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Real-time imaging of mechanically and chemically induced ATP release in human lung fibroblasts

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ABSTRACT

Extracellular adenosine 5′-triphosphate (ATP) acts as an inflammatory mediator of pulmonary fibrosis. We investigated the effects of mechanical and chemical stimuli on ATP release from primary normal human lung fibroblasts. We visualized the ATP release from fibroblasts in real time using a luminescence imaging system while acquiring differential interference contrast cell images with infrared optics. Immediately following a single uniaxial stretch for 1 s, ATP was released from a certain population of cells and spread to surrounding spaces. Hypotonic stress, which causes plasma membrane stretching, also induced the ATP release. Compared with the effects of mechanical stretch, ATP-induced release sites were homogeneously distributed. In contrast to the effects of mechanical stimuli, application of platelet-derived growth factor caused ATP release from small numbers of the cells. Our real-time ATP imaging demonstrates that there is a heterogeneous nature of ATP release from lung fibroblasts in response to mechanical and chemical stimuli.

1. Introduction

Irreversible or progressive pulmonary fibrosis is an important characteristic of several lethal lung diseases, specifically idiopathic pulmonary fibrosis (IPF) and severe acute respiratory distress syndrome (ARDS) (Pelham and Wang, 1997; Raghu et al., 2011; Wynn and Ramalingam, 2012; Barkauskas and Noble, 2014). In mechanically ventilated patients with respiratory failure due to ARDS or acute exacerbation of IPF, the lung is exposed to excessive stretch, which often causes further damage and fibrosis (Slutsky and Ranieri, 2013). Recent studies have suggested that mechanical forces such as stretch and shear stress are involved in the mechanisms underlying the pathogenesis of pulmonary fibrosis by activating lung fibroblasts (Hinz, 2012; Marinkovic et al., 2013; Ellson et al., 2014). Lung fibroblasts play a central role in production and deposition of extracellular matrix associated with pulmonary fibrosis (Phan, 2008; Hinz, 2012).

Extracellular ATP is an important ubiquitous mediator of intercellular signaling in the body, and it induces a wide range of physiological responses by activating purinergic P2X and P2Y receptors (Burnstock et al., 2012). In addition to its physiological roles, extracellular ATP is considered to participate in the pathophysiology of various pulmonary diseases, including pulmonary fibrosis, as a ‘danger-associated molecular pattern’ (DAMP) (Kunzelmann and Mall, 2003; Idzko et al., 2007; Mortaz et al., 2010; Riteau et al., 2010; Burnstock et al., 2012; Ellson et al., 2014; Pelleg et al., 2016). In the lung and airway, ATP is released from airway epithelial cells, alveolar epithelial cells, vascular endothelial cells, and airway smooth muscle cells in response to physical and chemical stimuli (Okada et al., 2006; Ramsingh et al., 2011; Burnstock et al., 2012; Grygorczyk et al., 2013; Okada et al., 2013; Takahara et al., 2014; Ito et al., 2016). We previously reported that ATP is released from normal human lung fibroblasts in response to cyclic mechanical stretch (Murata et al., 2014). However, the details of duration of ATP release and spatial distribution of ATP releasing cells remain poorly defined.

This study was designed to visualize how mechanical stimuli induce ATP release from primary human lung fibroblasts. To this end, we used an image analysis system and a cell stretching device that allowed us to visualize extracellular ATP released following mechanical stretch, hypotonic stress, and platelet-derived growth factor (PDGF) (Grygorczyk et al., 2013; Furuya et al., 2014; Takahara et al., 2014).

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2. Materials and methods

2.1. Cell culture

Primary cultures of normal human lung fibroblasts from three different donors were obtained from Lonza (Walkersville, MD). The cells were maintained in culture medium (FGM-2 BulletKit; Lonza) in an atmosphere of 5% CO_2 and 95% air at 37 °C.

2.2. Application of uniaxial cyclic mechanical stretch

Cells were cultured in 4-cm^2 silicone chambers (STB-CH-04; Strex, Osaka, Japan) coated with type I collagen. A uniaxial sinusoidal cyclic stretch (30 cycles/min) was applied to the chamber using a stretching apparatus (ST-140; Strex) (Iwaki et al., 2009; Morioka et al., 2011; Murata et al., 2014; Takahara et al., 2014). Strain was calculated by elongation of the silicone chamber. Either 4% or 20% stain was applied to the chamber.

2.3. Application of a single uniaxial stretch for real-time ATP imaging

Uniaxial mechanical stretch was applied to chambers made of a Silpot 184W/C silicone elastomer (Dow Corning, Midland, MI) with a 2-mm wide groove in the center where cells were seeded using a stretching apparatus (NS-600W; Strex) (Grygorczyk et al., 2013; Furuya et al., 2014; Takahara et al., 2014). The silicone chamber was coated with type I collagen. Strain was calculated from observations of displacement of the same cells before and after the stretch in infrared differential interference contrast (DIC) imaging. In the present study, 22% strain of 1 s duration was applied to the chamber.

2.4. Measurement of ATP concentrations

The concentrations of ATP of cell supernatants were measured by a luminometer (Berthold LB9506; Wildbad, Germany) using a luciferin-luciferase reagent (Lucifere250; Kikkoman Biochemifa, Tokyo, Japan) (Furuya et al., 2005; Murata et al., 2014; Takahara et al., 2014).

2.5. Real-time imaging of extracellular ATP

We visualized ATP release during cell stretch in real time using a luminescence imaging system under an upright microscope (BX51WI, 4 NA0.28 objective; Olympus, Tokyo, Japan) equipped with high-sensitivity EM-CCD cameras (Cascade 512F; Photometrics, Tucson, AZ) and a water-cooled image intensifier (C8600-04; Hamamatsu Photonics, Hamamatsu, Japan) (Grygorczyk et al., 2013; Furuya et al., 2014; Takahara et al., 2014). ATP bioluminescence was detected while infrared DIC images of the cells were simultaneously obtained. The imaging system and data acquisition were controlled by MetaMorph ver.7.5 (Molecular Devices, Downingtown, PA), and data were acquired every 100 ms or 500 ms. To generate ATP luminescence, we used high-sensitivity, glycine-buffered luciferin-luciferase stock (LuciferHS; Kikkoman Biochemifa) containing apyrase, which lowered a background luminescence and enabled high sensitive imaging. The effect of apyrase in the solution on released ATP is restrictive. It reduces ATP luminescence with a decay time constant of several minutes after the faster decay due to the diffusion of ATP. Thus, the kinetics of the raising phase and temporal pattern of ATP release are little affected by the apyrase because of a large difference of the time constant. ATP imaging was performed at 32 ± 3 °C.

2.6. Solution

To measure ATP, the FGM-2 BulletKit cell culture medium was changed to phenol-free DMEM/F-12 (Invitrogen, Carlsbad, CA) without FBS 1 h prior to stretching. The hypotonic solution (30%) was made by replacing phenol-free DMEM/F-12 with an equal amount of distilled water containing 2 mM $CaCl_2$ and 1 mM $MgCl_2$. The osmolality of the solutions was checked with a freezing point osmometer OM801 (Vogel, Giessen, Germany).

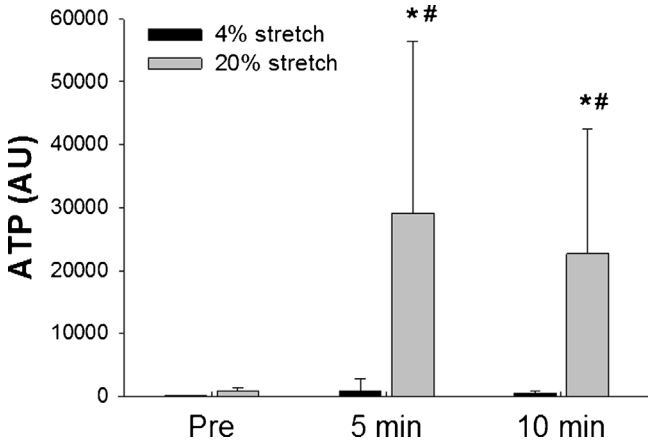

Fig. 1. Effects of uniaxial mechanical stretch on ATP release from normal human lung fibroblasts. Cyclic mechanical stretch (4% or 20% strain, 30 cycles/min for 10 min) was applied to 4-cm^2 silicone chambers coated with type I collagen in which cells were grown. Bulk concentrations of ATP in the cell supernatant were measured by a luciferin-luciferase luminescence (AU; arbitrary unit). Values are means (± SD) ($n = 6$). Significantly different ($^*P < 0.05$ *vs.* pre-stretched values, $^{\#}P < 0.05$ *vs.* 4% stretch).

2.7. Statistical analysis

Data are expressed as means (± SD). Two-way ANOVA was used to evaluate the statistical significance (SigmaPlot11.0; Systat Software Inc., San Jose, CA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. ATP release induced by cyclic mechanical stretch

Cyclic mechanical stretch (either 4% or 20% strain, 30 cycles/min) was applied for 10 min to the 4-cm^2 silicone chamber in which the cells were grown. The bulk concentrations of ATP in the cell culture supernatant were significantly elevated by 20% strain but not by 4% strain (Fig. 1). The concentrations of ATP induced by 20% stretch for 5 and 10 min were not significantly different. Cell viability as assessed by trypan blue extrusion was not affected after applying 20% stretch at 30 cycles/min for 10 min.

3.2. Visualization of ATP release by mechanical stretch

ATP release from the cells was visualized in real time. Fig. 2A shows representative luciferin-luciferase bioluminescent images for ATP (red) and infrared-DIC images for the cells at rest, during substrate stretching, and after unloading. The chamber was held in the stretched position for one second, and returned to the initial unstretched state. Following a single uniaxial stretch (22% strain) of 1 s duration, releases of ATP continued and increased in intensity and number, and spread to their surrounding spaces heterogeneously. ATP was released from multiple sites (24 active sites in the viewing field) (Fig. 2A). ATP releases were started at less than 500 ms after stretching (Fig. 2B). The time course (4 min) of luminescence intensity of the 24 active sites and the average values of their ATP release are shown in Fig. 2C. The time to the peak of ATP release from individual cells was heterogeneous in the range from 4 to 35 s with the average of 18 s after stretching (Fig. 2C). The duration of ATP release was 15–70 s. A movie of the ATP release due to 22% stretch is shown in Movie S1 (luminescence (red) and infrared-DIC (green) images) in the online supplement.

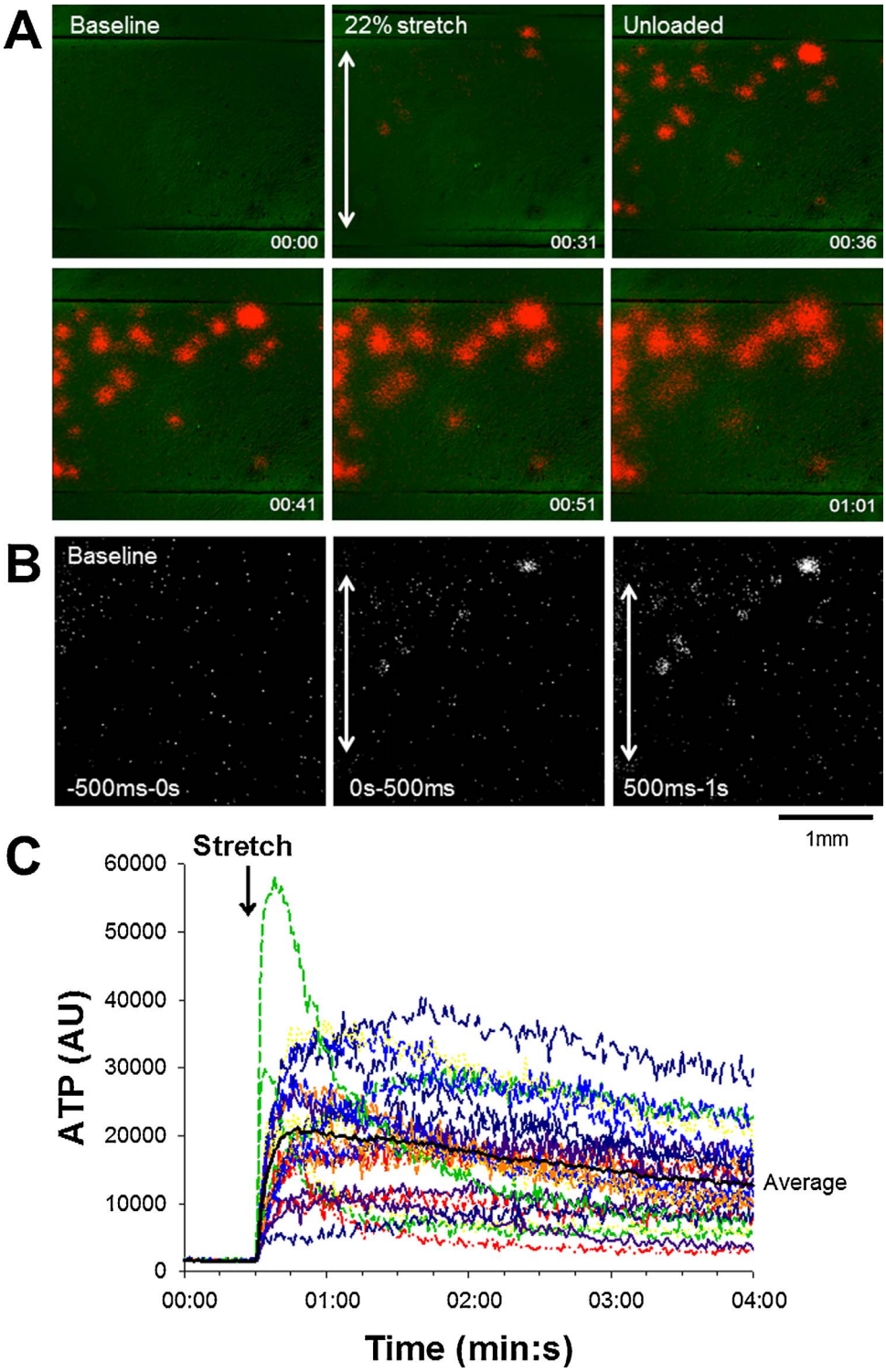

Fig. 2. Real-time imaging of ATP release induced by a single stretch. The chamber on which cells were cultured was mechanically stretched 1-s and then unloaded. (A) Sequence of luminescence images of ATP release (red) and infrared DIC images (green) of the cells at rest (baseline; upper left), during 1-s uniaxial stretch of 22% strain (upper middle, 31 s) along the vertical axis indicated by the black arrow, and in the unloaded state. See also Movie S1 in online supplement. (B) Luminescence of released ATP was observed even during 1-s stretching of 22% strain. (C) Time course of local luminescence intensities at 24 release sites (lines) and average (thick black line) of 24 data points after 22% stretch (arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Visualization of ATP release by hypotonic stress

Next, we visualized how stretching the plasma membrane by hypotonic stress induces ATP release. Following application of hypotonic solution (30%), releases of ATP increased in intensity and numbers, and spread to their surrounding spaces (Fig. 3A). Compared with the effects of mechanical stretch, the ATP release sites were homogeneously distributed (Fig. 3A). Kinetic analysis of luminescence intensities of ATP release for 12 min and 3 min is shown in Fig. 3B and C. Each response was transient with short duration of about 10–30 s. A movie of the luminescence of the ATP release in response to 30% hypotonic stress for 12 min duration is shown in Movie S2 in the online supplement.

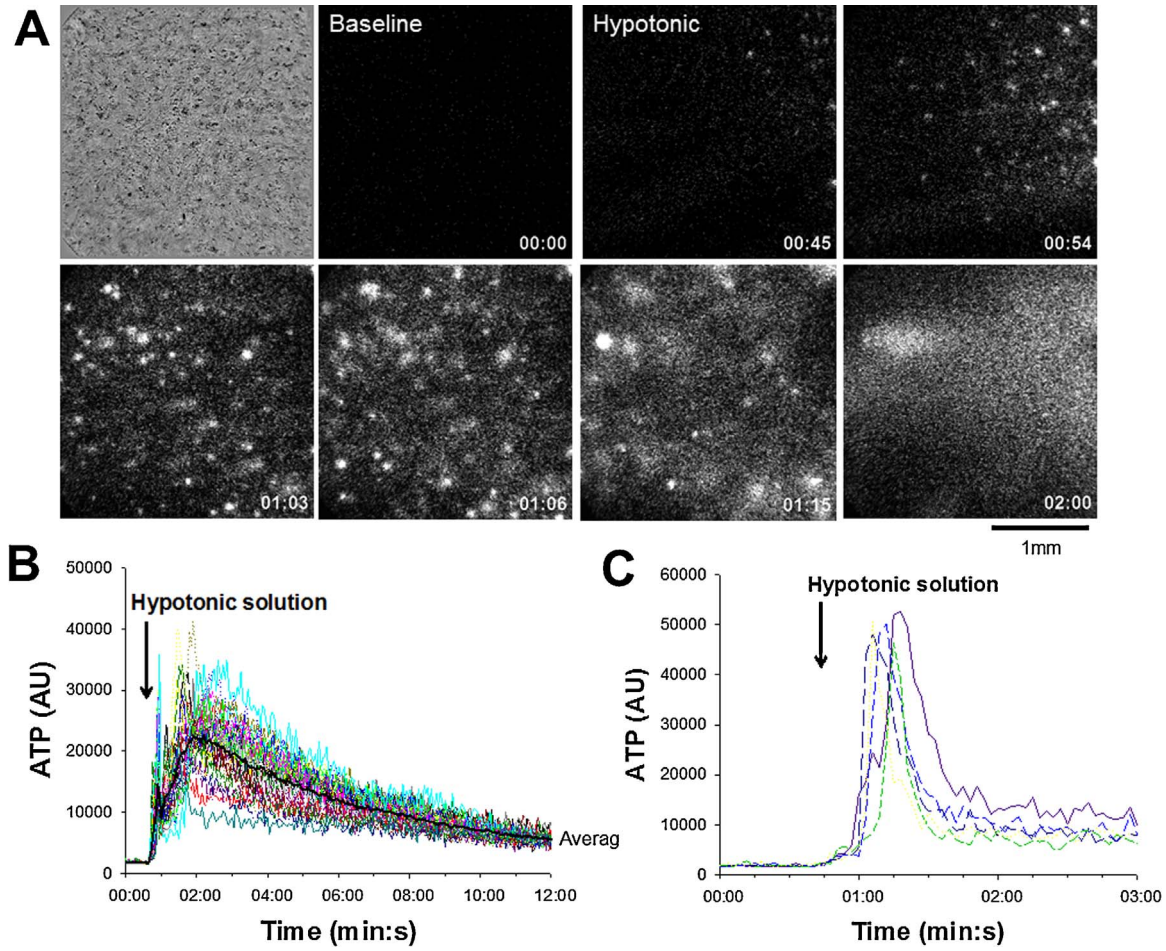

Fig. 3. Effects of stretch of the plasma membrane due to hypotonic stress on ATP release from fibroblasts. (A) Examples of luminescence images of ATP release (white) after application of 30% hypotonic solution at 30 s. (B) Time courses of local luminescence intensities of ATP release at 30 sites (lines) and average (thick black line) of 30 data points after application of hypotonic stress (arrow). See also Movie S2 in online supplement. (C) Typical time courses of ATP release at five sites (lines) for 3 min duration.

3.4. Visualization of ATP release by platelet-derived growth factor on

Next, the effects of chemical stimulation on ATP release were examined. Following application of PDGF-BB (10 ng/ml, Sigma–Aldrich, St. Louis, MO), ATP was transiently released from nine sites in the viewing field (Fig. 4A). Unlike the responses to stretch and hypotonic stress, the reaction to PDGF-BB did not occur immediately and the timing and duration of ATP release were heterogeneous. The time course of luminescence intensity of the nine active sites and their average values due to ATP release are shown in Fig. 4B. A movie of the ATP release in response to PDGF-BB is shown in Movie S3 in the online supplement.

4. Discussion

The bioluminescence of the ATP-dependent luciferase-mediated oxidation of luciferin is widely used as the standard technique to assess extracellular bulk ATP (Wood et al., 1989; Praetorius and Leipziger, 2009) as shown in Fig. 1. In this study, we used highly-sensitive luciferin-luciferase bioluminescence imaging coupled with infrared DIC imaging and a cell stretching apparatus (Grygorczyk et al., 2013; Furuya et al., 2014). This is the first study that directly visualized the extracellular ATP released from lung fibroblasts in response to uniaxial stretch, hypotonic stress, and PDGF at the cellular level. Importantly, ATP responses to stretch and PDGF are heterogeneous with respect to the release amplitude and duration and spatial distribution of releasing cells at the single-cell level (Figs. 2 and 4, Movie S1 and S3 in online supplement). Limited numbers of cells released ATP in response to a single stretch, consistent with previous findings in other cell types (Grygorczyk et al., 2013; Furuya et al., 2014; Takahara et al., 2014; Ito et al., 2016). In contrast to effects of stretch and PDGF, sites releasing ATP induced by hypotonic stress were homogeneously distributed. It is considered that hypotonic solution is able to stretch the plasma membrane homogeneously and in all directions. Moreover, there were various temporal patterns in the time when fibroblasts started releasing ATP following application of hypotonic stress (Fig. 3B and C) and PDGF (Fig. 4B). Taken together, our real-time ATP imaging has an advantage in assessing cell populations releasing ATP of a heterogeneous nature at the single cell level.

It is widely recognized that ATP is released from various kinds of cells in the respiratory system both as physiological and abnormal phenomena (Taylor et al., 1998; Burnstock et al., 2012; Ito et al., 2016; Pelleg et al., 2016). The present findings indicate that lung fibroblasts are another cellular source of extracellular ATP in the lung. In the physiological condition, the concentration of extracellular ATP *in vivo* is strictly controlled at a baseline steady state by balancing the amounts of ATP release and hydrolysis of the ATP by ecto-ATPases (Praetorius and Leipziger, 2009). Increased extracellular ATP levels act as a DAMP in the pathophysiology of pulmonary fibrosis and lung injury (Willart and Lambrecht, 2009; Riteau et al., 2010). Riteau et al. (2010) demonstrated that concentrations of ATP in bronchoalveolar lavage fluid from patients with IPF and bleomycin-treated mice were significantly higher than those of control subjects. PDGF is known as a mediator for promoting pulmonary fibrosis (Vignaud et al., 1991; Raghu et al., 2011). Although the PDGF-induced ATP release response was smaller than that induced by mechanical stimuli, the ATP release may partially

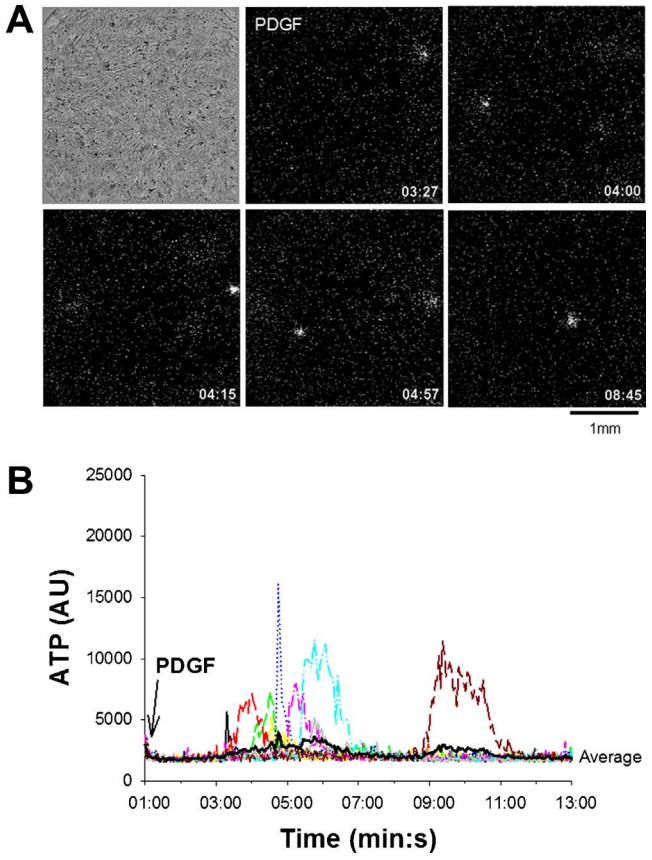

Fig. 4. Effects of chemical stimulation by PDGF on ATP release. (A) Example of luminescence due to extracellular ATP (white) at different time points after application of PDGF-BB (10 ng/ml) at 30 s. (B) Time courses of local luminescence images of ATP release at nine sites (lines) and average (thick black line) of nine data points after application of PDGF-BB (arrow). See also Movie S3 in online supplement.

be involved in the effects of PDGF on fibrotic responses of the lung. Taken together, although the ATP release responses are different between applied stimuli, ATP release from lung fibroblasts due to mechanical forces and PDGF may contribute to the pathophysiology of pulmonary fibrosis and severe lung injury.

In the present study, continuous stretching of the plasma membrane due to hypotonic stress *in vitro* is consistent with disease conditions *in vivo*, such as near drowning in freshwater, which cause severe pulmonary edema and injury (Rumbak, 1996). We applied a small (4%) or large (20% and 22%) uniaxial strain, which mimic tidal breathing in the physiological condition and hyperinflation during mechanical ventilation *in vivo*, respectively, to lung fibroblasts (Roan and Waters, 2011; Murata et al., 2014). The structure and stiffness of lung parenchymal tissue are regionally heterogeneous specifically in pulmonary fibrosis and ARDS (Matthay and Zemans, 2011; Carloni et al., 2013). Due to their heterogeneous nature, the exact strains and stresses may differ between the lesions in the lung. Recently, using intact rat lung tissues, Furuya et al. demonstrated that inflation of the lung induced ATP release and that the release sites were distributed heterogeneously (Furuya et al., 2016). The results of our *in vitro* experiments are consistent with those of the rat lung *in situ*. However, it is still unknown how ATP released from lung fibroblasts affects the respiratory system *in vivo*. The intracellular mechanisms and autocrine/paracrine mechanisms of ATP release were not investigated in our study. Multiple mechanisms and pathways underlying ATP release have been proposed (Praetorius and Leipziger, 2009; Adamson and Leitinger, 2014; Dahl, 2015). Future studies are needed to address mechanisms of a heterogeneous nature and intracellular regulation of ATP release.

In summary, using a real-time ATP imaging system, we demonstrated that ATP was heterogeneously released by a certain population of cells and spread to surrounding spaces in response to mechanical stimuli and PDGF. Future research will be important to determine how extracellular ATP in the lung released from fibroblasts and other cells contributes to the pathophysiology of pulmonary fibrosis and lung injury.

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Appendix A. Supplementary data

Supplementary data (Movie S1, S2, and S3) associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.resp.2017.04.008.

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