Role of Heat Shock Protein 70 in Induction of Stress Fiber Formation in Rat Arterial Endothelial Cells in Response to Stretch Stress

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We investigated the mechanism by which endothelial cells (ECs) resist various forms of physical stress using an experimental system consisting of rat arterial EC sheets. Formation of actin stress fibers (SFs) and expression of endothelial heat-shock stress proteins (HSPs) in response to mechanical stretch stress were assessed by immunofluorescence microscopy. Stretch stimulation increased expression of HSPs 25 and 70, but not that of HSP 90. Treatment with SB203580, a p38 MAP kinase inhibitor that acts upstream of the HSP 25 activation cascade, or with geldanamycin, an inhibitor of HSP 90, had no effect on the SF formation response to mechanical stretch stress. In contrast, treatment with quercetin, an HSP 70 inhibitor, inhibited both upregulation of endothelial HSP 70 and formation of SFs in response to tensile stress. In addition, treatment of stretched ECs with cytochalasin D, which disrupts SF formation, did not adversely affect stretch-induced upregulation of endothelial HSP 70. Our data suggest that endothelial HSP 70 plays an important role in inducing SF formation in response to tensile stress.

Key words: heat-shock protein 70, histochemistry, mechanical stretch stress, rat arterial endothelial cells, stress fibers

I. Introduction

Vascular wall cells are continually exposed to various forms of mechanical stress. These forms of stress include the stretch stress accompanying growth or muscular motion, cyclic pressure surges and circumferential stretch stress due to blood pressure and pulsation, and wall shear stress from blood flow. At physiological ranges of intensity, these forms of stress may act as modulators that maintain homeostasis within the vessel wall. For example, mechanical stress, such as shear and stretch stress, regulates the production of several vasoactive mediators, including nitric oxide, prosta-cyclin, endothelin, and thromboxane A₂, by the endothelial cells (ECs) in the vessel wall [4, 11]. However, when the intensity of these stimuli exceeds the normal range, or when the biological activity of the ECs themselves is reduced for some reason, cardiovascular diseases such as atherosclerosis and hypertension may arise [29].

One of the most intensively investigated morphological responses of ECs to mechanical stress is the development of actin stress fibers (SFs). SFs are composed primarily of actin filaments running through the basal cytoplasm of the cell, and are bound at both ends to the membrane protein integrin via adhesion proteins [2]. The integrins also bind specifically to extracellular matrix components such as fibronectin and thus strengthen cellular adhesion to the extracellular matrix [8]. Since ECs respond to high shear stress loading [7, 19] or stretching [6, 15, 30] by forming SFs, SFs are believed to play a fundamental role in maintaining cellular or epithelial integrity.

In addition to SF proteins, ECs also produce heat shock proteins (HSPs, stress proteins) in response to stress. Many kinds of stimuli, including high temperature, oxygen radicals, cytokines, and high shear stress, have been reported to
induce expression of HSPs [9, 42]. The HSPs are divided into four major families, according to their molecular weight; these families include the 90-kDa (HSP 90), 70-kDa (HSP 70), and 60-kDa (HSP 60) families, as well as the small HSP family, which contains HSPs of 15–42 kDa [41]. HSPs serve as molecular chaperones that interact with other cellular proteins to assist in their assembly, disassembly, stabilization, or transport [5, 12]. Some HSPs are believed to associate intimately with the cytoskeleton. For example, HSP 27 and αB-crystallin cooperate to suppress the production of aggregates of denatured intermediate filaments [27], and HSP 90 functions in microtubule dynamics by inhibiting tubulin polymerization [10]. In addition, some HSPs, in particular some members of the small HSP family, also influence actin filament dynamics [21].

Most of the data indicating that HSPs modulate actin dynamics were obtained in experiments using cultured cells in vivo rather than vascular ECs in situ. In the present study, we therefore investigated the mechanism by which ECs resist mechanical stress using an ex vivo system. Using ECs taken from various vascular segments excised from the rat arterial tree, we analyzed the relationship between stretch-induced SF formation and stretch-induced expression of HSPs 25, 70, and 90. The results of this study provide additional insight into the functional roles of endothelial HSPs in normal physiology and in the pathogenesis of vascular disorders.

II. Materials and Methods

Animals

Procedures involving animals and their care were conducted according to the Guide for the Care and Use of Laboratory Animals of Nippon Medical School, Japan. Virgin Wistar-Imamichi rats aged 9–12 weeks were purchased from the Institute for Animal Reproduction (Ibaragi, Japan). The rats were housed and bred under a 12-hr/12-hr light-dark cycle, and were provided with food and water ad libitum.

Stretch-stress procedure, EC sheet preparation, and analysis of SF formation

Stretch treatment and EC sheet preparation were performed as described previously [35]. Briefly, rats were perfused with saline solution containing 0.1% heparin and then with L15 incubation medium (Gibco, BRL Technologies, NY, USA) containing 10% calf serum (Gibco). Vessel segments from the straight portions of specific rat arteries were excised and placed in ice-cold L15 incubation medium containing 10% calf serum. These segments, which were selected based on our preliminary studies of SF formation along the arterial tree, were from the proximal part of the thoracic aorta (Th-p) and the middle parts of the abdominal aorta (Ab), the common iliac artery (Ci), and the common carotid artery (Cc). The excised vessel segments were cut open lengthwise and then pinned flat onto a black silicone rubber plate using tiny steel pins (Iken Kogyo Ltd., Kanagawa, Japan).

Mechanical stretch stress was applied in the direction of the vessel long axis at 5–30% amplitude for 1 hr at 37°C. Stretch amplitude was based on their original length in vivo, since shrinkage in each of the excised segments was different, varying from 19% in Th-p to 30% in Ab. Some vessel segments were incubated without stretching for 1 hr at 37°C and served as control samples. The specimens were subsequently fixed in cold 95% ethanol. Both the tunica externa and most of the tunica media were removed using fine-tipped forceps. The resulting arterial EC sheets were then transferred onto 4% gelatin-coated slides with the endothelial side facing downward. The slides containing the EC sheets were subsequently refixed with 3% paraformaldehyde for 15 min at 4°C to strengthen binding of the EC sheet to the gelatin layer. EC sheets were stained with 0.16 μM rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 20 min at 25°C to visualize actin SFs [31].

SF-positive ECs were quantified using fluorescence microscopy as described previously [35]. ECs with three or more SFs that were longer than ~4 μm and crossed the short axis of the cell were counted as SF positive. Measurements were carried out on 500–1500 cells per EC sheet using 8–16 sheet samples for each experimental condition.

Immunofluorescence analysis of the effect of mechanical stretch stress on HSP expression

Mechanical stretch stress was applied in the direction of the long axis of the excised vessels at 5–30% amplitude for 1 hr at 37°C as described above. After stretching, the specimens were fixed with 3% paraformaldehyde for 15 hr at 4°C, permeabilized with acetone for 20 min at 4°C, and then incubated with 3% skim milk for 1 hr at 4°C to block non-specific immunoreactions. Subsequently, they were incubated with a mouse monoclonal antibody against inducible HSP 70 (5 μg/ml; Medical Biological Laboratory, Nagoya, Japan) or against HSP 90 (1:200 dilution; Affinity Bioreagents, Golden, CO, USA), or with a rabbit polyclonal antibody against HSP 25 (1:500 dilution; StressGen Biotechnologies Corp., Victoria, Canada) for 15 hr at 4°C. Finally, they were incubated with fluorescein isothiocyanate-conjugated secondary antibodies (Sigma, St. Louis, MO, USA) for 6 hr at 25°C. Immunohistochemical control samples received the same treatment, except that the primary antibody was either omitted or replaced with non-immune IgG. Eight to sixteen EC sheets were examined for each experimental condition.

To examine HSP expression in arterial ECs in vivo, some rats were fixed by perfusion with 3% paraformaldehyde. The vessel segments of interest were then excised, cut open lengthwise, and immersed in the same fixative for 1 hr at 4°C. They were then immunostained in the same manner as described above, and EC sheets were prepared as described above. Eight to sixteen EC sheets were examined for each experimental condition.

Immunofluorescence images of the EC sheets were collected with an Olympus BX60F5 microscope and captured with an Argus-20 image processor (Hamamatsu Photonics,
Hamamatsu, Japan). The fluorescence intensity in a given area was measured using Adobe Photoshop V.3.0J or NIH Image V.1.6 to determine the level of HSP expression.

**Effects of inhibitors of HSP expression or SF formation**

We next examined the effects of HSP and actin filament inhibitors on HSP expression and SF formation in arterial ECs *ex vivo*. The inhibitors were SB-203580 (1–50 µM; Calbiochem, San Diego, CA, USA), an inhibitor of p38 MAP kinase [17]; geldanamycin (5 µg/ml; Sigma), an inhibitor of HSP 90 [23]; quercetin (1–100 µM; Extrasynthese, Genay, France), an inhibitor of HSP 70 [13, 37, 39]; or cytochalasin D (2 µg/ml; Sigma), an actin filament disruptor. After the vessel segments were exposed to the inhibitors, they were stretched, fixed, and immunostained in the same manner as described above. Since dimethyl sulfoxide (DMSO) was used as the inhibitor solvent, some arterial ECs were incubated with DMSO only as a vehicle control. Immunofluorescence images of the EC sheets were collected as described above.

The effects of the various HSP inhibitors on SF formation in the ECs in response to stretch stress were quantified using fluorescence microscopy as described above. Measurements were carried out on 500–1500 cells per EC sheet using 8–16 sheet samples for each experimental condition. The effects of the various inhibitors described above in the ECs in response to stretch stress were also evaluated by calculating the ratio of the integrated fluorescence intensity of HSP expression in stretched ECs with inhibitors to that in stretched ECs without inhibitors (vehicle control). The fluorescence intensity was measured as described above. Eight to sixteen EC sheets were examined for each experimental condition.

For quantitative analysis in this study, the means and standard deviations for the parameters examined were calculated. Statistical analysis was also performed using Fisher’s PLSD method, and differences were considered statistically significant at p values <0.01.

**III. Results**

**Formation of stress fibers in ECs in response to stretch stress**

Under non-stretch-stress conditions, fewer than 25% of the ECs in all the straight portions of the rat arteries examined were SF positive (Figs. 1 and 2). In the middle parts of the abdominal aorta and common iliac artery, formation of long SFs in response to stretch stress applied at more than ~20% amplitude was clearly evident (data for 30% ampli-
tude shown in Figs. 1 and 2; other amplitude data not shown). Most of SFs were aligned parallel to the direction of stretching (Fig. 1). In contrast, in the proximal part of the thoracic aorta and the common carotid artery, stretching induced only a slight increase in SFs under the same conditions (Fig. 2). For the remainder of this report, all of the data for stretch-stressed ECs were from arterial segments subjected to application of mechanical stretch stress for 30% amplitude for 1 hr.

The quantitative results for SF-positive ECs before and after exposure to stretch stress at 30% amplitude for 1 hr are summarized in Figure 2. The frequency of SF-positive ECs before and after application of stretch stress increased by 3.67- and 4.60-fold for cells from the middle part of the abdominal aorta and the common iliac artery, respectively. On the other hand, in the proximal part of the thoracic aorta and the common carotid artery, the frequency of SF-positive ECs increased by only 1.46- and 1.28-fold, respectively.

Expression of HSPs in response to stretch stress
Each of the endothelial HSPs examined (HSPs 25, 70, and 90) was constitutively expressed in arterial ECs in vivo at a constant level for all four anatomical locations examined (Fig. 3). The effects of stretching on expression of HSPs 25 and 70 followed one of two patterns, depending on anatomical location. In ECs from the middle parts of the abdominal aorta and the common iliac artery, expression of HSPs 25 and 70 markedly increased after stretch stimulation (Figs. 4 and 5), and the increase was greater for HSP 70 than for HSP 25. In contrast, stretching of ECs from the proximal part of the thoracic aorta and the common carotid artery caused little change in the level of expression of HSPs 25 and 70, which remained the same or slightly decreased (Figs. 4 and 5). Notably, the second group also exhibited poor formation of SFs in response to mechanical stretch stress (see Fig. 2). Endothelial HSP 90 was not upregulated by stretch stress in any of the arteries examined (Fig. 5).

Relationship between stretched-induced formation of SFs and HSP expression
The relationship between SF formation and HSP expression was investigated by treating the arterial ECs with one of the following inhibitors before stretching: SB203580 (1–50 µM), a p38 MAP kinase inhibitor that acts upstream of the HSP 25 activation cascade; geldanamycin (5 µg/ml), an inhibitor of HSP 90; and quercetin (1–100 µM), an inhibitor of HSP 70. The number of SFs formed upon tensile stress loading in ECs from all the sources examined was unaffected by treatment with SB203580 or geldanamycin (Fig. 6A–C). In contrast, quercetin appeared to inhibit SF formation in a concentration-dependent manner (Fig. 6D–F). In ECs in the middle parts of the abdominal aorta and the common iliac artery, SF formation was blocked when quercetin was present at 100 µM during stretching, and the SF-positive frequency in stretched, quercetin-treated ECs was about the same as that of non-stretched control ECs (compare Figs. 2 and 7). The HSP 70 immunofluorescence signal was clearly diminished in quercetin-treated ECs, as compared to non-treated ECs under stretch stress conditions (Figs. 8C and 9).

Finally, we examined the effect of cytochalasin D on HSP 70 expression in stretch-stressed ECs. Cytochalasin D is a cell-permeable, fungal toxin that disrupts actin filaments and inhibits actin polymerization. Although treatment of the ECs with cytochalasin D (2 µg/ml) almost completely inhibited SF formation (data not shown), it did not adversely affect the upregulation of endothelial HSP 70 that was induced by mechanical stretch stress (Fig. 8D). In the abdominal aorta and the common iliac artery, no significant differences were observed in the upregulation of HSP 70 by stretch stress in cytochalasin D-treated vs. non-treated ECs (Fig. 9).

IV. Discussion
The results of this study show that rat arterial ECs respond to mechanical stretch stress by upregulating HSP
expression and SF formation. Our results strongly suggest that stretch-stress-induced SF formation is mediated by HSP 70 and not by HSP 25 or HSP 90. Accurate evaluation of the expression and distribution of macromolecules in ECs within ex vivo or in vivo vessel structure may be difficult because endothelial profiles in histological cross-sectioned vessels contain all three component layers of blood vessels (i.e., tunica intima, media, and adventitia) and have extremely small volume. In the present study, we used EC sheets to perform histochemical analyses of HSP expression and SF formation in ECs. Since the prepared samples were EC monolayers, IF microscopy in combination with the EC sheet technique could efficiently visualize the two-dimensional morphological and cytochemical dynamics of vascular endothelium.

The presence of intracellular actin SFs in vivo has been reported for various types of cells, including scleroblasts [8], vascular ECs [8], peritoneal mesothelial cells [32], and epi-

Fig. 4. HSP 70 immunohistochemistry in rat arterial ECs from Th-p (A, B) and Cil (C, D) under non-stretch-stressed (A, C) and stretch-stressed (B, D) conditions. HSP 70 expression was upregulated by application of stretch stress (30% amplitude, 1 hr) in Cil ECs (D) but not in Th-p ECs (B). The abbreviations used here are the same as those used in Fig. 2. Images A–D are shown at the same magnification; bar=20 µm.

Fig. 5. Quantitative analysis of HSP upregulation in response to stretch stress. Rat arterial ECs were subjected to application of mechanical stretch stress at 30% amplitude for 1 hr. The relative activities of HSPs 25 (gray bar), 70 (black bar), and 90 (white bar) are shown as the ratios of fluorescence intensities before and after cells were subjected to stretch stimulation. In Ab and Cil ECs, expression of HSPs 25 and 70 increased after stretch stimulation, and the level of HSP 70 induction was greater than that of HSP 25. In Th-p and Cc ECs, expression levels of HSPs 25 and 70 after stretch stimulation was about the same as or slightly less than those in the non-stretched controls. Endothelial HSP 90 was not upregulated by tensile stress in any of the samples. The abbreviations used here are the same as those used in Fig. 2. Values shown are means±SD. *Differences in HSP 70 upregulation between Ab and Th-p, Ab and Cc, Cil and Th-p, and Cil and Cc segments are significant (p<0.01). †Differences in HSP 25 upregulation between Ab and Th-p, Ab and Cc, Cil and Th-p, and Cil and Cc segments are significant (p<0.01).
these cells of the renal proximal tubule [26]. These cells are constantly exposed to mechanical stresses due to fluid flow, and they encounter cyclic or constant stretch stress. SF formation and orientation in vascular ECs in vitro [7] and in vivo [19] are greatly influenced by wall shear stress. Stretch stress also affects the formation and arrangement of SFs in ECs in vitro [5, 15, 30], ex vivo [34, 35], and in vivo [36], as well as in mesothelial cells excised from the small intestine [33]. Our results are consistent with these earlier findings and suggest that SFs are formed as an emergency countermeasure against intense mechanical stress. Furthermore, they probably play a role in maintaining cellular structural integrity by strengthening cell-to-matrix adhesion.

HSPs, which were originally identified as special proteins that protect against and are induced by heat stress, are also induced by certain chemicals, such as heavy metals and arsenious acid, oxidative stress, and some kinds of mechanical stress, including high osmotic pressure, shear stress, and
Role of HSP70 in SF Formation in ECs

A relationship between HSPs and actin filaments was suggested by Huot et al. [14], who speculated that an HSP 25/MAP kinase-activated protein kinase 2/3/p38 MAP kinase cascade may regulate SF formation in response to oxidative stress [14]. HSP 25 is also phosphorylated in response to other kinds of stimulation, including shear stress [20].

The MAP kinase cascade mentioned above may play a role in rearrangement of cell shape and SF redistribution upon shear stress [1], but the degree of phosphorylation of HSP 25 induced by shear stress is rather low compared to that induced by oxidative stress. In the present study, stretch stress applied \textit{ex vivo} induced arterial ECs to upregulate expression of HSPs 25 and 70 and to form SFs, and the upregulation of HSP 25 was much less pronounced than that of HSP 70 (see Fig. 5). Moreover, inhibition of p38 MAP kinase activity by SB203580 had no apparent effect on the induction of SF formation upon application of stretch stress (see Fig. 6). These findings indicate that the proposed HSP 25/MAP kinase-activated protein kinase 2/3/p38 MAP kinase cascade does not play a significant role in stretch-stress-induced SF formation in our system. In addition, the possibility that stretch-induced SF formation requires significant participation by HSP 90 was eliminated by our finding that geldanamycin did not affect this process (see Figs. 5 and 6).

Fig. 7. Quantitative analysis of the effect of quercetin on stretch-stress-induced SF formation. Quercetin was administered to the arterial segments while they were in the stretched state (30% amplitude, 1 hr). Gray and black bars indicate the relative frequencies of SF-positive ECs observed in DMSO- and 100 µM quercetin-treated samples, respectively. Quercetin treatment significantly reduced the frequency of SF-positive ECs in Ab and Cil ECs. The abbreviations used here are the same as those used in Fig. 2. Values shown are means±SD. *Differences between with quercetin and with DMSO in Ab segment, and in Cil segment are significant (p<0.01).

Fig. 8. Effects of quercetin and cytochalasin D on upregulation of HSP 70 in stretch-stressed ECs from the common iliac artery of rat. Results shown are from HSP 70 immunohistochemistry of ECs that were (A) DMSO-treated and non-stretched, (B) DMSO-treated and stretch-stressed (30% amplitude 1 hr), (C) 100 µM quercetin-treated and stretch-stressed, or (D) 2 µg/ml cytochalasin D-treated and stretch-stressed. Upregulation of HSP 70 was blocked by quercetin but not by cytochalasin D. Images A–D are shown at the same magnification; bar=20 µm.
In contrast to the above-described results for HSPs 25 and 90, inhibition of HSP 70 upregulation by quercetin resulted in significant suppression of stretch-induced SF formation in ECs (see Fig. 7). Moreover, disruption of SF formation by cytochalasin D treatment had no apparent effect on HSP 70 expression (see Fig. 9). These results suggest that endothelial HSP 70 plays an important role in SF formation in response to stretch stress. This hypothesis is also supported by our finding that upregulation of HSP 70 was barely detectable in ECs originating from the arterial regions where SF formation was not induced by mechanical stretch stress (see Figs. 2 and 5). Taking all of these findings into consideration, we hypothesize that stretch-induced SF formation is mediated by the upregulation of HSP 70. The specific pathway(s) that correlate HSP 70 expression with SF formation in vascular ECs under mechanical stretch stress remain to be elucidated.

Evidence for HSP involvement in the pathogenesis of atherosclerotic vascular diseases is accumulating [24, 25, 40]. Anti-microbial HSP antibodies produced in response to infection cross-react with HSPs in stressed ECs owing to the high degree of sequence homology that exists between human and microbial HSPs. This immunological cross-reaction causes endothelial damage and early atherosclerotic lesions. Many data using experimental animal models show that HSP 60 and anti-HSP 60 may play an important role in the initiation of atherogenesis. However, HSP 70 and anti-HSP 70 in cardiovascular diseases are still uncertain. Chan et al. found that there was a significant correlation between anti-HSP 70 antibody and different vascular diseases (i.e., lower limb claudicants, lower-limb critical ischaemia, and abdominal aortic aneurysms), suggesting that HSP 70 and anti-HSP 70 might be involved in the pathogenesis and propagation of atherosclerosis [3]. Interestingly, in the present study, we revealed that the HSP 70-upregulation response to stretch stress differed among the anatomical regions within the rat vascular tree tested (see Fig. 5). ECs from the middle parts of the abdominal aorta and the common iliac artery mounted a significant response to stretch stress, dramatically increasing HSP 70 expression and SF formation. On the other hand, ECs from the proximal part of the thoracic aorta or the common carotid artery were only weakly sensitive to stretch stress. The positional differences in stretch stress-induced SF formation correlate well with the upregulation of HSP 70. Details on the mechanisms of these positional differences in HSP responsiveness within the vascular tree are unclear. Tangirala et al. reported that in apoE- and LDL receptor deficient mice with extensive atherosclerosis, the additional atherosclerosis site was found predominantly in the abdominal aorta but not in the thoracic aorta [38]. The differential responsiveness of HSP 70 expression and SF formation within the vascular tree may affect the pathogenesis of vascular disorders.

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VI. References

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