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Vascular Endothelial Barrier Dysfunction Mediated by Amyloid- β Proteins

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Abstract

Neuronal inflammation is very common in Alzheimer's disease (AD). This inflammation can be caused by infiltration of neutrophils across the blood brain barrier (BBB). Endothelial permeability changes are required for the infiltration of high molecular weight components to the brain. Deposition of toxic amyloid- β (A β) fibrils in the cerebral vasculature, as well as in brain neurons has been implicated in the development of AD. This study investigates the effect of A β fibrils on the permeability of the endothelium and the mechanism for the observed permeability changes. A β (1–40) and (1–42) fibrils, but not monomers, were found to increase permeability of bovine pulmonary arterial endothelial cells in a dose- and time-dependent manner as detected by transendothelial electrical resistance. This increase in permeability is only partially (25%) inhibited by catalase and is not associated with an increase in cytosolic Ca⁺² or tyrosine phosphorylation. These results indicate that hydrogen peroxide is not the primary mediator for the permeability changes. Treatment of cells with both amyloid fibrils resulted in stress fiber formation, disruption and aggregation of actin filaments and cellular gap formation. The results of this study reveal that A β increases the permeability of endothelium by inducing change in the cytoskeleton network.

Keywords

Amyloid- β ; Endothelial cells; Alzheimer's disease; Permeability

INTRODUCTION

Alzheimer's disease (AD) is a progressive neuronal degenerative disorder of the central nervous system that ultimately results in the loss of cognitive function [1]. This disease is characterized by the accumulation of toxic amyloid- β (A β) proteins in the extracellular space of the brain and on the wall of blood vessels in the brain [2,3].

The endothelium of brain blood vessels provides a semi-permeable barrier between blood components and brain tissue. Maintenance of the integrity of this barrier is crucial for vessel wall homeostasis. Evidence for dysfunction in the endothelial barrier of the brain vascular system in AD subjects is provided by the prevalence of an increase in microvascular permeability and disruption of cerebral vascular endothelial cell (EC) tight junctions [4,5] and

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cerebrovascular inflammation in Alzheimer's disease[6]. Impaired integrity of the blood brain barrier has also been reported in animal models of AD [7].

In animal models the impaired integrity of the BBB has been shown to be restored following immunization with antibodies against A β implicating vascular A β in the impaired BBB of these animals [8]. A role for A β in barrier dysfunction is further implied by studies involving the intracarotid infusion of A β into rats [9] and guinea pigs [10]. These studies demonstrate that infused A β penetrates the blood brain barrier and is found in the brain parenchyma. Amyloid β induced damage also increases the permeability of cerebral vessels and results in the transfer of T lymphocytes into brain [11].

The mechanism for A β induced barrier dysfunction has, however, not been elucidated. The barrier functions of ECs are known to be regulated by signal transduction systems of junctional and cytoskeletal proteins[12]. Elevated concentrations of reactive oxygen species (ROS) in the range of 50 μ M to 100 μ M alter the signal transduction process increasing the permeability of the endothelium and promoting leukocyte adhesion leading to transmigration of leukocytes to underlying tissue [13]. This process generates inflammation leading to impairment of the endothelial barrier function [12,14]. Amyloid- β fibrils are known to generate ROS causing lipid peroxidation and protein damage [15–17]. It is, however, not known to what extent A β induced changes in barrier function are associated with the production of ROS. In this study, we characterize the effect of A β proteins on vascular ECs that can be associated with changes in permeability and investigate the contribution of ROS to the observed changes in permeability.

MATERIALS AND METHODS

Synthetic A β (1–40), (40–1), (1–42) and (42–1) were obtained from BioSource International, Inc. (Camarillo, CA). Peptides were suspended in phosphate buffered saline, pH 7.4 without calcium and magnesium at a concentration of 250 μ M and incubated at 37° C for 36 hrs to form fibrils. Under these conditions the (1–40) and (1–42) A β form fibrils but the (40–1) and (42–1) reverse sequence A β do not form fibrils.

Cell Culture

Bovine pulmonary arterial endothelial cells (BPAECs) were cultured in minimum essential medium (MEM) with 10% fetal calf serum. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air. Cells grown in culture flasks were detached with 0.05% trypsin and resuspended in fresh medium and cultured on gold electrodes for electrical resistance determinations, or on glass coverslips for calcium and immunocytochemistry studies, or in 100-mm dishes for Western blotting.

Measurement of Transendothelial Electrical Resistance (TER)

TER was measured in an electrical cell-substrate impedance sensing system, Applied Biophysics, Inc. (Troy, NY) [18]. The cells were cultured on gold microelectrodes (8 electrodes per plate) until reaching ~95% confluence. At this stage tight cell-cell junctions are formed. One hour before TER measurements, the cells were rinsed with serum-free MEM media and 0.38 ml of serum-free media was added to each electrode. Electrodes were placed into an electrical cell-substrate impedance incubator for 30 min-1 h to stabilize basal electrical resistance. The tightness of the junctions is reflected by their electrical resistance. The resistance is expressed as normalized resistance (1×10^3 Ohms) in time course experiments. Once cells attained stable TER, A β with or without SOD or catalase were added into electrodes and the TER was monitored for a designated time. A decrease in TER is an indication of weakened cell-cell junctions and increased permeability.

Measurement of Intracellular Ca^{2+} Concentration

BPAECs were plated on glass coverslips (Hitachi Instruments) pretreated with 0.1% gelatin solution and grown to ~95% confluence in complete MEM. All procedures for Ca^{+2} measurement were carried out in basic media: 116 mM NaCl, 5.37 mM KCl, 26.2 mM NaHCO_3 , 1.8 mM CaCl_2 , 0.81 mM MgSO_4 , 1.02 mM NaHPO_4 , 5.5 mM glucose, and 10 mM HEPES/HCl, pH 7.40. Cells were loaded with 5 μM Fura-2 AM (acetoxymethylester of Fura-2) in 1 ml of the above media in the presence of 0.1% bovine serum albumin and 0.03% pluronic acid at 37°C in a cell culture incubator, as recommended by the manufacturer's protocol. Cells were rinsed twice, and a coverslip was inserted diagonally into 1.0 cm acrylic cuvettes (Sarstedt, Newton, NC), which were filled with 3 ml of the incubation media at 37°C. Fura-2 fluorescence was measured with an Aminco-Bowman Series 2 luminescence spectrometer (SLM/Aminco, Urbana, IL) at excitation wavelengths of 340 and 380 nm and emission wavelength of 510 nm. Intracellular free calcium, $[\text{Ca}^{2+}]_i$, was calculated from the 340/380 ratio using a Ca^{2+} calibration curve and manufacturer software.

Preparation of Cell Lysates and Western Blotting

BPAECs were grown on 100-mm culture dishes to ~95% confluence in complete MEM. Subsequent incubations were carried out in serum-free media. Cells were incubated with 10 $\mu\text{g/ml}$ of $\text{A}\beta(40-1)$, $\text{A}\beta(1-40)$, $\text{A}\beta(42-1)$, and $\text{A}\beta(1-42)$ for 1 hr. After incubation with $\text{A}\beta$, the reaction was stopped by rinsing with ice-cold PBS containing 1 mM orthovanadate. ECs were lysed with 0.5–1 ml of non-denaturing cell lyses buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA , 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g/ml}$ leupeptin and 5% protease inhibitor mixture (Roche Applied Science). The lysed cells were scraped off the culture dish and sonicated on ice with a probe sonicator (15s $\times$ 2) followed by centrifugation at 5000 $\times$ g in a microcentrifuge at 4°C for 5 min. Protein concentrations of the supernatants were determined using the Pierce protein assay kit. The supernatants, adjusted to 0.5–1 mg protein/ml, were denatured by boiling in 1 $\times$ SDS sample buffer for 5 min and analyzed on 10% SDS-PAGE gels. Protein bands were transferred overnight (25 V, 4°C) onto polyvinylidene difluoride (Millipore) membranes, probed with primary antibody, anti-phosphotyrosine, clone 4G10 (Upstate Biotechnology Inc, NY, USA) and secondary antibody, peroxidase conjugated goat anti-mouse IgG (Upstate Biotechnology Inc, NY, USA) and immunodetected by using the enhanced chemiluminescence kit (Amersham Biosciences) according to the manufacturer's protocol. The blots were scanned (UMAX Power Look II) and quantified by the automated digitizing system UN-SCAN-IT GEL (Silk Scientific Corp.).

Immunofluorescence Microscopy

BPAECs grown on coverslips to ~95% confluence were treated with $\text{A}\beta(1-40)$ and $\text{A}\beta(1-42)$ for 6 hrs, and rinsed twice with PBS, and treated with 3.7% formaldehyde in PBS for 10 min at room temperature and then permeabilized for 4 min with 0.25% Triton X-100 prepared in 3.7% formaldehyde. After washing, cells were incubated for 30 min at room temperature in TBST blocking buffer containing 1% bovine serum albumin. Actin stress fibers were determined by staining cells on coverslips with Alexa Fluor phalloidin 568. Cells were visualized by Nikon Eclipse TE 2000-S immunofluorescence microscopy with a Hamamatsu digital camera (Japan) using a 60x oil immersion objective and MetaVue software (Universal Imaging Corp.).

RESULTS

Effect of A β (1–40) and A β (1–42)- fibrils on the permeability of BPAECs

Both A β fibrils (1–40 and 1–42) formed by incubation of A β peptides for 36 hr at 37°C decreased the TER of EC monolayers in a time- and concentration-dependent manner (Fig. 1). The effect of the (1–42) fibrils (Fig. 1B) is, however, significantly faster than the effect of (1–40) fibrils (Fig. 1A). Thus, the decrease in TER with 10 μ M fibrils had leveled off after 3 hrs for the (1–42) fibrils, while 10 hrs was required to produce a similar decrease in TER for the (1–40) fibrils. The decrease in the TER of cells by both types of A β s is an indication of weakened cell-cell junctions and increased permeability.

Effect of H₂O₂ and inverted sequence A β monomers on the Permeability of BPAECs

The effect of hydrogen peroxide, a positive control, and the inverted sequence amyloids A β (40-1) and (42-1), which do not form fibrils, on the TER was investigated. As shown in Figure 2 the TER of the confluent endothelial cell monolayer was decreased following the addition of 100 μ M hydrogen peroxide in a time dependent manner. The decrease in TER is an indication of increased permeability of the BPAECs monolayers. Treatment of cells with A β 40-1 or 42-1 of inverted sequence, which remain as monomers did not change the TER of ECs. A β (1–42) has a greater tendency to form fibrils than A β (1–40) [19]. This finding indicates that fibril formation is required for the decrease in TER and, thus, explains the more effective decrease in TER by A β (1–42) than A β (1–40) fibrils (Fig.1).

Effect of SOD and catalase on A β (1–40) and A β (1–42) mediated vascular permeability

The neural and vascular toxicity of A β fibrils has been shown to be associated at least in part by the generation of ROS such as superoxide and hydrogen peroxide [20]. ROS are known to alter the TER of endothelial cell monolayers [21–23]. To determine whether A β mediated permeability (Fig. 1) is dependent on ROS, the effect of superoxide dismutase (SOD) and catalase, a hydrogen peroxide scavenger, on the changes of TER were investigated. As shown in Figure 3, SOD had no significant effect on the decrease of TER by A β (1–40) or A β (1–42). Furthermore, catalase only partially (~25%) inhibited the decrease of TER by both amyloid fibrils.

Effect of A β on cellular calcium levels

Cytosolic calcium levels were measured (Fig. 4) to understand the mechanism for decreasing the TER of ECs by A β . The increase in cytosolic calcium in response to ROS and other edemagenic agents has been shown to be associated with endothelial barrier dysfunction [12, 24,25]. In our experiments, thrombin was used as a positive control to check the release of Ca²⁺ into cytosol of EC monolayers. Treatment of confluent ECs with A β (1–40) did not have any effect on cytosolic Ca²⁺ (Fig.4). The effect of A β fibrils formed from (1–42) also had no effect on Ca²⁺ (data not shown). The decrease in the TER of EC monolayers by A β fibrils is, therefore, not primarily associated with the release of calcium.

Total protein tyrosine phosphorylation in A β treated endothelial cells

Endothelial barrier functions are regulated by phosphorylation of adherens and tight junction proteins [12,26]. ROS are known to increase the tyrosine phosphorylation of various proteins involved in endothelial barrier functions [12] [14]. Several studies have shown that cellular gap formation in endothelial monolayers due to oxidative stress is dependent on an increase in the phosphorylation of junctional proteins [27,28]. Therefore, total protein tyrosine phosphorylation in an endothelial lysate was assessed by the immunocytochemistry method. Treating ECs with both A β proteins for 60 min did not increase the phosphorylation of proteins (Fig.5). In fact, a decrease in protein tyrosine phosphorylation was observed with A β (1–40)

and A β (1–42) fibrils. These results indicate that the increase in permeability of EC monolayers by A β is not due to altering the phosphorylation of junctional proteins.

Cytoskeletal protein changes

The endothelial barrier functions are maintained by a highly dynamic network of actin filaments through their association with actinin, myosin, and tropomyosin. The effect of A β on the cytoskeleton network was investigated to determine if the change in barrier function by A β involves the cytoskeleton. Treatment of ECs with varying concentrations of A β for 6 hrs and staining with Alexa fluor Phalloidin to visualize F actin by immunofluorescence microscopy is shown in Figure 6. Lower concentrations of amyloid (3.125–6.25 μ M for A β (1–42) and 6.25–12.5 μ M for A β (1–40)) resulted in an increase in stress fiber formation (see vertical arrows in Fig. 6), while higher concentrations (12.5 μ M for A β (1–42) and 25 μ M for A β (1–40)) resulted in aggregation of actin filaments (parallel arrows in Fig. 6), which produce gaps in the cell monolayer (Fig. 6). The formation of stress fibers, actin cytoskeleton aggregation and gap formation at lower concentrations for A β (1–42) than for A β (1–40) is consistent with a reduction in TER at lower concentrations for A β (1–42) (Fig. 1). These results show that A β causes cytoskeleton remodeling in endothelial cell. The initial step in this remodeling that involves an increase in stress fibers corresponds to the A β concentration that produces the decrease in electrical resistance. The formation of these stress fibers are expected to alter the interactions between adjacent ECs that are responsible for the barrier function retaining the endothelial cells intact. The higher concentrations of A β resulting in aggregation and the disruption of the actin network may indicate the beginning of a necrosis affect on the ECs.

DISCUSSION

Amyloid peptides (1–40) and (1–42) are formed from the cleavage of the amyloid β precursor protein (A β PP) by β and γ secretases [29]. They are found primarily in the brain, but are also found at low nanomolar concentrations in the human plasma. [30]. These plasma amyloids can be sequestered from the luminal side to the abluminal side of the blood brain barrier for fibrils formation [9] [10]. Deposition of fibrils formed by these peptides in the brain has been implicated in the development of AD. In addition, A β deposits are found in brain capillaries, small arteries and arterioles producing cerebral amyloid angiopathy (CAA) [31–33] in spite of an anti-amyloidogenic effect of BBB by preferential sequestration of the soluble A β -apoJ complex and excluding pro-amyloidogenic isoforms of apoE3 and apoE4 [34]. About 80 to 90% of Alzheimer's subjects are diagnosed with CAA, implying a role for vascular A β in AD subjects.

Pulmonary arterial endothelial cells produce tight junctions that restrict the passage of substances across the pulmonary endothelium. This barrier is, however not as restrictive as the BBB with very tightly junctional proteins linking the endothelial cells. In addition BBB ECs shares a common basement membrane with astrocytes and pericytes making even tighter contacts. These same differences may explain why we were unable to grow human brain endothelial cells to confluence on gold electrodes. We therefore were forced to use bovine pulmonary arterial endothelial cells (BPAECs) for the present in vitro study of barrier function.

Neuronal inflammation is commonly found in most Alzheimer's subjects [35,36]. It usually occurs when pro-inflammatory mediators interact with the endothelium resulting in adhesion and a compromised BBB that permits the migration of blood neutrophils and plasma proteins into the brain [6,35,37–40]. In AD subjects the large pool of A β fibrils, consisting primarily of A β (1–40), in the vascular system [32] suggest that A β may play a role in the breaching of the BBB that facilitates the migration of neutrophils considered a major source for inflammation in AD subjects.

In this investigation, we studied the effect of A β fibrils (1–40) and (1–42) on barrier dysfunction by measuring TER of bovine pulmonary arterial endothelial cells. Exposure of these ECs to A β fibrils (1–40) and (1–42), but not monomers in the concentration range of (1–10 μ M) decreased the TER indicating that A β fibrils increase endothelial permeability (Fig.1).

The effect of non-monomeric amyloids on endothelial barrier function has recently been reported [40]. That study, however, emphasized the nature of the amyloid aggregate and showed that the dominant effect is due to small aggregates. The nature of the amyloid species in our study is limited to a comparison of monomers (no effect) and the difference between aggregates formed from (1–40) and (1–42) amyloids. We observed enhanced effects on the barrier function with A β (1–42), which can be attributed to a greater tendency to form fibrils. However, in our study we have a distribution of aggregates and fibrils. Consistent with the results of Gonzalez et al.[40], the enhanced effect that we observe (Fig. 1) for A β (1–42) can be attributed to greater toxicity of the (1–42) aggregates than the (1–40) aggregates of a similar size distribution and not to the formation of larger fibrils.

Our study instead of being directed at the nature of the A β aggregate, has investigated the mechanism for A β induced changes in barrier function. An increase in vascular permeability that does not involve EC death (necrosis or apoptosis) can be generated by ROS. Hydrogen peroxide, a ROS produced *in vivo*, is known to increase the vascular endothelial permeability (Fig.2) [28]. The mechanism for hydrogen peroxide-induced changes in permeability of ECs involves signal transduction and redox-regulated transcription factor pathways [12]. Hydrogen peroxide induces protein kinases and cytosolic calcium, which increases the endothelial permeability through the tyrosine phosphorylation of junctional proteins [28,41] and the regulation of myosin light chain kinase [12,24,42]. An effect of A β on endothelial permeability was expected to involve ROS, because of studies that have shown that the toxicity of A β is associated with the generation of ROS [43]. Our studies, however, rule out a primary role for ROS generation in A β induced permeability. We, thus find (Fig.3) that SOD which reacts with superoxide has no effect on A β induced permeability and catalase produces only a 25% decrease in the A β induced permeability.

The catalase effect suggests a secondary role for ROS. However, the cytosolic calcium (Fig. 4) and the tyrosine phosphorylation results indicate that the amyloid induced ROS effect implied by the catalase experiment does not involve the same pathway involved in hydrogen peroxide mediated permeability changes. The absence of any increase in cytosolic calcium and an actual decrease in phosphorylation instead of the expected increase in phosphorylation also rules out an edemagenic function of A β where the increase in permeability coincides with the phosphorylation of adherens and tight junction proteins [26].

Our studies instead implicate changes in the cytoskeletal proteins of endothelial cells. It is known that the cytoskeleton network is maintained by linking the actin filaments directly to tight junctional proteins and via actin binding proteins which interact with catenins to other junctional proteins [12,14]. A dramatic effect on the organization of the F-actin filaments, formation of stress fibers and intracellular gap formation in the presence of β (1–40) and β (1–42) (Fig.6) is indicative of cytoskeleton rearrangement. The damage to these actin and junctional protein bonds results in the dissociation and redistribution of proteins, which affect the cell to cell barrier functions.

This finding is consistent with Amyloid- β induced morphological changes of astrocytes produced by inducing reorganization of the cytoskeleton [44]. Multiple pathways for an effect of ROS on the cytoskeleton have been proposed[12], which may help to explain the partial inhibition by catalase (Fig. 3) despite the absence of an effect on calcium and tyrosine phosphorylation (Fig. 4 & Fig. 5).

Cytotoxic compounds and certain drugs, which cause tissue necrosis also change the permeability of the endothelium [45–47]. Amyloid- β fibrils known to have a cytotoxic effect [48,49] and to cause cell apoptosis [50,51] can, therefore, be expected to increase the permeability. However, apoptosis and cell death generally requires concentrations $> 10 \mu\text{M}$ and longer incubation times (24 to 72 hrs) [49,50]. In our experiments the permeability changes were observed at concentrations $< 10 \mu\text{M}$ and within an hour of incubation indicating that necrotic effects of A β should not be involved in the permeability changes that we have observed. However the gross cytoskeleton changes observed at the highest A β concentrations may be indicative of the beginning of necrosis. The cytoskeletal changes observed under these conditions are actually similar to the reported changes observed during necrosis. These reported cytoskeletal changes also did not produce an increase in calcium, although they were associated with an increase in MAP kinases, which have also been reported to increase with A β [50,51].

Our results implicate the cytoskeleton in the amyloid induced barrier dysfunction. However, additional studies are necessary to delineate the actual mechanism for this process. The possible roles of the different MAP kinases need to be investigated. In addition modulation of cellular thiol redox status, which is regulated by the redox status of glutathione, can alter signal transduction pathways of EC barrier function.

In conclusion, the A β proteins found in the circulatory system and in brain cells have been shown to alter the endothelium barrier function resulting in increased permeability by modifying the cytoskeletal proteins. These modifications can increase the adhesion and diapedesis of monocytes into the subendothelium causing inflammation that is seen in Alzheimer's subjects.

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Figure 1. Decrease in the permeability of endothelial cells by Aβ proteins

The endothelial cells were grown in gold microelectrodes to 95% confluence in MEM. Before the experiment, media in the microelectrodes was replaced with 0.38 ml serum free MEM. Electrodes were placed into an electrical cell-substrate impedance folder for 30 min to stabilize basal electrical resistance. After stabilization of resistance 20 μl Aβ were added and the TER was monitored as mentioned in the materials and methods section. A: Varying concentrations Aβ (1–40) (1.25 to 10 μM) fibrils were added into microelectrodes and the TER was monitored for 10 hrs. The changes in the TER at the end of 10 hrs was measured and compared with control. B: Varying concentrations of Aβ (1–42) fibrils were added into microelectrodes and the TER was monitored for 3 hrs. The decrease in the TER was measured at the end of 3 hrs and compared with control. Values are mean of 4 independent experiments. * Significantly different from control $p < 0.05$. ** Significantly different from control $p < 0.001$.

Figure 2. Effect of A β monomers and hydrogen peroxide on endothelial barrier function
Endothelial cells were grown in gold microelectrodes as mentioned in figure 1. Cells were stimulated with 10 μ M A β monomers (40-1 or 42-1) or 100 μ M hydrogen peroxide and monitored TER as mentioned in the materials and methods section

Figure 3. Effect of SOD and catalase on Aβ mediated endothelial resistance

Cells were grown in electrodes as described in Fig 1. A, SOD (100 U) and catalase (100U) were added prior to addition of 10 μM Aβ (1–40). B, SOD and catalase were added prior to addition of 10 μM Aβ (1–42). The decrease in the resistance was calculated by the subtraction of the resistance obtained at the end of experimental period (Fig.1) from the beginning of normalized resistance.

Figure 4. Effect of A β (1-40) on cytosolic calcium level

Cells were grown on coverslips to 95% confluence and loaded with fura-2 AM (5 μ M) for 15 min in a cell culture incubator. Cells were washed and challenged with thrombin, as a positive control, and varying concentration of A β (1-40). The intracellular concentration of Ca^{+2} was then measured as described in the materials and methods section. Values are mean of 3 independent experiments.

Figure 5. Effect of A β fibrils on total protein tyrosine phosphorylation in ECs

Cells were grown on 100 mm dishes to 95% confluence and treated with A β (1–40 or (1–42) for 1 hr. Cell lysates were subjected to 10% SDS-PAGE and probed with ant-phosphotyrosine antibody as described in the materials and methods section. B. Phosphorylation of total tyrosine groups (% control) was calculated by image analysis of blot. Values are mean of 3 independent experiments.

Figure 6. Amyloid induced actin fiber rearrangement in ECs

Cells were grown on glass coverslips to 95% confluence. Cells were incubated with varying concentrations of A β (1–40) or (1–42) for 6 hr. F-actin cytoskeletal organization was visualized following the fixation with 3.7% formaldehyde and staining with Alexa Fluor phalloidin 568. Stained cells were examined as mentioned in the materials and methods section. (↓) Indication of stress fiber formation, (→) Indication of aggregated actin filaments, (insert) gaps formation relative to control image.