ATP Release from Cultured Endothelial Cells and Intercellular Calcium Signaling during Shear Stress Exposure*

Susumu KUDO**, Kaoru HOSOE**, ***, Makoto HOSOBUCHI****, Naoto KAWASAKI** and Kazuo TANISHITA*****

**Department of Mechanical Engineering, Shibaura Institute of Technology, 3-7-5 Toyosu, Koto-ku Tokyo 135-8548, Japan
E-mail: kudous@sic.shibaura-it.ac.jp

***TERUMO, 44-1-2 Hatagaya, Shibuya-ku, Tokyo, 151-0072, Japan

****NIKON CORPORATION 471 Nagaodai-cho, Sakae-ku, Yokohama, Kanagawa 244-8533, Japan

*****Department of System Design Engineering, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan

Abstract
Intracellular calcium ([Ca²⁺]) is a second messenger molecule critical for numerous intracellular signaling pathways in endothelial cells (ECs). Direct mechanical stimulation imposed on single ECs by a microprobe has been demonstrated to increase [Ca²⁺] levels in ECs. After an initial delay time, this Ca²⁺-signal propagates from the mechanically stimulated cell to its neighboring cells in the form of an intercellular Ca²⁺-wave, a process termed intercellular communication. Although intercellular communication is a fundamental property of many multicellular systems, it remains unclear as to whether intercellular communication following shear stress of ECs actually occurs, and if so, whether this communication occurs via gap junctions or the release of extracellular mediators including ATP. In the current study, we investigated ATP release from ECs during periods of shear stress and measured intercellular Ca²⁺-waves using adenosine 5'-triphosphate, P₃-(1-(2-nitrophenyl)ethyl)ester and disodium salt (NPE-caged ATP) stimulation. In addition, we investigated intercellular communication in ECs during shear stress using chemical inhibitors of both gap junctions and various components of the ATP paracrine signaling pathway. ECs subjected to shear stress loading released ATP. Using NPE-caged ATP, local increase of extracellular ATP induced a Ca²⁺-wave. Furthermore, [Ca²⁺] responses in ECs under shear stress loading was inhibited by the purinergic receptor blocker, ATPase, and several metabolic inhibitors including FCCP and rotenone. These results suggest that [Ca²⁺] communication mediated by ATP exists in ECs under shear stress loading in vitro.

Key words: Endothelial Cell, Shear Stress, ATP, Autocrine/Paracrine, Calcium Signaling

1. Introduction
It is well established that endothelial cells (ECs) line the inner surface of blood vessels, and that these cells are responsible for many of the important functions of blood vessels including maintenance of the vascular permeability barrier, establishment of a nonthrombogenic luminal surface and the secretion of vasodilatory factors such as nitric
oxide. ECs are constantly subjected to shear stress induced by blood flow, a stress that affects many of the physiological and biochemical processes controlled by these cells \(^1\). In addition, shear stress is also thought to exert effects on endothelial signal-transduction systems that in turn initiate numerous cellular responses \(^4\).

Intracellular calcium ([Ca\(^{2+}\)]\(_i\)) is an important second messenger that mediates critical intracellular pathways following stimulation of ECs by a variety of agonists and forces. Shear stress has been reported to stimulate an increase in [Ca\(^{2+}\)], in cultured EC monolayers \(^6\) and induce [Ca\(^{2+}\)] oscillations in single ECs \(^7\). Direct mechanical stimulation imposed on single cells by a microprobe has been shown to increase [Ca\(^{2+}\)], (also called a Ca\(^{2+}\)-signal) in ECs \(^10\). After an initial period of delay, this Ca\(^{2+}\)-signal propagates from the mechanically stimulated cell to neighboring cells in the form of an intercellular Ca\(^{2+}\)-wave, a process termed intercellular communication. Intercellular communication is considered to be a fundamental property of multicellular systems and a method by which ECs coordinate localized responses and thereby enhance changes in vascular tone and permeability.

Ca\(^{2+}\)-waves have been observed not only in cultured ECs but also within the endothelium in vivo. Uhrehnolt et al. \(^14\) have previously examined the propagation of Ca\(^{2+}\)-waves along the endothelium of hamster feed arteries using confocal laser microscopy. They were able to demonstrate that acetylcholine triggers an increase in [Ca\(^{2+}\)], in the ECs localized to the site of stimulation, and that the ensuing Ca\(^{2+}\)-wave propagated bi-directionally along the endothelium. Ying et al. \(^15\) have also reported the generation of Ca\(^{2+}\)-waves in lung capillary endothelium. The study imaged venular capillaries of isolated blood-perfused rat lung, and indicated the presence of intercellular Ca\(^{2+}\)-waves that spontaneously arose from a single EC and then extended for short distances along the capillary wall.

In several cell types, intercellular communication is considered to largely occur via gap junctions \(^16\) or through the release of extracellular diffusible substances including ATP (also called paracrine signaling) \(^10\). During periods of stimulation by shear stress, ECs release endogenous ATP \(^26\). However, it remains unclear as to whether intercellular communication upon shear stress of ECs does actually occur, and if so, whether this form of communication occurs through gap junctions or via the release of an extracellular mediator. Unlike direct mechanical stimulation using a microprobe, it is difficult to impose fluid shear stress on a single cell in a flow chamber system. Thus, in the current study we investigate ATP release from ECs during shear stress and intercellular Ca\(^{2+}\)-wave using adenosine 5’-triphosphate, P\(_r\)-(1-(2-nitrophenyl)ethyl)ester, disodium salt (NPE-caged ATP). In addition, although the intercellular communication is not measured directly, we investigate intercellular communication in ECs during shear stress using inhibitors of both gap junctions and numerous components of the ATP paracrine signaling pathway.

### 2. Materials and methods

#### 2.1 Cell culture

Bovine aortic endothelial cells (BAECs; Dainippon Pharmaceutical) were cultured and grown to confluence in Dulbecco’s Modified Eagles Medium (DMEM), containing 10% fetal bovine serum (FBS; SIGMA), 10000 U/ml penicillin, 10000 \(\mu\)g/ml streptomycin, 25 \(\mu\)g/ml anfotelin B and 25 mM HEPES (pH 7.4). For flow experiments, BAECs (passage 6-10) were cultured on glass-base dishes (IWAKI) pretreated with 0.1% collagen (Koken). BAECs cultured in DMEM with 10% FBS and additives reached confluence within 4 days.

#### 2.2 Flow system

A parallel plate flow chamber was used to apply shear stress to ECs as described previously \(^24\), with the exception that BAECs were cultured in glass-base dishes. The flow
chamber consisted of a polycarbonate base and a glass-base dish, separated by a silicone gasket. HEPES buffered saline solution (HBSS; 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 10 mM D-glucose, 1.8 mM CaCl₂, 15 mM HEPES) flowed from a polycarbonate reservoir mounted 60 cm above the microscope platform through polypropylene tubing into the flow chamber that was mounted on the microscope stage. From the out port of the flow chamber, HBSS was collected into a lower polycarbonate reservoir and was pumped back to the upper reservoir. The temperature of the flow circuit and flow chamber was maintained in a constant temperature controlled enclosure. The cells were exposed to 2 Pa of shear stress for 600 s.

2.3 ATP measurement

The BAECs were exposed to 2 Pa shear stress at 37°C and ATP that was released from BAECs measured by a luciferin-luciferase assay. In order to do this, the perfusate containing ATP was collected from the outlet tube of the flow chamber and 100-μl aliquots applied to 100 μl of the ATP assay mix (luciferase, luciferin, MgSO₄, DTT, EDTA; Sigma). The ATP assay mix was injected into each sample and its relative light intensity recorded for 60 s in a luminometer (AB220; ATTO) at room temperature. A calibration curve for ATP concentrations was obtained for each experiment using the same batch of ATP assay mix.

2.4 Caged compound loading and UV spot illumination

Adenosine 5'-triphosphate, P₃-(1-(2-nitrophenyl)ethyl)ester, disodium salt (NPE-caged ATP; Molecular Probes) was used for the investigation of the effect of extracellular ATP on single BAECs. To remove ATP that may be photoreleased at optical wavelengths, NPE-caged ATP was dissolved in HBSS containing apyrase. The solution was centrifuged at 4000 g for 40 min using an Ultrafree-MC centrifuge (Amicon) and the purified NPE-caged ATP dissolved in HBSS at a concentration of 25 μM. NPE-caged ATP was in the HBSS over the BAECs and released locally into the bath by a UV laser. UV spot illumination for photo release in [Ca²⁺], imaging experiments was performed using the AQUA COSMOS System (Hamamatsu) mounted on an inverted microscope (TMD 300, Nikon) with a 20× objective lens. The 400-nm UV beam had a diameter of 5 μm. The images were represented as the relative calcium green 1 fluorescence changes (F/F₀). F values represented the measured fluorescence intensity of calcium green 1 and F₀ represented the fluorescence intensity prior to photoliberation of NPE-caged ATP.

2.5 Fluorescence recovery after photobleach

Fluorescence recovery after photobleach (FRAP) was conducted in accordance with the method of Cotrina et al. BAECs were incubated with 10 μg/ml 6-carboxyfluorescein diacetate (CF; Sigma) for 6 min, whereby excitation of CF was provided by the 488 nm line of the confocal laser scanning microscope (CLSM). Emission was long pass-filtered (515 nm) and detected with the confocal aperture appropriately set. After obtaining a baseline fluorescence image of the BAECs, the area of laser scanning was reduced to include only one target cell. Complete or almost complete photo bleaching occurred after five scans at full laser power. Subsequently, the microscope settings were returned to the recording configuration and the refill of the BAECs were monitored for 6 min.

2.6 [Ca²⁺], measurement and analysis

BAECs cultured on glass-base dishes were incubated in DMEM with 10% FBS containing 4 μM Calcium Green-1/AM (Molecular Probes) for 40 min (at 24°C). The BAECs were then rinsed in DMEM and set in the flow chamber. For measuring [Ca²⁺], in BAECs, fluorescent and phase contrast images of the BAECs were obtained using a CLSM.
(MRC-1000; Bio-Rad) mounted on an inverted microscope (TMD 300; Nikon) with a 20× objective lens. An excitation wavelength of 488 nm was emitted from a 25-mW argon laser. 

\[ [Ca^{2+}] \]

was visualized using a 510 nm band-pass excitation filter, a 510-nm dichroic mirror and a 540-nm long-pass emission filter. The image sampling rate was 1 frame every 3 s. A total of 200 frames were acquired in 1 single experiment. After obtaining images using a CLSM, all individual BAECs were manually outlined from phase contrast images using Scion Images (Scion Corporation). The fluorescent intensity was represented on a 256-gray scale and the fluorescent intensity of \([Ca^{2+}]\) on individual cells were analyzed as a normalized value. The normalized values were calculated from the ratio of fluorescent intensity at a time to the mean intensity for 10 min (total measuring time). \([Ca^{2+}]\) response was defined as the value 30% larger than the mean intensity.

2.7 ATP paracrine signaling pathway antagonists

Thapsigargin, 3β-Hydroxy-11-oxo-18α, 20β-olean-12-en-29-oic acid (18α-GA), apyrase, suramin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and rotenone were used at a concentration of 75 nM, 10 μM, 10 units/ml, 100 μM, 20 μM and 4 μM, respectively. All agents were purchased from Sigma.

2.8 Statistical analysis

Statistical significance was tested using a one-way analysis of variance (ANOVA), and the differences between two groups were calculated using post hoc tests. A difference of \(P < 0.05\) level was considered significant. The data are presented as mean ± standard error of the mean (SEM).

3. Results

In the current study, we examined the effect of 2 Pa of shear stress on the release of ATP from BAECs (Fig. 1). Following 20 s of exposure to shear stress, ATP release increased to 10 pM \((P < 0.05)\). After 40 s of exposure to shear stress, ATP release began to decrease to levels similar to those that were initially measured (almost 0 pM).

![Fig. 1](image-url)  

**Fig. 1** The release of intracellular adenosine triphosphate (ATP) by endothelial cells under shear stress loading over time. The results are presented as the mean ± SEM of 5 experiments. *, statistically significant difference from 0 min at each time \((P < 0.05)\).

As BAECs subjected to shear stress released ATP, we next investigated the effects that a local increase in extracellular ATP concentration had on BAEC monolayers, assuming that extracellular ATP concentrations increase locally when a single BAEC releases ATP. In order to achieve this, we utilized NPE-caged ATP to induce extracellular ATP increase within a 5-μm diameter in the BAEC monolayer. Figure 2 demonstrates that \([Ca^{2+}]\)-waves form following induction with NPE-caged ATP. The cell indicated by the arrow represents
an [Ca\(^{2+}\)]\(_i\) increase following exposure to UV irradiation. Once the indicated cell an [Ca\(^{2+}\)]\(_i\) -increase, a subsequent [Ca\(^{2+}\)]\(_i\) increase was also observed in the adjacent cells, a phenomenon known as an intercellular [Ca\(^{2+}\)]\(_i\) wave. However, even when similar regions were observed and when the same NPE-caged ATP concentrations were utilized, the number of responding cells demonstrating an intercellular [Ca\(^{2+}\)]\(_i\)-wave was shown to differ. Although only a single cell demonstrated an increase in [Ca\(^{2+}\)]\(_i\) in Fig. 2A, approximately 10 cells surrounding a stimulated cell showed an increase in [Ca\(^{2+}\)]\(_i\) in Fig. 2B. Table 1 outlines the number of cells involved in [Ca\(^{2+}\)]\(_i\) wave generation and demonstrates a differing sensitivity to ATP among ECs. Ninety percent of total cells demonstrated [Ca\(^{2+}\)]\(_i\) increase following exposure to UV irradiation, while 8% of total cells did not represent an [Ca\(^{2+}\)]\(_i\) increase. These results demonstrated that the number of cells involved in [Ca\(^{2+}\)]\(_i\) wave varied even within the same EC monolayer.

![Fig. 2 ATP triggered an [Ca\(^{2+}\)]\(_i\) response in BAECs. Time sequences of intercellular [Ca\(^{2+}\)]\(_i\) waves triggered by photoliberation of NPE-caged ATP in a confluent EC monolayer. The images represent the relative calcium green 1 fluorescence changes (F/F\(_0\)) and are color coded as indicated in the color bar. The numbers at the bottom of each image indicate the time following the start of photostimulation. The arrows indicate the position of the UV photostimulation. A and B are from similar regions of the monolayer. After the [Ca\(^{2+}\)]\(_i\) response disappeared in part A, photostimulation was conducted at a different position and is shown as the arrow in part B. Scale = 50 \(\mu\)m.](image)
Table 1 The frequency of the number of cells involved in [Ca$^{2+}$], wave induced by photoliberation of NPE-caged ATP.

<table>
<thead>
<tr>
<th>Number of Cells</th>
<th>0</th>
<th>1-4</th>
<th>5-9</th>
<th>more than 10</th>
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<tbody>
<tr>
<td>Frequency (%)</td>
<td>8 ± 5</td>
<td>32 ± 13</td>
<td>29 ± 5</td>
<td>30 ± 14</td>
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</tbody>
</table>

Results are expressed as mean ± SEM. Number of experiments is 82.

To investigate intercellular Ca$^{2+}$ communication during shear stress exposure, we examined the effect of shear stress on [Ca$^{2+}$], increase in BAEC monolayers subjected to 2 Pa of shear stress by flowing HEPES buffered saline solution without ATP through the flow chambers. We demonstrate that approximately 35% of all BAECs exhibited an [Ca$^{2+}$], response when exposed to 2 Pa of shear stress, in 51 experiments, each of which involved the measurement of at least 200-260 cells. When individual BAECs were monitored during shear stress exposure, we found that the general result was similar in all response cells. That is, the [Ca$^{2+}$], rose rapidly to a peak level and then decayed to its basal value (Fig. 3A and Fig. 3B). Although this general behavior was characteristic of all cells, individual cell responses demonstrated some differences over the time course experiments. For example, following 27 s cell 1 increased its [Ca$^{2+}$], then after 36 s cell 2 increased its [Ca$^{2+}$], and after 69 s cell 3 and the adjacent cell 4 increased their [Ca$^{2+}$], levels simultaneously. Several adjacent cells were also shown to respond to shear stress at almost the same time. However, if the [Ca$^{2+}$], wave occurred under shear stress exposure, we were unable to distinguish between the cell that emitted the signals and the cell that received them. Thus, we investigated BAEC [Ca$^{2+}$],-increase during shear stress exposure using inhibitors of gap junctions and inhibitors of various members of the ATP paracrine signaling pathway.

Firstly, to block the gap junctions, we utilized the well characterized gap junction inhibitor 18α-GA. Figure 4 shows the effect of 18α-GA on intercellular diffusion of the cell junction tracer, CF, using the FRAP technique (25, 26). In the control BAEC monolayer, fluorescence was successfully recovered following the photobleaching process. However, in the BAEC monolayer treated with 18α-GA, the fluorescence was reduced to refill levels. From these images, we calculated percentage refill of fluorescence intensity (Table 2). In control cells, the percentage of the refill of fluorescent intensity increased over time. The
percentage of refill was determined to be 50, 70 and 90% at 2, 4 and 6 min, respectively. However, in the cell monolayers treated with 18α-GA, the percentage of refill did not show a statistically significant change ($P>0.05$) over the course of the experiment. These results suggest that 18α-GA inhibits gap junctions and we then examined the effect of 18 α-GA on the number of BAECs demonstrating an increase in [Ca$^{2+}$]$_i$ during exposure to 2 Pa of shear stress (Fig. 5A). We show that there was no significant difference in the number of BAECs demonstrating an increase in [Ca$^{2+}$]$_i$ between the cells treated with 18α-GA and the untreated cells.

![Image](image1.png)

Fig. 4  FRAP in matched control (left panel) cultures and cultures treated with 18α-GA (right panel). ECs from both cultures were loaded with the gap junction fluorescence tracer CF. The top panel displays the culture immediately after bleaching. The photobleached cells are indicated by the arrows. The bottom panel indicates the refill of fluorescence 6 min later (arrowhead). Scale = 20 μm.

Table 2 The effect of 18α-GA on gap junction coupling

<table>
<thead>
<tr>
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<th>0 min</th>
<th>2 min</th>
<th>4 min</th>
<th>6 min</th>
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<tr>
<td>Control (%)</td>
<td>24 ± 5</td>
<td>57 ± 8*</td>
<td>74 ± 9*</td>
<td>88 ± 9*</td>
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<tr>
<td>18α-GA (%)</td>
<td>23 ± 6</td>
<td>28 ± 6</td>
<td>34 ± 7</td>
<td>40 ± 7</td>
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</table>

Gap junction coupling was calculated as percentage refill of fluorescence intensity at 2, 4 and 6 min following photobleaching. Percentage refill was calculated from the ratio of the fluorescent intensity at each time point to the mean intensity prior to photobleaching. *, statistically significant difference from 0 min at each condition ($P < 0.05$). The results represent data from seven experiments.

Next, we investigated inhibitors of the ATP paracrine signaling pathway (Fig. 5B). We initially examined the effect of suramin, a purinergic receptor blocker, on the number of BAECs increasing their [Ca$^{2+}$]$_i$ levels. We show that suramin reduced the number to approximately 40% to the control cells. In order to remove extracellular ATP, we added apyrase, ATPase, to the extracellular buffer. The addition of apyrase was also proved to be an effective inhibitor of reducing the number to 25% of the controls. As we have demonstrated previously (27) that FCCP and rotenone inhibit ATP synthesis, we examined the metabolic inhibitors of FCCP and rotenone on BAEC number. The number of cells treated with FCCP and rotenone decreased to 20% of that of controls. These results demonstrate that ATP affects the number of BAECs increasing [Ca$^{2+}$]$_i$ during 2 Pa shear stress exposure.
Fig. 5  A) The effect of shear stress and 18α-GA on the number of the cells that increase their [Ca^{2+}]. B) The effect of shear stress and the addition of inhibitors on the number of the cells that increase their [Ca^{2+}]. Each result is normalized to control values and is compared with the control value for statistical significance. *, statistically significant difference from control conditions (P < 0.05). The number of experiments were 13 for 18α-GA, 12 for suramin, 13 for apyrase and 10 for FCCP and rotenone treatments.

We also investigated the effects of thapsigargin on the number of BAECs increasing [Ca^{2+}], during shear stress exposure (results not shown). Thapsigargin is an inhibitor of Ca^{2+}-pump activity and prevents the sequestering of cytoplasmic Ca^{2+} into inositol 1,4,5-triphosphosphate (IP_{3})-sensitive intracellular stores. The Ca^{2+} response during shear stress loading was completely inhibited by thapsigargin treatment (a total cell number of 951 in three experiments, suggesting that IP_{3} dependent Ca^{2+} release from intracellular Ca^{2+} pools is more dominant than extracellular Ca^{2+} entry for Ca^{2+} response during shear stress loading).

4. Discussion

The present study demonstrates that ATP is released from BAECs during shear stress loading alone and that intercellular Ca^{2+}-waves are generated following photoliberation of extracellular NPE-Caged ATP. Furthermore, the present study demonstrates that the number of BAECs increasing their [Ca^{2+}], during shear stress exposure is reduced following the addition of the purinergic receptor blocker suramin, ATPase (apyrase), and the metabolic inhibitors FCCP and rotenone. [Ca^{2+}] did not appear to change after treatment with the gap junction inhibitor 18α-GA. Although the intercellular Ca^{2+} communication is not measured directly, these results suggest that in BAECs, intercellular Ca^{2+} communication during shear stress exposure exists and that it is mediated via the release of ATP.

In addition, we show that BAECs release ATP when subjected to 2 Pa of shear stress and then subsequently decrease to a steady-state level of ATP concentration. We represented extracellular ATP concentration as the unit of pM. However, since the number of endothelial cells differed at each experiment, using the unit of pM/cells would have permitted more precise analysis. Although the magnitude of shear stress differs slightly, a similar phenomenon has been previously observed (20-23). Yagutkin et al. (23) showed that during stimulation with shear stress, endothelial cells are able to release endogenous ATP. They demonstrated an initial transient increase in extracellular ATP concentration that then rapidly decayed to steady-state levels when shear stress of 2.5 Pa was exhibited. Although ECs were shown to release ATP in response to shear stress, the precise mechanism whereby shear stress induces ATP release remains unclear. One possible explanation is that ECs release ATP via exocytosis, a process whereby intracellular vesicles containing ATP are
transported to and then fuse with the plasma membrane, before their release into the extracellular space. Bodin and Burnstock (28) found that two inhibitors of vesicular transport, monesin and N-ethylmaleimide, produced a significant reduction in ATP released from vascular endothelial cells. They therefore suggested that the release of ATP from ECs was achieved by vesicular exocytosis. Another plausible explanation suggested was the activity of cell-surface ATP synthase. Yamamoto et al. (29) found that the inhibition of cell-surface ATP synthase with angiostatin, piceatannol or anti-ATP synthase antibody markedly reduced the flow-induced ATP release from human pulmonary artery ECs (HPAECs). They therefore suggested that cell-surface ATP synthase was involved in flow-induced ATP release by HPAECs.

Although the present study did not investigate the effect of suramin, apyrase, and FCCP and rotenone on ATP release, it is considered that these agents except apyrase affect ATP release. Suramin has been shown to behave as both purinergic receptor blocker and ecto-ATPase inhibitors (30). Yagutkin et al. showed the effect of shear stress and suramin as ecto-ATPase inhibitors on the release of ATP from endothelial cells. They showed there was a transient increase of extracellular ATP when a shear stress of 2.5 Pa was imposed on the endothelial cells. Furthermore, suramin increased the shear-stress-dependent ATP release from endothelial cells because ecto-ATPase activity was partially inhibited by suramin. In the present study, extracellular ATP concentration of the BAECs treated by suramin is considered to be higher than that of untreated cells. Even in high concentration of extracellular ATP, the Ca\(^{2+}\) response during shear stress loading is considered to decrease because suramin blocks the purinergic receptors of BAECs. FCCP inhibits not only intracellular ATP synthesis but also intracellular transport (31). As discussed in the preceding paragraph, the release of ATP from ECs was suggested to be achieved by vesicular exocytosis. Thus, FCCP is considered to suppress the shear-stress-dependent ATP release from BAECs. The decrease of extracellular ATP suggests Ca\(^{2+}\) response during shear stress loading decreases.

We showed that ATP release from BAEC monolayer, but we could not show the ATP release from a single BAEC during shear stress. Thus, we could not quantitatively analyze the relationship between ATP release from single BAEC and [Ca\(^{2+}\)] signals during shear stress exposure. However, in the present study, we demonstrate that [Ca\(^{2+}\)] signals are propagated to neighboring cells following photoliberation of extracellular NPE-caged ATP with a UV laser. These results suggest that a local increase of extracellular ATP levels induces an [Ca\(^{2+}\)] \textit{wave}. Although some clusters of ECs increased [Ca\(^{2+}\)], at similar times under shear stress loading (Fig. 3), [Ca\(^{2+}\)] \textit{waves} as shown in Fig. 2 were not always evident. There are numerous explanations for this observation. Firstly, there are different sensitivities to extracellular ATP levels between the ECs outlined in Table 1 and those outlined in the same region in Fig. 2. Thus, even when one cell increases [Ca\(^{2+}\)] levels and releases ATP, it is possible that the direct neighboring cells do not respond but rather more distant cells do respond. Thus, although [Ca\(^{2+}\)]-wave generation occurred, it was not always observed in the EC monolayers subjected to shear stress exposure. However it is considered that intercellular Ca\(^{2+}\) communication does exist. Secondly, it is hypothesized that the cell that is stimulated and actually releases the ATP does not always increase [Ca\(^{2+}\)]. Morenhout et al. (13) have reported that stimulated cells do not demonstrate an increase in [Ca\(^{2+}\)], whereas the adjacent cells do. In the presence of extracellular Ca\(^{2+}\), such as when an individual EC within the monolayer was mechanically stimulated with a microprobe, [Ca\(^{2+}\)] increased in the stimulated cell and spread in the form of a wave from the site of contact to the cell edge. However, in the absence of extracellular Ca\(^{2+}\), there was no increase in [Ca\(^{2+}\)] in the stimulated cell, yet a wave of increased [Ca\(^{2+}\)], occurred in neighboring cells. In the present study, it was impossible to locate the precise EC that released the ATP in response to shear stress exposure. Even when an EC did not increase [Ca\(^{2+}\)], levels the EC was still able
to release ATP, which was in turn transported to downstream ECs. Thus, changes in $[\text{Ca}^{2+}]_i$ levels in downstream ECs appeared to be independent of ATP released from upstream ECs given that the upstream EC did not increase their $[\text{Ca}^{2+}]_i$ levels.

In the present study, we demonstrate that approximately 35% of the total cells exhibited an $[\text{Ca}^{2+}]_i$ response when exposed to 5 min of shear stress at 2 Pa. Helminger et al. (9) investigated the $[\text{Ca}^{2+}]_i$ response in single BAECs exposed to various levels of steady flow shear-stress. In particular, they investigated the relative percentage of cells exhibiting 0, 1-2, or 3-8 $[\text{Ca}^{2+}]_i$ oscillations during the first 9 min following initiation of flow, for different levels of steady shear stress, and showed that approximately 40% of the cells exhibited 1-2 oscillations, and that about 20% exhibited 3-8 $[\text{Ca}^{2+}]_i$ oscillations at a shear stress of 2 Pa, the level of shear stress utilized in the present study. The $[\text{Ca}^{2+}]_i$, response achieved differs to that in the present study (35%) and their study (totally 60%). This difference may be attributed to an alteration in the buffers utilized during the two protocols. In the current study, we used HBSS buffer without serum as the preferred perfusion medium in the flow chamber system. Helminger et al. (9) used serum-supplemented DMEM. The difference in perfusion medium may result in differences in $[\text{Ca}^{2+}]_i$, response levels during shear stress exposure.

Several studies have demonstrated that direct mechanical stimulation imposed on a single cell by a microprobe may increase $[\text{Ca}^{2+}]_i$ in ECs, and subsequently induce an $[\text{Ca}^{2+}]_i$ wave spreading to adjacent cells in the monolayer (10-13). Moerenhout et al. (13) investigated intra- and intercellular $[\text{Ca}^{2+}]_i$ signaling during mechanical stimulation by a microprobe in calf pulmonary artery endothelial cells. They demonstrated that $[\text{Ca}^{2+}]_i$ release in the mechanically stimulated cell and its adjacent cells was mediated by the phospholipase C and inositol 1,4,5-triphosphosphate (IP3) signaling pathway. They also showed that intercellular propagation of the $[\text{Ca}^{2+}]_i$, wave evoked by a mechanical stimulus was unaffected by gap junction blockers, and was mediated via the release of nucleotides into the extracellular space (12). Several investigations failed to observe a significant flow-induced $[\text{Ca}^{2+}]_i$ signal in the absence of ATP (32,33). They suggested that the flow signal was not directly related to shear stress but rather to the dynamic balance between receptor binding and hydrolysis of ATP. However, the present study showed a flow induced $[\text{Ca}^{2+}]_i$ response in the HEPES buffer that was ATP free before shear stress loading because ATP was released from the endothelial cells affected by shear stress. The present study showed that ECs under shear stress loading released ATP, and confirmed in a separate experiment using NPE-caged ATP that $[\text{Ca}^{2+}]_i$ waves were induced by a localized increase of extracellular ATP. Furthermore, $[\text{Ca}^{2+}]_i$ response of ECs to shear stress loading was inhibited by suramin, apyrase, and rotenone and FCCP. These data suggest that intercellular $[\text{Ca}^{2+}]_i$, signaling mediated via ATP exists in endothelial monolayers subjected to shear stress loading.

In conclusion, the results from this study suggest that intercellular communication occurs via ATP released from ECs under shear stress loading and is mediated by an increase in $[\text{Ca}^{2+}]_i$, in response to local flow changes. Such endothelial communication may play key roles in the coordination of several important vascular functions including vasomotion, adaptation of vessel diameter, inhibition of thrombosis and adjustment of vascular permeability.

**Acknowledgement**

This study was supported in part by a Grant-in-Aid for Scientific Research (A) from the Japan Society for the Promotion Science.
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