mechanisms involved in these changes.

MATERIALS AND METHODS

Antibodies and Reagents—The following antibodies were used: proliferating cell nuclear antigen and smooth muscle α-actin (Sigma), signaling antibodies p38, phospho-p38, p44/p42, and phospho-p44/p42 (Cell Signaling, Beverly, MA), nuclear pore protein antibodies mAb414 and NUP 153 (BabCO, Richmond, VA), and loading control glyceraldehyde-3-phosphate dehydrogenase (Abcam, Cambridge, UK). Secondary antibodies used were anti-mouse IgG (Chemicon, Temecula, CA) and Alexa488 anti-mouse IgG (Molecular Probes/Invitrogen). Other reagents purchased included PD-98059 and SB-202190 (Calbiochem) calcein AM and...
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Alexa<sub>488</sub> BSA conjugate (BODIPY-FL conjugate-BSA) (Molecular Probes/Invitrogen).

Cell Culture—Smooth muscle cells were isolated from the thoracic aorta of New Zealand White rabbits, as described previously (21). Briefly, aortic rings were placed in a culture plate containing DMEM, 20% fetal bovine serum, and 10% fungizone. The rings were incubated for several days then transferred to a new plate to allow the migration of smooth muscle cells (SMCs). The aortic tissue was then removed to allow the cells to grow to confluency (DMEM plus 5% fetal bovine serum plus 2% fungizone). Cells were trypsinized and seeded at a density of 40,000 cells per well in 6-well bioflex plates coated with Type 1 collagen. Cells were left to attach to the membranes for 24 h and maintained in starvation media (DMEM, 5 μg/ml holo-transferrin, 1 mm sodium selenite, 200 μM ascorbate, 10 mm insulin, 2.5 μM sodium pyruvate, and 2% fungizone) for 3 days prior to stretch.

Mechanical Stretch—Mechanical stimulation was performed using a Flexcell 4000 cell stretch vacuum system. VSMCs plated on bioflex plates were supplemented with media (DMEM plus 5% fetal bovine serum plus 2% fungizone). Stoppers were inserted under the membranes of non-stretched controls. Pulsatile stretch was performed using a frequency of 1 Hz and an elongation of 20% for 24 or 48 h.

Microinjection and Measurement of Nuclear Protein Import by Confocal Microscopy—Thin walled glass capillary tubes (1.0 mm, 3 inch) were used to fashion micropipettes for cell injection. The capillary tubes were made using a Flaming/Brown micropipette puller (Sutter Instruments, Model p-97). After stretch, the bioflex membranes containing the cells were carefully removed with a scalpel and placed in a Leyden dish containing 1 ml of pre-warmed perfusate buffer (6 mM KCl, 1 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 10 mM dextrose, 6 mM HEPES, pH 7.4). Cells were maintained at 37 °C using a microperfusion chamber. An Alexa-BSA NLS fluorescent substrate was prepared in our laboratory by conjugating BODIPY-BSA to a SV40 large T antigen NLS (CGGGPKKKRKVED) (21, 22). The substrate was added to a micropipette, which was then inserted into the cell cytoplasm using an MS314 micromanipulator (Fine Science Tools). Cells were injected using a PV830 Pneumatic PicoPump (World Precision Instruments) and the pipette was slowly removed. In control experiments, cells remained viable for >10 h. Images of the cell after microinjection were acquired on a Bio-Rad MRC600 CLSM. Images were taken of the pre- and post-injected cells to observe the rate of nuclear import of the Alexa-BSA NLS fluorescent protein for each cell over time. There is no movement of the fluorescent marker into the cell nucleus when it does not contain an NLS. As discussed previously (21, 23), the use of this marker protein is advantageous because, instead of measuring the import of one specific protein, it identifies regulatory factors and pathways applicable to the nuclear import of all proteins. Final images were analyzed and processed on an SGI workstation using the Molecular Dynamics Imagespace image analysis software.

Immunoblotting—Protein samples were prepared using 30 μg of protein in 2× Sample Buffer (0.15 M Tris, pH 6.8, 1.2% SDS, 30% glycerol, 15% β-mercaptoethanol, 1.8% bromphenol blue) and separated on SDS-polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes and placed in blocking buffer (10% skim milk powder, 0.05% Tween 20 in Tris-buffered saline) overnight at 4 °C, followed by incubation with the 1° antibody in Tris-buffered saline, 1% skim milk, and 0.05% Tween 20. The membrane was then incubated for 1 h in a solution containing 1% skim milk powder, 0.05% Tween 20, and the 2° antibody (i.e. a horseradish peroxidase-conjugated goat anti-mouse Ig). The membrane was then visualized with the ECL detection system (Pierce), and images were created using a Fluores-max instrument and quantified using Quantity One imaging software.

Immunocytochemistry—For the detection of mAb414, SM α-actin, and NF-κB, cells grown on Flex membranes were fixed with 3.7% paraformaldehyde for 15 min and permeabilized with 0.1% Triton-X. Cells were washed and incubated with blocking buffer (10% skim milk powder, 1× Tris-buffered saline) overnight at 4 °C followed by incubation with the 1° antibody in Tris-buffered saline and 1% skim milk overnight at 4 °C. Cells were then washed and incubated with 1× Tris-buffered saline and the 2° antibody (i.e. Alexa 488-conjugated goat anti-mouse Ig) for 1 h in the dark. The cells were rinsed, treated with Hoechst for 5 min, and inverted on a microscope slide using 10 μl of Fluorosave reagent. Images were collected on a Zeiss fluorescent microscope using a 63× oil immersion objective. Total fluorescence was measured to determine any differences in nuclear pore density and nuclear area was calculated by staining DNA with Hoechst. Each experiment consisted of averaging data from ~75–100 cells for both stretched and non-stretched groups.

Mechanical Stretch of VSMCs Stimulates Cell Growth—After stretching, control and stretched cell groups were microinjected with specific MAPK inhibitors (i.e. SB202190 and PD98059). Micropipettes contained the Alexa-BSA NLS substrate and either 1 μM SB-202190 or 1 μM PD98059, and injected into VSMCs to measure nuclear protein import rate. Control and stretched cell groups were also treated with media (DMEM supplemented with 5% fetal bovine serum and 2% fungizone) containing 1 μM PD98059 during the 48-h stretching regimen. Cell lysates were collected, and Western blots were analyzed to determine changes in nuclear pore protein expression as well as the phosphorylation status of phospho-p42.

Statistical Analysis—Variation between means was determined using one-way analysis of variance followed by Duncan’s Posthoc test and significance was set at p < 0.05.

RESULTS

Mechanical Stretch of VSMCs Stimulates Cell Growth—VSMCs were mechanically stretched to stimulate cell growth. After 48 h of stretch, the cells exhibited a significant 5-fold increase in proliferating cell nuclear antigen expression when compared with non-stretched control cells (Fig. 1A). This would suggest that the stretched cells were actively entering the cell cycle. Cell counts confirmed that the cells were completing the cell cycle. There was a significant increase in cell number in a time-dependent manner as a function of the stretch stimulus (Fig. 1B). Both of these effects were blocked in the presence of a MAPK inhibitor PD98059 (Fig. 1, A and B).

Cell hypertrophy is another type of cell growth that may be stimulated by cell stretching. Cell hypertrophy was determined using the cytoplasmic probe calcein AM to measure total cell...
volume. Cells stimulated by stretching exhibited a significant increase in cell size compared with the non-stretched control group (Fig. 1C). This too was blocked in the presence of 1 μM PD98059. These results demonstrate that mechanical stretching is capable of inducing both hyperplasia and hypertrophy in the SMCs and that this appears to occur in an MAPK-dependent manner.

Involvement of the MAPK Pathway in the Stretch-induced Stimulation of Cell Growth—Using antibodies against the phosphorylated form of p44/42, Western blots were performed to further identify the activation of this pathway. Stretched cell lysates exhibited a significant increase in the levels of phospho-p44/42 when compared with the non-stretched control groups (Fig. 2). This was reversed after treatment with 1 μM PD98059 during stretching (Fig. 2). PD98059 induced no effect when stretching was not present.

Effects of Mechanical Stretching on Nuclear Protein Import—VSMCs were subjected to stretch and microinjected with Alexa488-BSA NLS-conjugated fluorescent substrate to visualize nuclear import rate in cells over time. Nuclear protein import increased in cells after stretching in a time-dependent manner (Fig. 3). We examined the maximal rate of import during the initial period of transport. For the first 10 min after injection, import occurred in a linear fashion ($R^2$ value = 0.98, 0.98, and 0.97 in control cells or cells after 24 or 48 h of stretch, respectively). Cells stretched for 48 h had the largest increase in nuclear import; therefore, subsequent experiments were focused on this time point.

Involvement of the MAPK Pathway in the Stretch-induced Stimulation of Nuclear Protein Import—The mechanisms mediating the stretch-induced effects on nuclear import were unknown. The data in Figs. 1 and 2 would suggest that the MAPK pathway may be important in mediating the effects of stretching on cell growth. In addition, previous work in our...
laboratory has identified the MAPKs as an important regulatory pathway in nuclear protein import (21, 24, 25). Therefore, MAPK inhibitors were again used to determine the role of the MAPK pathway as a potential mechanism for the stretch-induced effects on nuclear protein import. Both the p38 and MEK inhibitors SB202190 and PD98059, respectively, were able to reverse the effects of stretching on nuclear protein import (Fig. 4). The changes in nuclear fluorescence were apparent whether the measurements were expressed as total fluorescence or as nuclear/cytosolic fluorescence. There was also a small decrease in fluorescent intensity with the use of PD98059 below control values. These data would suggest that there may be a modest activation of the MAPK pathway in control cells as they adhere and “mildly stretch” onto the surface of the culture plate. It is important to note, however, that this is not a statistically significant decrease below control values. PD98059 is an MEK inhibitor. We also used the more selective p38 inhibitor SB202190 to determine if it could reverse the effects of stretch on nuclear protein import (Fig. 4, C and D). Its effects were similar to those of PD98059.

Effects of Stretch on Nuclear Pore Density—Nuclear protein import may be altered through a direct increase in the number of nuclear pore channels. Immunocytochemistry revealed a significant increase in nuclear pore protein (as detected by mAb414 fluorescence) in stretched cells when compared with non-stretched controls (Fig. 5). This was evident if the fluorescence was plotted as absolute values or as a percentage of control. To confirm and quantitate this, cell lysates were collected from stretched and control groups and run on SDS-PAGE gels. Western blots were probed with antibodies against two different nuclear pore proteins using mAb414 and NUP153. Both p62 protein expression (using the mAb414 antibody) (Fig. 6A) and NUP153 (Fig. 6B) were significantly increased in stretched cells when compared with control. The presence of PD98059 blocked these effects.

The Influence of Nuclear Size on Nuclear Pore Density—As shown in Fig. 1, stretching induced an increase in cell size. It is possible that this increase in cell size may have also induced an increase in nuclear size as well. If so, it is also possible that the increase in nuclear pore density was simply a reflection of the greater size of the nuclei. To determine if this was true, nuclear volume was measured by confocal microscopy and then this was plotted as a function of nuclear pore expression as indicated by p62 expression. As shown in Fig. 7, nuclear size was significantly increased in the cells after 48 h of stretch. However, the increase in p62 expression was still significantly ele-
vated in comparison to control even when expressed as a ratio to nuclear size.

**DISCUSSION**

Hypertension can increase overall cyclic mechanical strain on the arterial wall in the range of 15–30% (1, 4), leading to the pulsatile stretch of vascular SMCs within the medial layer of the vascular wall (1, 4). Cell growth is a hallmark response in adaptive remodeling of vascular arteries in response to hypertension (2, 10, 26). VSMCs within the arterial wall are constantly exposed to a variety of flow-related mechanical stimuli (27, 28). These mechanical stimuli such as intermittent flow, pressure, turbulent flow, and stretching can induce significant changes in cell growth and alter cell shape (1, 29–32). The pathophysiological mechanism underlying these changes is not well understood and remains to be determined. It has been suggested that VSMCs in the vessel wall can adapt in response to the mechanical forces induced by hypertension, and that mechanical stress may be involved in the progression of arteriosclerosis and medial vascular hypertrophy (5).

In the present study, we used a physiological pulsatile stretch of 20% elongation at a frequency of 1 Hz (60 cycles/min) (33, 34). This amount of stretch was adequate to induce the cells to

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**FIGURE 4.** Treatment of VSMCs with the specific MAPK inhibitors PD98059 (A and B) and SB202190 (C) during the stretching regimen induced a significant down-regulation of nuclear protein import after 48 h of stretching. (*, \( p < 0.05 \) versus control cells that were not exposed to stretch stimulus, \( n = 4 \) independent experiments).
actively enter and complete the cell cycle. This stretching stimulus not only induced cell hyperplasia but also caused cellular hypertrophy. Our results are consistent with other studies that have shown this as well (29–32).

Despite considerable research effort (35, 36), the exact sequence of events within the cell that leads to vascular cell growth caused by stretching is not fully elucidated. To investigate the underlying intracellular mechanisms that lead to the increase in cell growth, we monitored the rate of nuclear protein import in VSMCs. Nuclear protein import can be an important regulatory step in gene expression (21, 37). For example, Perez-Terez et al. (38) reported that a depletion in Ca\textsuperscript{2+} or ATP/GTP in cardiac cells resulted in a closed state within the NPC and an inhibition of nuclear protein import. Maul et al. (39) reported that the prevention of ATP synthesis also leads to the inhibition of nuclear pore formation. Disturbances in ion channel homeostasis are common during cardiac hypertrophy leading to the depletion of Ca\textsuperscript{2+} stores that can directly result in a higher incidence of ischemic heart disease and heart failure (38). Perez-Terez et al. (38) concluded that the inhibition in nuclear protein import directly contributes to the progression of disease. However, the import of proteins into the nucleus is but one step in the process of gene expression and may or may not be changed by a physiological stimulus like mechanical stretching. Although it is clear that complete blockade of import will have drastic changes on cell viability and growth, the effects of more subtle changes as may occur during pathological conditions are unknown. Surprisingly little is known about the plasticity of nuclear protein import in cells in situ. Therefore, it was important to study whether mechanical stretching of VSMCs leads to a direct increase in the rate of nuclear protein import.

In our study, mechanical stretching of VSMCs resulted in an increased rate of nuclear protein import. Thus, nuclear protein import may represent an important regulatory point in the control of cell growth in the vasculature during mechanical stretching.

The mechanism responsible for this adaptive response in nuclear protein import requires further study. One potential mechanism for the increased rate of nuclear protein import may be due to a direct increase in the number of nuclear pores present on the nuclear envelope. Mechanical stretching did
cause an increase in nuclear size but the increase in pore expression was significantly greater than the increase in nuclear size, thus suggesting that pore density increased as well. The increase in NPC expression was shown by both immunocytochemical analyses and Western blots. This finding is consistent with recent data that have shown that cells contain more NPCs when the nucleus is more active in transcription (20, 39–42).

NPCs have been described as both mobile and dynamically adaptable structures, especially under metabolic and hypertrophic stress (38, 43, 44). Studies using green fluorescent protein-tagged nucleoporins have revealed free moving NPCs within the nuclear envelope as well as the formation of NPC clusters (45). Our data argue strongly that mechanical stretching can also induce a significant increase in nuclear pore number. Therefore, we can conclude that an increase in nuclear pore number may be at least one of the underlying mechanisms for the increase in the rate of nuclear protein import.

It was hypothesized that an intracellular signaling mechanism may also be involved in the increase in nuclear protein import, NPC expression, and vascular cell growth. MAPKs are a group of signal transduction pathways (6) that may play a role in the progression of vascular diseases such as hypertension and ischemic heart disease (5, 46). Previous studies have shown that MAPKs are rapidly activated in response to elevated levels of mechanical stretching (2, 5, 6, 46). Our results as well as others (4) show a significant increase in the phosphorylated state of p44/42 (ERK2) due to mechanical stretching, and the effects of stretching on cell growth were blocked by inhibitors of the MAPK pathway. Previous work in our laboratory using permeabilized cell assays and microinjection techniques has shown that ERK2 has the capacity to increase nuclear protein import (24, 25). It was reasonable, therefore, to assess the MAPK pathway as a mechanism for the stretch-induced changes in nuclear protein import. The ERK and p38 MAPK inhibitors, PD98059 and SB202190, respectively, blocked the stimulatory effects of mechanical stretching on nuclear protein import.

![Figure 6](image6.png)

**Figure 6.** Nuclear pore protein expression increased in cells stimulated by stretch. Western blots probed with two monoclonal nuclear pore protein antibodies and mAb414 for p62 (A) and NUP153 (B) revealed an increase in nuclear pore protein expression. Stretched cells exhibited a significant increase in p62 expression, which was blocked in both cases by the treatment with PD98059 during stretch (B). (*, p < 0.05 versus control, n = 3–5 independent experiments).

![Figure 7](image7.png)

**Figure 7.** Nuclear size is increased by cell stretching, but this does not explain the increase in nuclear pore expression. Nuclear size was significantly increased by cell stretching. Expression of p62 was significantly increased in stretched cells despite the increase in nuclear size in comparison to control cells. (*, p < 0.05 versus control, n = 3–5 independent experiments).
import and cell proliferation (24, 25, 47, 48). It may be concluded from these results that the MAPK pathway plays an active role in the stretch-induced increase in nuclear protein import and cell proliferation and that the two processes are closely integrated in response to mechanical stretching. In addition, although it was not statistically significant, the dramatic down-regulation in NPC expression induced by PD98059 would suggest that it may have a role to play in this process as well.

In summary, these results suggest that mechanical stretching induces a high level of activation of the MAPK pathway and this is directly involved in the up-regulation of nuclear protein import and NPC expression. The increased import of specific proteins into the nucleus (i.e. transcription factors) could directly lead to the activation of genes that cause cell proliferation and hypertrophy. If uncontrolled, this could lead to the progression of proliferative diseases like hypertension, atherosclerosis, and cancer (9). These results, therefore, may have additional significance to identify targets for the treatment of cellular hypertrophy and hyperplasia.

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