Visualization of flow-induced ATP release and triggering of Ca\(^{2+}\) waves at caveolae in vascular endothelial cells

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Summary
Endothelial cells (ECs) release ATP in response to shear stress, a fluid mechanical force generated by flowing blood but, although its release has a crucial role in controlling a variety of vascular functions by activating purinergic receptors, the mechanism of ATP release has never been established. To analyze the dynamics of ATP release, we developed a novel chemiluminescence imaging method by using cell-surface-attached firefly luciferase and a CCD camera. Upon stimulation of shear stress, cultured human pulmonary artery ECs simultaneously released ATP in two different manners, a highly concentrated, localized manner and a less concentrated, diffuse manner. The localized ATP release occurred at caveolin-1-rich regions of the cell membrane, and was blocked by caveolin-1 knockdown with siRNA and the depletion of plasma membrane cholesterol with methyl-beta-cyclodextrin, indicating involvement of caveolae in localized ATP release. Ca\(^{2+}\) imaging with Fluo-4 combined with ATP imaging revealed that shear stress evoked an increase in intracellular Ca\(^{2+}\) concentration and the subsequent Ca\(^{2+}\) wave that originated from the same sites as the localized ATP release. These findings suggest that localized ATP release at caveolae triggers shear-stress-dependent Ca\(^{2+}\) signaling in ECs.

Key words: Endothelial cells, Shear stress, ATP release, Calcium (Ca\(^{2+}\)) signalling, Caveolae

Introduction
Adenosine 5’-triphosphate (ATP) is the source of the energy that drives virtually all cell functions; it also functions as an autocrine and paracrine regulatory signaling molecule. It is generally acknowledged that many different cell types release ATP in response to mechanical or biochemical stimulation, and that the released ATP modulates cell function by activating nearby purinoceptors, such as ion channel P2X receptors and G-protein-coupled P2Y receptors (Khakh and Burnstock, 2009; Milner et al., 1990; Yegutkin, 2008). However, it remains unclear how cells release endogenous ATP into the extracellular space and, so far, a main hurdle in ATP research has been the difficulty of directly visualizing ATP release by living cells.

The vascular endothelial cells (ECs) that line the inner surface of blood vessels are exposed to shear stress – a biomechanical force generated by flowing blood – and they alter their morphology, function and gene expression in response to changes in shear stress (Ando and Yamamoto, 2009). These EC responses to shear stress play important roles not only in the homeostasis of the circulatory system but in blood-flow-dependent phenomena, such as angiogenesis, vascular remodeling, aneurysm formation and atherosclerosis. Numerous studies have been undertaken to clarify how ECs sense shear stress and transmit the signal into the cell interior, but the mechanism has only partially been revealed (Davies, 1995). Our previous studies have shown that ATP-mediated Ca\(^{2+}\) signaling plays an important role in shear stress mechanotransduction. Shear stress increases intracellular Ca\(^{2+}\) concentration dose-dependently by causing an influx of extracellular Ca\(^{2+}\) through a subtype of P2X purinoceptors, the P2X4 receptors (Yamamoto et al., 2000a; Yamamoto et al., 2000b). Activation of P2X4 receptors requires ATP, which is supplied in the form of endogenous ATP released by ECs (Yamamoto et al., 2003). In addition, our recent study in P2X4-knockout mice revealed that the Ca\(^{2+}\) signaling triggered by shear stress has a crucial role in vascular physiology and pathophysiology, because P2X4-knockout mice were found to exhibit impaired vasodilator responses to acute increases in blood flow and to have higher blood pressure than wild-type mice, both of which were attributable to reduced endothelial nitric oxide (NO) production (Yamamoto et al., 2006). Adaptive vascular structural remodeling in response to a chronic decrease in blood flow was also impaired in the knockout mice (Yamamoto et al., 2006). The mechanism of ATP release as an early response to shear stress, however, is still unknown, and whether ATP release occurs at a specific cellular location and whether it is sufficient to activate nearby purinoceptors remain unclear.

To analyze the dynamics of ATP release, we developed a novel chemiluminescence imaging method by utilizing a biotin–luciferase chimera protein that can be stably immobilized on a
biotinylated cell surface with streptavidin, and an intensified charge-coupled device (CCD) camera. This method allowed us to visualize ATP release at the cell surface in real time and at high resolution. Since caveolae have been implicated as plasma membrane microdomains that sense or transduce altered shear stress into biochemical signals, thereby regulating EC function (Yu et al., 2006), we investigated the relationship between ATP release and caveolae by immunostaining with an antibody against caveolin-1, the primary structural protein of caveolae. In addition, to investigate the role of ATP release in shear stress Ca²⁺ signaling we performed Ca²⁺ imaging by using a fluorescent probe Fluo-4 in the same cells as examined by ATP imaging.

**Results**

**Distribution and ATP sensitivity of cell-surface-attached luciferase**

The distribution of cell-surface-attached luciferase on cells immunostained with a FITC-labeled antibody against luciferase was examined with a confocal laser scanning microscope. En-face images showed an even and dense distribution of biotin–luciferase over the entire cell surface, and longitudinally cut images showed that the biotin–luciferase was localized uniformly on the apical cell membrane (Fig. 1A). Application of shear stress caused no significant change in the distribution of cell-surface-attached luciferase, indicating that this method can be used even under flow conditions.

To assess the ability of cell-surface-attached luciferase to detect ATP, various concentrations of ATP were added to luciferase-labeled human pulmonary artery ECs (HPAECs), and luminescence emitted as a result of the ATP-triggered luciferase–luciferin reaction was measured with a CCD camera. The intensity of the luminescence in response to the addition of ATP increased in a concentration-dependent manner, and there was a clear correlation between the luminescence intensity and ATP concentration (Fig. 1B). These findings indicate that this method of ATP detection allows to determine quantitatively the extracellular ATP concentrations at the cell surface.

**Visualization of shear-stress-induced ATP release by ECs**

Luminescent ATP signals at the cell surface were monitored with a CCD camera before and after shear stress application, and the signals were transformed into pseudo-color images. As soon as the cells were exposed to shear stress, ATP was released from the entire surface of the cell membrane that was monitored, and the ATP signals were particularly strong at localized regions at the edge of the cell (Fig. 2A), thereby indicating the existence of two distinct manners of ATP release, a diffuse manner and a highly concentrated, localized manner.

The temporal changes in ATP signals were quantified in the regions of diffuse ATP release and the regions of localized ATP release (Fig. 2B). ATP release began simultaneously in both regions, but the peak of the ATP signal in both regions was markedly different. The ATP concentration estimated from the luminescence intensity reached more than 10 μM in the regions of localized ATP release, but it remained below 1 μM in the regions of diffuse ATP release (Fig. 2C). The ATP signal in both regions increased further when the shear stress was raised from 10 dynes/cm² to 40 dynes/cm², indicating that the amount of ATP release is shear-stress dependent (Note: 1 dyne = 10 μN).

Secondary application of shear stress to the same cells induced an ATP release that was almost spatiotemporally identical to that of the initial stimulation (data not shown).

**Localized ATP release occurs in caveolin-1-rich regions of the cell edge**

To examine the relationship between the regions of localized ATP release and caveolae (cholesterol-rich plasma membrane microdomains) after visualizing ATP release, cells were immunostained with an antibody to caveolin-1, a marker protein for caveolae. Caveolin-1 was unevenly distributed over the cell surface and was concentrated at specific parts of the cell edge. Comparison between the sites of ATP release and caveolin-1 distribution revealed that the localized ATP release occurred in the caveolin-1-rich cell edge regions (Fig. 3).

To investigate the role of caveolae in shear-stress-induced ATP release, we used small interfering RNA (siRNA) in order to specifically knockdown the expression of caveolin-1. In clear contrast to the control cells, which had been subjected to transfection conditions alone, marked suppression of localized ATP release was observed in the HPAECs transfected with caveolin-1 siRNA (Fig. 4A,B). Caveolin-1 siRNA had no
significant effect on diffuse ATP release. Next, we treated HPAECs with methyl-β-cyclodextrin (MβCD), which disrupts caveolae and lipid rafts by depleting plasma-membrane cholesterol. Treatment with MβCD significantly inhibited shear-stress-induced localized ATP release but did not have a significant effect on diffuse ATP release (Fig. 4A,B). The inhibitory effect of MβCD on localized ATP release was partially prevented by pretreatment with cholesterol. These findings suggest that caveolae are involved in shear-stress-induced, localized ATP release.

Colocalization of localized ATP release and the subsequent initiation of Ca2+ waves
Luciferase-labeled HPAECs were exposed to shear stress and examined for changes in intracellular Ca2+ concentration ([Ca2+]i) by using the Ca2+ indicator Fluo-4 and a fluorescence microscope. Shear stress evoked a rapid increase in [Ca2+]i, that started at a single site in the cell and propagated throughout the entire cell in the form of a Ca2+ wave (Fig. 5A). The Ca2+ wave also propagated into the cell nucleus. After Ca2+ imaging, ATP imaging was performed on the same cells. The regions of localized ATP release coincided exactly with the initiation sites of Ca2+ waves. Comparison between the start of localized ATP release and increase in [Ca2+]i, showed that ATP release always preceded the increase in [Ca2+]i. Treatment of HPAECs with angiostatin, a known blocker of ATP release, almost completely abolished both the shear-stress-induced ATP release and the increase in [Ca2+]i (Fig. 5B). These results suggest that the localized release of ATP at caveolae triggers the increase in [Ca2+]i by activating nearby purinoceptors.

**Discussion**
The novel ATP imaging method described here clearly demonstrated that HPAECs release ATP in response to shear stress in two distinct manners; i.e. a highly concentrated, localized manner and a diffuse manner. A variety of methods can be used to detect ATP that is released at the surface of living cells, including biosensor techniques (Bell et al., 2003; De Proost et al., 2009; Hayashi et al., 2004; Hazama et al., 1998; Llaudet et al., 2005; Schneider et al., 1999) and methods that induce luminescence or measure fluorescence by using ATP-sensitive proteins added to the extracellular space (Arcuino et al., 2002;
Corriden et al., 2007; Wang et al., 2000) or targeted to the plasma membrane (Beigi et al., 1999; Joseph et al., 2003; Okada et al., 2006; Pellegatti et al., 2005). However, some of these methods provide only semi-quantitative information on ATP release, others are unsuitable for the visualization of ATP release, mainly because of weak signals. In our study, however, we were able to visualize ATP release of ECs by using a cell-surface-targeting luciferase and a high-resolution CCD camera. To obtain stronger signals, we generated a biotin–luciferase fusion protein that can attach to biotinylated cell surfaces through interaction with streptavidin (Nakamura et al., 2006). Since various plasma membrane proteins can bind to many biotins and one streptavidin molecule has four biotin-binding sites, a large amount of luciferase can be bound to the cell surface. In addition, by using the PicaGene reagent, which emits several times more luminescence than other luciferase–luciferin reaction reagents, it became possible to obtain luminescence strong enough for high-resolution imaging of ATP release. This imaging method should prove useful for studying ATP release mechanisms and the functional roles of ATP in various cell types.

Caveolae are small flask-shaped invaginations of cholesterol-rich cell membranes. They are characterized by the presence of the protein marker caveolin-1 and are known to play crucial roles in multiple signal transduction events at the surface of various cell types (Shaul and Anderson, 1998). Recent studies have demonstrated that caveolae sense or transduce shear stress into biochemical signals that regulate EC functions. Shear stress has been found to activate extracellular-signal-regulated kinase (ERK) in bovine aortic ECs that was blocked by the delivery of polyclonal caveolin-1 antibody into the cells (Park et al., 2000). The production of a potent vasodilator, nitric oxide (NO), by ECs increases in response to shear stress, and one of the mechanisms of shear-stress-induced NO production is dissociation of endothelial NO synthase (eNOS) from caveolae followed by activation of eNOS (Rizzo et al., 1998). A more recent study comparing caveolin-1 knockout mice with wild-type mice showed that lack of caveolin-1 impaired blood-flow-dependent eNOS activation, vasodilation, and vascular remodeling and that these abnormalities were rescued by reconstituting caveolin-1 into the vascular endothelium of the knockout mice (Yu et al., 2006). The present study revealed that localized ATP release induced by exposing HPAECs to shear stress occurs in caveolin-1-rich regions of the plasma membrane. The localized ATP release was markedly suppressed by knockdown of caveolin-1 expression with siRNA and by depletion of membrane cholesterol with MβCD, which disrupts caveolae/lipid rafts. Although the possibility remains that the inhibition of the localized ATP release by caveolin-1 siRNA and MβCD is attributable to secondary rather than primary effects, these findings suggest that caveolae are involved in the localized ATP release that occurs in response to shear stress.

It remains unclear why the localized ATP release occurs preferentially at caveolin-1-rich regions of the cell membrane. Since shear stress causes microscale deformation/displacement of cell surface membrane proteins, the lipid bilayer itself, and the cytoskeleton and connected proteins, it seems likely that the sites of shear-stress-induced deformation are linked to the localization of ATP release. Caveolae/lipid raft microdomains are characterized by a unique lipid composition that contains high concentrations of cholesterol and sphingolipids in places where the lipid bilayer is more rigid and lipid movement is more restricted than in other parts of the membrane (Gaus et al., 2003). These differences in the physical properties of the membrane may be responsible for the localization of ATP release. On the other hand, caveolae/lipid rafts are closely associated with cytoskeleton filaments, and shear stress may affect the caveolae and associated molecules through cytoskeleton networks. Helmke et al. showed that strain in the intermediate filament was heterogeneous, with small regions of high-strain concentrations located at the cell periphery and at several sites in the cell interior, and that the hot spots of strain concentration were repositioned by shear stress (Helmke et al., 2003). These hot spots of cytoskeletal strain may coincide with the locations of ATP release. It requires further research to clarify whether the localization of ATP release is a plasma membrane domain phenomenon or influenced by interference with cytoskeleton function or both.

Depending on the cell type and extrinsic stimulus, ATP is released into the extracellular space by ATP-permeable membrane channels, including connexin hemichannels (Stout et al., 2002) and volume-regulated anion channels (Sabirov and Okada, 2004), by diffusion facilitated by ATP-binding cassette transporters, such as the cystic fibrosis transmembrane...
conductance regulator (Schwiebert et al., 1999), or by vesicular transport and exocytotic secretion (Bodin and Burnstock, 2001). Our previous study revealed that ATP synthase is localized in caveolae and involved in shear-stress-induced ATP release by HPAECs (Yamamoto et al., 2007). However, it remained unclear whether caveolar ATP synthase is involved in any of the above-mentioned ATP-releasing pathways or in an unknown pathway. In view of the discovery of the two different manners of ATP release in this study, HPAECs may use dual pathways to release ATP in response to shear stress. Further study will be needed to identify the pathway responsible for each manner of ATP release.

In the present study we performed intracellular Ca$^{2+}$ imaging and ATP imaging of the same cells. Shear stress evoked an increase in [Ca$^{2+}$], that originated at a specific site and propagated throughout the entire cell in the form of a Ca$^{2+}$ wave in a manner that resembled to the Ca$^{2+}$ responses previously observed in bovine fetal aortic ECs (Ishiki et al., 1998). The sites where the increase in [Ca$^{2+}$], originated coincided with the sites of localized ATP release. Comparisons between the start of the [Ca$^{2+}$] increase and ATP release revealed that ATP release always preceded the Ca$^{2+}$ increase. The ATP concentration at the sites of localized ATP release reached more than 10 μM, which is sufficient to activate purinoreceptors. Thus, it seems that shear stress first triggers ATP release, which activates nearby P2X and/or P2Y receptors, and, in turn, leads to Ca$^{2+}$ responses. Many studies have been devoted to investigation of shear stress mechanotransduction and have demonstrated the involvement of various membrane molecules and cellular microdomains in its mechanisms, including ion channels, growth factor receptors, G proteins, caveolae, adhesion proteins such as integrin, VE-cadherin, and platelet endothelial cell adhesion molecule-1, the cytoskeleton, the glycocalyx, and primary cilia (Ando and Yamamoto, 2009; Davies, 1995). However, it is unclear how these molecules and microdomains enable ECs to sense shear stress and transmit the signal to downstream effectors to allow cells to respond. Our previous studies in ECs demonstrated that the P2X4 receptor contributes to shear stress mechanotransduction through Ca$^{2+}$ signaling, which plays a crucial role in the control of vascular function in vivo (Yamamoto et al., 2000a; Yamamoto et al., 2003). Clarification of the mechanisms of ATP release as an early response to shear stress should lead to a better understanding of the mechanotransduction of shear stress.

Materials and Methods

Cell culture

Human pulmonary artery endothelial cells (HPAECs) were obtained from Clonetics and grown on a 1% gelatin-coated tissue culture flask in M199 supplemented with 15% FBS, 2 mM L-glutamine (Gibco), 50 μg/ml heparin, and 30 μg/ml EC growth factor (Becton Dickinson). The cells used in the present experiments were in the 7th and 10th passage.

Production and purification of biotin–luciferase

Biotin–luciferase protein was produced and purified as previously reported (Nakamura et al., 2006). Briefly, biotin acceptor peptide (BAP) was fused to thermostabilized firefly luciferase, and the biotin–luciferase gene fusion plasmid, pET-NHis-BAP-Luc, was constructed. For purification, six repeats of the histidine sequence (His-tag) were coded to the N terminus of the fusion gene. The pET-NHis-BAP-Luc plasmid was transfected into Escherichia coli BL21 (DE3) competent cells and the cells were grown and lysed by sonication. The soluble fraction of the bacterial lysates was subjected to metal-ion affinity chromatography in order to purify biotin–luciferase protein by using the His-tag.

Cell-surface labeling with biotin–luciferase

HPAECs cultured on coverslips were incubated with 250 μg/ml EZ-Link® Sulfo-NHS-Biotin (Thermo Scientific Pierce, Rockford, IL) for 10 minutes at room temperature. After washing with HBSS, cells were treated with 2 μM streptavidin (Wako) for 30 minutes at 37°C and then, after another HBSS wash, incubated with
1 mg/ml biotin–luciferase for 30 minutes at room temperature. To observe the distribution of biotin–luciferase on the cell surface, cells were immunostained with FITC-conjugated anti-luciferase antibody (10 μg/ml, Rockland).

Shear-stress stimulation and ATP imaging
A coverslip on which luciferase-labeled cells had been cultured was placed in a parallel-plate flow chamber whose temperature can be controlled (FCS2, Flukum).
images were acquired sequentially with an exposure period of 100 milliseconds as (ImagEM C9100-13, Hamamatsu), extracellular ATP levels at the cell surface

40, 1.15 NA, Nikon) with a water-cooling electron multiplier CCD camera

breakdown of luciferin was detected through a water immersion objective (Fluor

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Statistical analysis

All results are expressed as the mean ± s.d. Statistical significance was evaluated

using ANOVA and Bonferroni adjustments applied to the results of Student’s t-test

performed with SPSS software (SPSS Inc). Values of P < 0.01 were regarded as

statistically significant.

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