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Intracellular Ca^{2+} Responses in Cultured Endothelial Cells to Mechanical Stimulation by Laser Tweezers*

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Abstract

It is well known that endothelial cells (ECs) respond to the fluid imposed shear stress and change their shapes and functions. We have focused on the importance of cytoplasmic micro-mechanical strain in mechano-sensing mechanism of ECs. To this end, the cytosolic Ca^{2+} responses of ECs to the mechanical stimulus by laser tweezers that can apply the micro-mechanical force to nano/micro-organisms without any physical contact, were investigated. When the laser spot focused on the nucleus of EC was slightly moved, the cytosolic Ca^{2+} increased immediately in the same EC, whereas there was no Ca^{2+} increase without laser spot movement. In the absence of extracellular Ca^{2+} in the medium or the blockade of stretch activated ion channels, there was also an increase of Ca^{2+} in stimulated ECs. Therefore, the increased Ca^{2+} in stimulated ECs is considered to be derived from intracellular Ca^{2+} store. The heterogeneous Ca^{2+} propagation from the stimulated EC to surrounding ECs was also observed. Two types of Ca^{2+} wave propagation were observed, the fast one that the velocity was more than 20μm/sec, and the slow one that the velocity was less than 1μm/sec. The micro-stress induced by the micro-movement of the nucleus can be a trigger of the cytosolic Ca^{2+} increase and the cytoplasmic micro-mechanical strain may play an important role in mechano-sensing mechanism of ECs.

Key words: Endothelial Cells, Mechano-Transduction, Calcium Ion, Laser Tweezers

1. Introduction

Local hemodynamic shear stress is one of the major factors for atherogenesis [1-3]. Therefore, endothelial cell (EC) responses to the fluid imposed shear stress have been well studied. Initial events of EC responses to shear stress are intracellular Ca^{2+} increase [4], activation of K^+ current [5] and G proteins [6]. Subsequently, stress fiber formation is occurred and it leads to the morphological change of ECs [7, 8].

For decades, the mechano-transduction in ECs has been discussed [3, 9, 10] and various candidates for the mechano-sensor have been proposed. For instance, activation of integrins and focal adhesion complex associated proteins are considered to be important in the mechano-transduction in EC [11, 12]. Another candidate for the mechano-sensor is a cell membrane. Deformation of the endothelial cell membrane or direct effects of shear stress

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on the cell membrane activate ion-channels and G-protein [5, 6, 13, 14]. Glycocalyx layer on the cell membrane is also considered as the mechano-transducer [15]. In this case, fluid shear stress is sensed with glycocalyx layer and signals are transmitted inside the cells via cytoskeletal structures. Osawa et al. reported the importance of PECAM-1 in the responses of EC to shear stress [16]. It is considered that the stress or strain transmitted to the portion of EC-to-EC contacts induces phosphorylation of PECAM-1 and the molecule plays an important role in the mechano-sensing mechanisms. With the relation to the intracellular Ca^{2+} increase in ECs exposed to shear stress, Yamamoto et al. reported the importance of P_2X_4 receptor in the ATP-induced Ca^{2+} influx in ECs under flow exposure [17].

Despite the fact that, many candidates for the mechano-sensor have been proposed with supporting evidences, the mechano-sensing mechanisms of endothelial cells are still being debated. Because of the complexity of the signaling transduction in living cells, it is necessary to investigate each candidate separately, i.e. simple experimental model, to understand the mechano-sensing mechanisms of ECs. Here, we have focused on the cytoplasmic micro-mechanical strain. To apply a micro-mechanical force to nano/micro biological specimens, such as proteins or cells, the laser tweezers is a powerful tool [18-20]. The force that the laser tweezers can apply to small specimens is still smaller than the fluid shear stress. Although, the strain generated by the laser tweezers in cells may be very small, as the first step, we have investigated the cytosolic Ca^{2+} responses, one of the very initial events in ECs exposed to shear stress, to the micro-mechanical stimulation by laser tweezers.

2. Materials and methods

2.1. Endothelial Cell Culture

Bovine aortic endothelial cells (BAECs) were purchased from Kurabo (Osaka, Japan) and cultured with DMEM contained with 10% heat-inactivated fetal bovine serum (JRH Biosciences, KS, USA), penicillin and streptomycin (GIBCO, CA, USA). When confluent, cells were subcultured with 0.05% trypsin-EDTA (GIBCO, CA, USA) and passaged at 1:4 split ratios. Fully confluent cultured endothelial cell populations seeded on glass bottom cell culture dish from fourth to eighth passage were used in this study.

2.2. Laser manipulation system

Figure 1 shows the laser manipulation system (Sigma Koki, Tokyo, Japan). The wavelength of the laser used in this study is 1064nm and the power is 2.5 W. Laser beam is led to the inverted microscope (IX70, Olympus, Tokyo, Japan) from the back port.

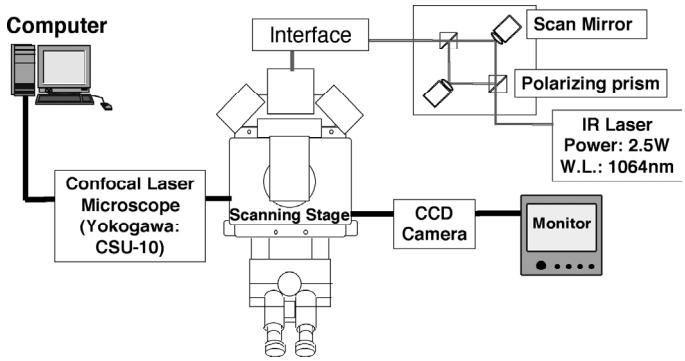


Fig. 1 Schematic diagram of the laser manipulation system in this study. Infra-red laser, the power is 2.5W and the wave length is 1064nm, is used as the laser source. The position of the laser spot can be monitored with a CCD camera which is sensitive to IR and connected to the right side port of the microscope, and controlled with scan mirrors. Fluorescence images were taken with the confocal microscope unit and loaded into the computer.

2.3. Observation of intracellular Ca^{2+}

In this study, the ratio metric method using the combination of Fluo-4 and Fura-red [21], was used to observe the changes of intracellular Ca^{2+} in ECs. The fluorescence intensity ratio of Fluo-4 to Fura-red is well agreed with the Ca^{2+} concentration. The emission wave length are 516nm for Fluo-4 and 655nm for Fura-red, respectively. The excitation wave length are almost the same for both Fluo-4 and Fura-red, 488nm and 494nm. Therefore, both fluorescent dyes can be excited with the single wave length. Using this method, we can estimate the cytosolic calcium with the visible light. ECs were loaded with Fura red (40μM) and Fluo-4 (20μM) for 30min. ECs in the glass bottom cell culture dish were set in the incubator on the stage of the inverted microscope and observed with an x40 oil immersion lens. After initiation of image scanning, the laser beam was focused to the endothelial cell nucleus. After 5 or 10 seconds, laser spot was moved to the cytoplasmic area from the nucleus as shown in Fig.2. The movement of laser spot was 5μm~8μm. Color fluorescence images were taken by a confocal laser scanning unit every second. Cytosolic calcium response in the endothelial cells was evaluated with the ratio of the green Fluo-4 image to the red Fura-red image.

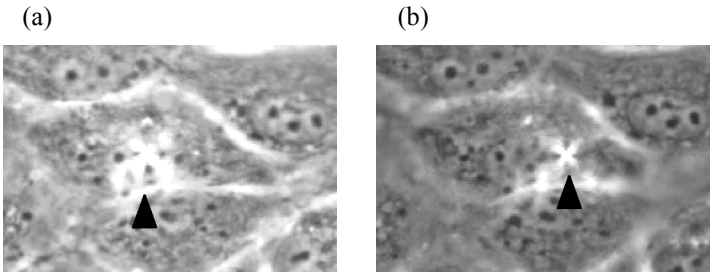


Fig. 2 The laser spot focused on the endothelial cell. (Arrow heads) (a) The laser spot was focused on the nucleus of EC. (b) The laser spot was moved out of the nucleus.

3. Results

The trapping force of this system to endothelial cells was estimated with trapping a floating EC in the flow chamber. A single floating EC was trapped with the laser in the flow chamber (18mm in width and 1mm in height) and applied the culture medium flow. We measured the maximum flow rate that the cell could not be kept as the trapped condition. From that flow rate, we estimated the trapping force of this system as approximately 100pN.

Figure 3 shows the cytosolic Ca^{2+} responses in endothelial cells with ((a); n=13) or without ((b); n=7) laser spot movement. After laser spot was moved out of the nuclues, the cytosolic Ca^{2+} increased in ECs. In contrast, in the endothelial cells without laser spot movement, cytosolic Ca^{2+} concentration remained unchanged. In most cases, the cytosolic Ca^{2+} concentration reached a plateau at 40 seconds after laser spot movement.

The cytosolic Ca^{2+} increase is caused by the influx from the outside the cells or the release from the Ca^{2+} store inside the cells. When stretch activated ion channels were blocked by 10 mM of Gadolinium (n=11), the cytosolic Ca^{2+} increased in the almost same pattern as the stimulated ECs without Gadolinium (Fig. 4). The cytosolic Ca^{2+} also increased when calcium in the medium was chelated with 2 mM of EDTA (n=6) (Fig. 5).

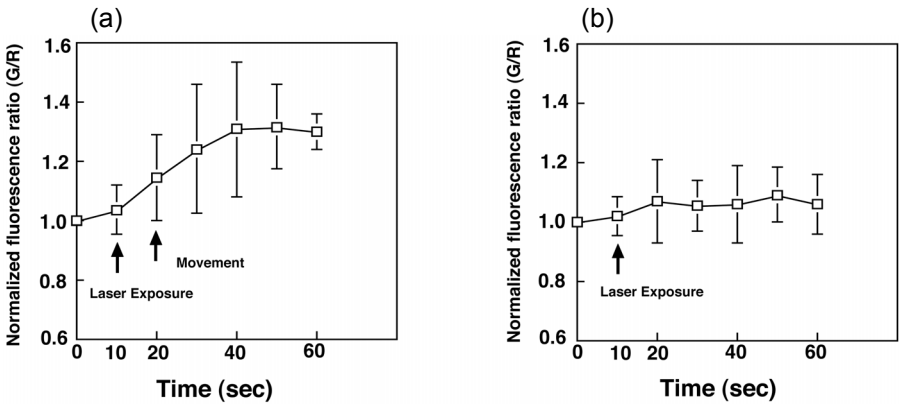


Fig. 3 Changes of intracellular Ca^{2+} concentration in stimulated ECs as indicated with normalized fluorescence ratio of Fluo-4 and Fura-red, with movement of the laser spot focused on the nucleus (a), and without movement of the laser spot (b).

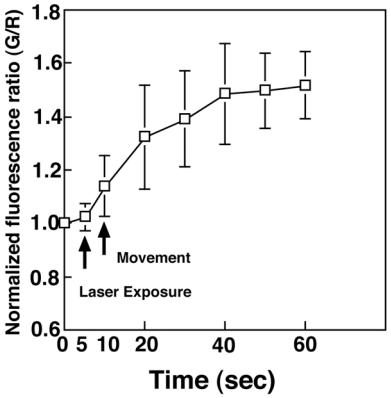


Fig. 4 Changes of intracellular Ca^{2+} concentration in stimulated ECs as indicated with normalized fluorescence ratio of Fluo-4 and Fura-red. ECs were treated with 10 mM of Gadolinium for blocking stretch activated ion channels.

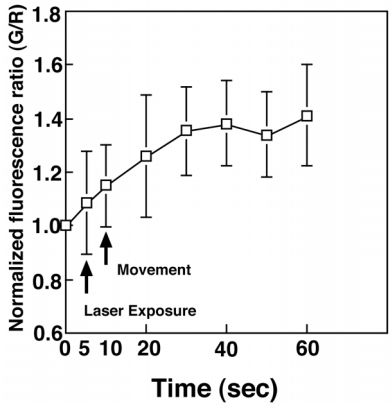
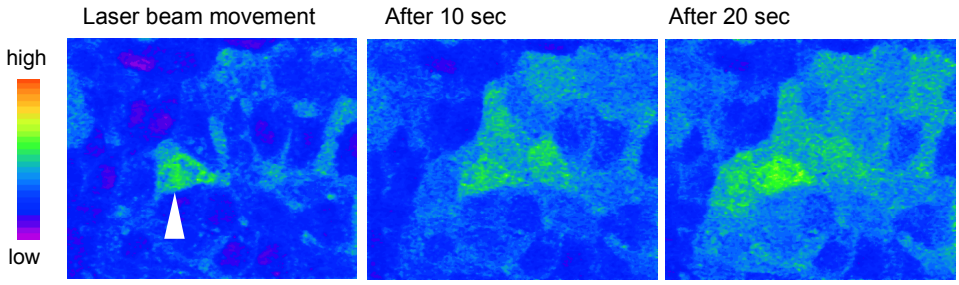


Fig. 5 Changes of intracellular Ca^{2+} concentration in stimulated ECs as indicated with normalized fluorescence ratio of Fluo-4 and Fura-red. ECs were stimulated in the absence of extracellular Ca^{2+} with EDTA.

In this study, the Ca^{2+} propagation from the stimulated ECs to the surrounding cells was observed. Figure 6 shows two examples of the Ca^{2+} propagation as indicated with pseudo-color images of cytosolic Ca^{2+} concentration. The Ca^{2+} in some surrounding ECs increased almost simultaneously with the stimulated cell. In this case, the Ca^{2+} propagation was very quick, within 1 second. We simply estimated the Ca^{2+} propagation speed with Ca^{2+} wave form in each point in some surrounding cells. The arrival time of Ca^{2+} wave was defined as increasing Ca^{2+} concentration from the baseline. The Ca^{2+} propagation speed in the case of fast propagation was estimated to be more than 20μm/sec. However, very slow Ca^{2+} propagation in more than 10 seconds was also observed. In that

case, the Ca^{2+} propagation speed was estimated to be less than 1μm/sec. In some cases, there was no increase of Ca^{2+} even in the neighboring ECs to stimulated EC. In addition, both of fast and slow Ca^{2+} propagations were observed in the same monolayer. Therefore, the Ca^{2+} propagation pattern is heterogenous.

Example 1



Example 2

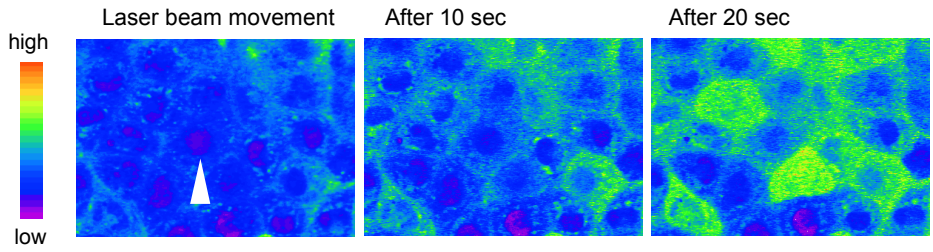


Fig. 6 Examples of the propagation of the intracellular Ca^{2+} from stimulated EC to surrounding ECs as indicated with pseudo-color images of Ca^{2+} concentration. Arrow heads indicate stimulated ECs.

4. Discussions

In this study, we have observed the cytosolic Ca^{2+} increase in ECs and its propagation to surrounding cells when the laser spot focused on the nucleus of EC was moved out of the nucleus. The Ca^{2+} propagation is caused by the propagation of Ca^{2+} itself and/or other signaling propagations. 1064nm laser is widely used for the optical manipulation for biological specimens, such as proteins and cells [18-21, 22], because that laser is reported that there is nontoxic to cells [22] and the thermal effect is negligible [23]. Therefore, the micro-mechanical stress generated around the nucleus can be a trigger of the cytosolic Ca^{2+} increase released from Ca^{2+} store inside the cells.

The intracellular Ca^{2+} increase in ECs is one of the earliest events when ECs are exposed to mechanical stress, such as shear stress or cyclic stretch, and Ca^{2+} plays an important role in the signaling transduction inside the cells [4, 24, 25]. The increase of Ca^{2+} is caused by the influx from outside of cells and release from the Ca^{2+} store inside the cells [24]. Both of them participate in the response to shear stress. In this study, Ca^{2+} increase in stimulated ECs seemed to be derived only from the Ca^{2+} store inside the cells. Therefore, our experimental model does not completely mimic the response of ECs to shear stress. Helmke, et al [26] reported that the displacement of fluorescence labeled intermediate filaments in ECs exposed to 1.2 Pa shear stress was nearby 1μm. When the laser spot was moved out of the nucleus of ECs in our experiments, we could not observe any movement of the nucleus. Moreover, we observed the movement of the cytosol with fluorescent micro beads whose diameter was 100 to 200 nm. There was not any movement of fluorescent micro beads in ECs when the laser spot was moved out of the nucleus (data not

shown). Compared this result with Helmke's results, the strain generated by the movement of laser spot in ECs in our experiments is much smaller than that in ECs by shear stress. On the other hands, it is known that stretching force to the plasma membrane activates ion channels known as the stretch activated ion channels [25]. Shear stress is also known to activate these channels [27]. Therefore, some level of strain inside the ECs causes both influx of Ca^{2+} from outside of cells via ion channel and the release of Ca^{2+} from the store inside the cells. However, the strain less than some level, such as in our experiments, induces only the release from the Ca^{2+} store inside the cells. On the other hands, there is possibility that the laser spot movement directly affects to the membrane of endoplasmic reticulum, Ca^{2+} store inside the cells, and induces the Ca^{2+} release.

The responses of EC to the mechanical stimulation to the single cell were also reported by Demer et al. [28] and Sigurdson et al. [29] almost simultaneously with the same methods. In their cases, EC was nudged with a micropipette and the Ca^{2+} increase was observed in the stimulated EC. However, there was no Ca^{2+} increase in the absence or very low concentration of extracellular Ca^{2+}. Therefore, the mechanism of Ca^{2+} increase in ECs was different from our experiment. In their experiments, the stretch activated ion channels play a major role for intracellular Ca^{2+} increase and the strain of the plasmamembrane or cytoskeletal structures is much higher than that in our experiments. Demer et al. [28] also reported the propagation of Ca^{2+} from the stimulated EC to surrounding cells. The Ca^{2+} propagation velocity in their study was 27.8 ± 13.6 μm/sec, almost the same value of the faster propagation in our experiments. The mechanism of Ca^{2+} propagation in Demer's results and ours might be the same, i.e. the electrical coupling of cells because of the fastness of propagation. Coincidentally, the very slow Ca^{2+} propagation was also observed. If the Ca^{2+} propagation is caused by the diffusion of some chemical agents, such as ATP or IP_3 [30], the propagation might be homogeneous. However, the propagation was heterogeneous in this study. Therefore, the slow propagation may be related with the chemical communication between cells via gap junctions that are heterogeneously distributed.

Using the laser tweezers, we can not apply the visible strain to the plasmamembrane of a cell in the monolayer, but can apply the quantitative force to a cell via microbeads adhered to the cell surface. In the future, we are planning to investigate the Ca^{2+} responses to the internal force and external force to the cells separately with the laser tweezers.

5. Conclusion

When the nucleus of EC is slightly moved by laser tweezers, the cytosolic Ca^{2+} increases and the Ca^{2+} propagates from the stimulated EC to the surrounding cells heterogenously. The increased cytosolic Ca^{2+} is released from the Ca^{2+} store inside the ECs. The micro-strain induced by the micro-movement of the nucleus can be a trigger of the cytosolic Ca^{2+} increase and the cytoplasmic micro-mechanical strain may play an important role in mechano-sensing mechanism of ECs.

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