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Role of ROS in Aβ42 mediated cell surface P-selectin expression and actin polymerization

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Abstract

P-selectin and actin cytoskeleton reorganization play an important role in vascular inflammation. In turn, there is increasing evidence that cerebrovascular factors contribute significantly to the development and progression of Alzhemer's disease. In this study we have evaluated the effects of Aβ42 oligomers on P-selectin expression and actin polymerization in mouse endothelial cells (bEnd3). Our results indicated that Aβ42 induced plasma membrane accumulation of P-selectin and promoted actin polymerization, and these events were correlated with increased reactive oxygen species (ROS) generation. The rapid, posttranslational cell signaling response mediated by ROS may well represent an important physiological trigger of the microvascular inflammation in Alzheimer disease.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, which affects approximately 10% of the population at age 65 and 40% of people over the age 80. There is evidence that the deposition of aggregated amyloid-beta peptide (Aβ) in brain parenchyma and brain vessels is the main cause of neuronal dysfunction and death in AD.[1-4] Aβ is derived from amyloidogenic sequential proteolysis of amyloid precursor protein and it is capable of aggregating into neuritic plaques and neurofibrillary tangles. It consists of 36-43 amino acids, but the most toxic form is Aβ1-42.[4-6] Aβ exhibits multiple cytotoxic effects to neurons, glial cells and causes dysfunction of the blood brain barrier (BBB). It has been shown previously that deposition of Aβ in the brain vessels promotes recruitment and transmigration of blood-born inflammatory cells into the brain parenchyma and induces neuroinflammation.[7-11]

Transmigration of inflammatory cells from the bloodstream into tissue is a sequential process which starts with interactions between inflammatory cells and endothelium. This intercellular interaction is controlled by cell adhesion molecules such as selectins and leucocyte-activating factors.[12] There are three members of the selectin family: P-, E-, and L-selectins. E-selectin and P-selectin are expressed on the surface of endothelial cells, while L-selectin is mainly presented on the surface of monocytes and leucocytes.[13] All selectins have similar functions, but P-selectin is stored in cytoplasmic Weibel-Palade bodies (WPb) of endothelial cells and can be mobilized on the endothelial cell surface within minutes upon exposure to different pro-inflammatory agents.[14,15] Rapid expression of P-selectin in the vasculature lumen is the earliest response to stimulation by different regulators of physiological processes such as thrombin, histamine, bradykinin etc.[12,16] P-selectin levels can peak as within 6-10 minutes following activation, and is then rapidly cleared through endocytosis.[14]

There is evidence that Aβ42 activates endothelial cells, induces translocation of P-selectin to the cell surface, promotes lymphocyte-endotheliocyte interaction, induces actin polymerization and increases cell stiffness.[10,17,18] However, regulatory mediators for P-selectin translocation and expression on the surface of brain endothelial cells following Aβ42 administration remain to be elucidated. In this study, we examined a role of ROS in actin polymerization and P-selectin expression on the surface of the endothelial cells that are activated by Aβ42.

METHODS

Cell culture and treatment

Mouse bEnd3 line (ATCC) was used in this research. bEnd3 cells respond to A β treatment similarly to human primary cerebral endothelial cells (CECs) and are a common model to

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investigate mechanisms of E- and P-selectin endothelial cells surface expression.[17,19,20]

BEnd3 cells were cultured in DMEM with 10% FBS and 1% antibiotic/antimycotic and maintained in humidified 5% CO_2 at 37ºC. Cells were grown on cover slips until confluence. Aβ42 oligomers were prepared using the globulomer protocol[21] with modifications. Briefly, 1 mg of the Aβ42 synthetic peptide (American Peptides) was suspended in 200 µl of 100% 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) and aliquot into 1.5ml centrifuge tubes. HFIP was removed by evaporation in a SpeedVac, Aβ42 was diluted in 2 µL DMSO to phenol free 100 µM in ice-cold culture Ham's medium, sonicated for 60 s, and incubated at 37ºC for 2 h. The presence of oligomeric form of Aβ42 was verified by Western blot analysis.

In this study we used immortalized bEnd3 cells as following: control; cells incubated with Aβ42 for 10, 30 and 60 min; cells incubated with 30 mM of antioxidant N-acetylcysteine (NAC) for 1 hr; cells pretreated with NAC followed by Aβ42 exposure. NAC doses have been chosen based on previously reported studies.[22,23] Fluorescent microscopy of anti-P-selectin, dihydroethidium (DHE), and Oregon-green phalloidin was used to quantify the surface P-selectin expression, ROS production, and actin polymerization.

Measurement of ROS generation

DHE staining was applied to determine superoxide anion production.[24] DHE reacts with O_2 •- to produce oxyethidium (oxy-E), a highly fluorescent product, which binds to DNA and causes an increased fluorescent intensity of the cell nuclei. For ROS measurements, cells were starved for 12 hr, rinsed twice with warm phenol free DMEM, and incubated with DHE (20µM) for 2 hr in parallel with Aβ42.

Immunofluorescence labeling

After treatment with Aβ42 cells were fixed immediately using 3.7% paraformaldehyde solution for 30 min. To block non-specific binding, 5% goat serum in PBS was applied to the cells for 1 hr. Cellular surface P-selectin was labeled with its primary antibody (R&D systems) without permeabilization at 4^0C overnight, followed by goat alexa Fluor 594 anti-rat secondary antibody (Invitrogen) labeling at room temperature for 1 hr. To confirm the specificity of the primary antibodies, cells were labeled by secondary antibodies alone. Secondary antibodies did not show immuno-staining in the absence of the primary antibody (data not shown). For measurement of actin polymerization cells were fixed by the same procedure as for P-selectin immunostaining. After fixation, cells were permeabilzed by 0.1% Triton X-100 in PBS for 5 min and labeled with Oregon-green phalloidin (250 mM) (Invitrogen) in PBS with 1% BSA at room temperature for 1 hr. Cells are then counterstained by incubation in 300 nM DAPI (4',6-diamidino-2-phenylindole, Sigma) for 5 minutes, rinsed and mounted on glass microscope slides in Fluoromount (Sigma).

Quantitative immunofluorescence microscopy (QIM)

Bright-field illumination and fluorescence microscopy were performed with Zeiss Axio Observer Z1 fluorescence microscope and 40X, NA 0.95 objective. Images were acquired using a cooled CCD camera controlled with a computer and MetaVue imaging software. The typical exposure time for fluorescence image acquisition was 400 msec. Background was subtracted for all images prior to analysis. Actin polymerization was quantified by calculating the intensity of Oregon-Green phalloidin-labeled F-actin per cell area. The intensity was then normalized by the intensity of the labeled F-actin in control cells (without any treatment). A similar approach was applied to quantify the relative expression of P-selectin and ROS production. A total of 300 images were analyzed.

Western blot analysis

Following the treatments, bEnd3 cells were harvested in 300 µL sample buffer containing 50 mM Tris-HCl, pH 7.4 1 mM EDTA, 100 mM NaCl, 0.1% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 1mM sodium *o*-vanadate, 1µg/mL leupeptin, 1 µg/mL pepstatin, and 10 µg/mL aprotinin. Extracts then were collected, sonicated, and equivalent amounts of each sample (40 µL) were resolved in 10% Tris-HCl gel electrophoresis. After electrophoresis, proteins were transferred to nitrocellulose membranes. Membranes were incubated in Tris-buffered saline, pH 7.4, with 0.5% Tween 20 (TBS-T) containing 5% non-fat milk for 1 hr at room temperature. The blots were then washed and reacted with rabbit anti-P-selectin (1:600; Cell Signaling Technology) overnight at 4ºC. After washing with TBS-T, they were incubated with goat anti-rabbit IgG – horseradish

peroxidase (1:5000; Santa Cruz) for 1 hr at room temperature. The blots then were washed 3 times with TBS-T. Immunolabeling was detected by chemiluminescence (SuperSignal West Pico and West Fempto). For quantification, blots were scanned and intensity of protein bands was measured as optical density using the Quantity One program (BioRAD, Hercules, CA, USA). P-selectin was detected at 140 kDa. Ratios of P-selectin to βactin were calculated for each sample and normalized to control.

Statistical analysis

Data from at least three independent experiments were normalized to control and reported as mean ± SD. Mean differences within experimental groups were tested with One Way ANOVA, while differences between the treatments were tested with unpaired t-test against control in each group separately. Values were considered significantly different if P≤0.05. Statistical analyses were performed with Sigma Plot 8.0 software.

RESULTS

Aβ42 stimulated ROS generation in the bEnd3 cells

To investigate intracellular ROS production, we measured DHE fluorescence. Figure 1A shows the images of DHE-stained bEnd3 cells treated with Aβ42 oligomers and the ROS scavenger NAC. Quantitative analysis was accomplished by integration of fluorescent intensity for each cell. Fig. 1B shows significant time-dependent ROS accumulation in each group exposed to 5 μM Aβ42. We observed 34.5%, 61%, and 83% average increases in fluorescence intensity after 10 min, 30 min and 60 min of Aβ42 treatment, respectively. Aβ42 stimulated ROS production in bEnd3 cells was attenuated by pretreatment with the antioxidant NAC.

Aβ42 stimulated mobilization of P-selectin on the bEnd3 cell surface

To investigate the effect of Aβ42 oligomers on the expression of P-selectin, quantitative immunofluorescence microscopy of P-selectin stained with primary and secondary antibodies and Western blot analysis for total P-selectin expression were performed. Aβ42 increased P-selectin fluorescence at the surface of bEnd3 cells in a time dependent manner and in parallel to ROS elevation (Figure 2 A, B). We observed 28%, 46%, 54% increased P-selectin plasma membrane expression after 10 min, 30 min and 60 min of Aβ42 treatment. However, total expression levels of P-selectin were not changed following exposure to Aβ42 (Figure 2 C). Pretreatment with NAC

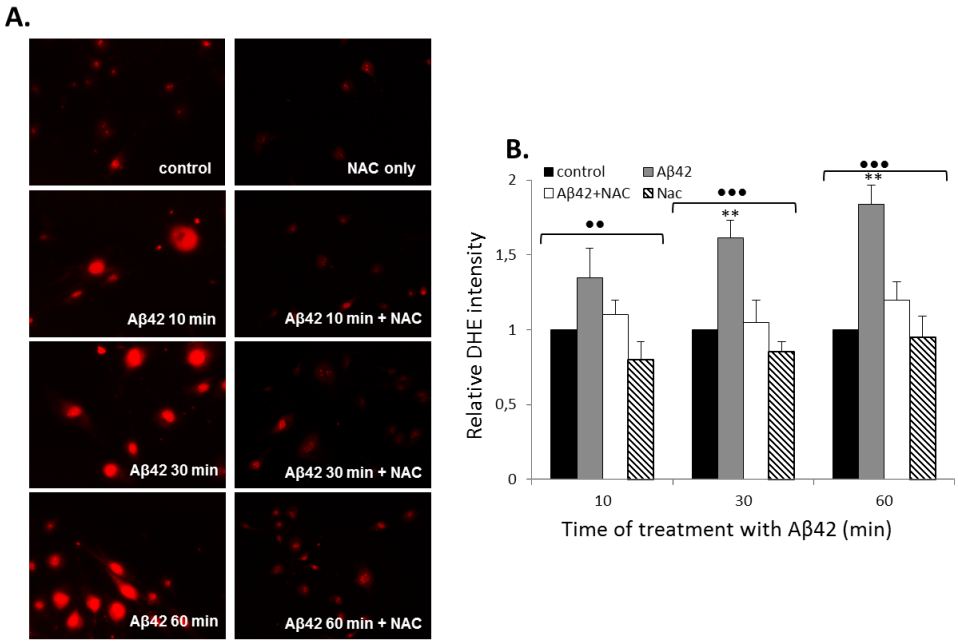

Figure 1. Aβ42 induced ROS generation in bEnd3. Images of DHE-stained bEnd3 (A); relative DHE fluorescent intensity (B). ** - p ≤ 0.01 compared to the control in each experimental group (unpaired T-test analysis). ••• - p ≤ 0.001, •• - p ≤ 0.01 (One Way ANOVA).

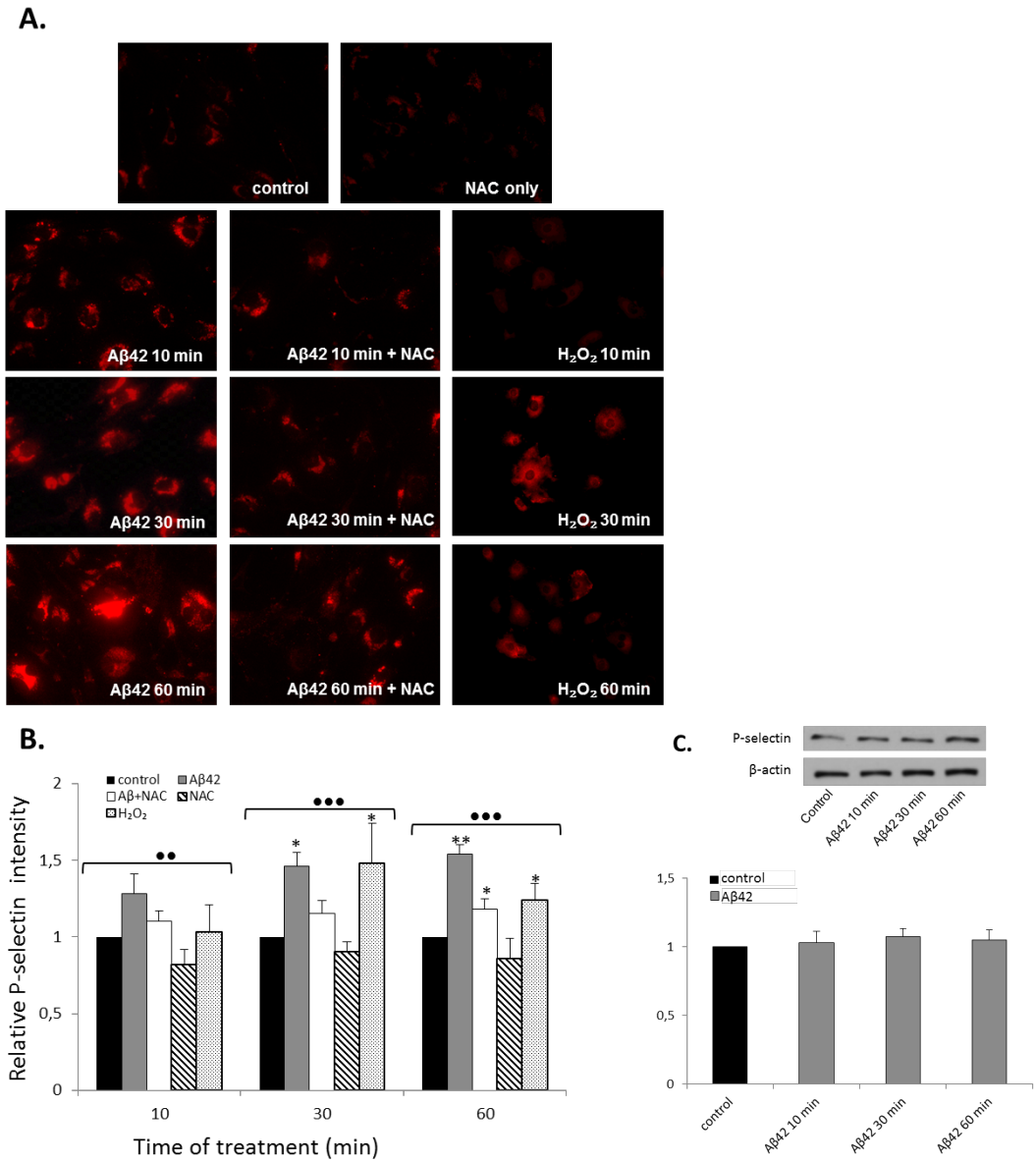

Figure 2. Aβ42 enhanced P-selectin fluorescence on the cell surface, but did not affect P-selectin expression on the translational level. Fluorescent images of anti-P-selectin labeled bEnd3 cells (A); relative P-selectin intensity in the mouse bEnd3 cells (B); Western blot analysis of total P-selectin (C). ** - $p \leq 0.01$, *- $p \leq 0.05$ compared to the control in each experimental group (unpaired T-test analysis) ••• - $p \leq 0.001$, •• - $p \leq 0.01$(One Way ANOVA).

attenuated Aβ42 induced P-selectin localization, while NAC alone did not significantly affect P selectin localization. As a positive control, H_2O_2 (0.5 mM) also increased P-selectin expression on the cell surface, which peaked after 30 min of H_2O_2 treatment. Likewise, Western blot analysis did not reveal any change in total P-selectin levels after 10 min, 30 min and 60 min of H_2O_2 treatment (not shown). These results support our hypothesis that, within this time frame Aβ42 regulates localization, but not total expression of P-selectin through elevated ROS.

Actin polymerization in bEnd3 cells treated with Aβ42

To investigate the effect of Aβ42 oligomers on the actin polymerization, we then measured cytoskeleton dynamics by quantitative

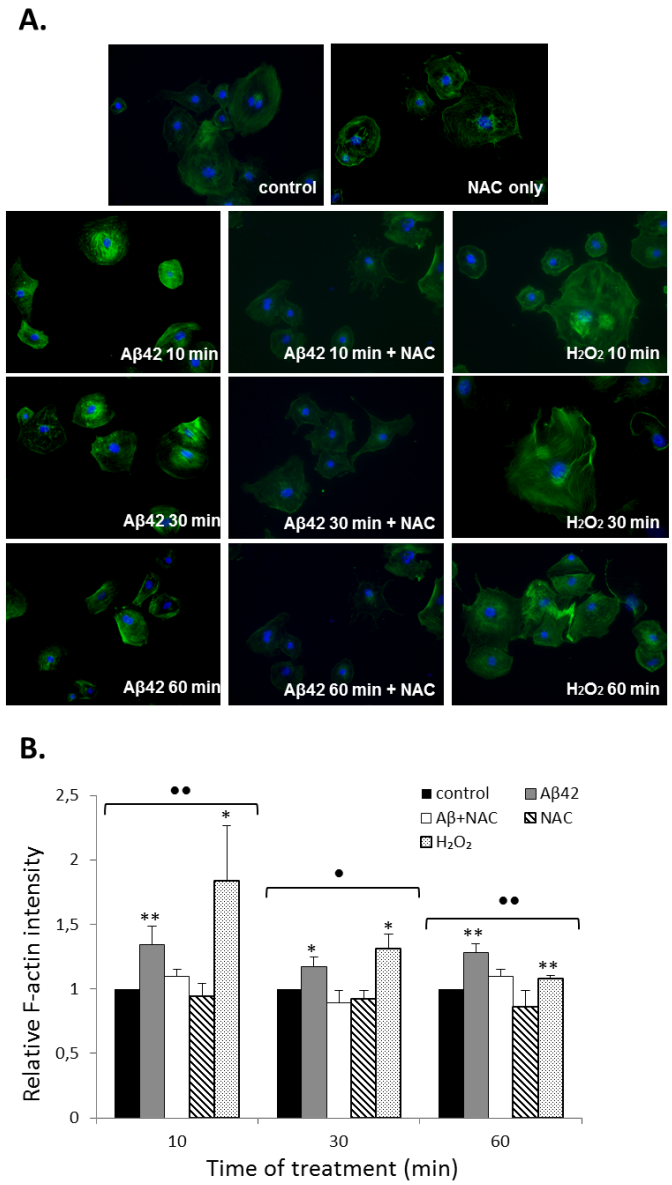

Figure 3. Aβ42 induced actin polymerization in mouse bEnd3 cells. Fluorescent images of Oregon-green phalloidin-stained bEnd3 cells (A); relative F-actin intensity in mouse bEnd3 cells (B). ** - $p \leq 0.01$; * - $p \leq 0.05$ compared to the control in each experimental group (unpaired T-test analysis). •• - $p \leq 0.01$, • - $p \leq 0.05$ (One Way ANOVA).

immunofluorescence microscopy experiments with Oregon-green phalloidin staining (Figure 3 A, B). Exposure of bEnd3 cells with Aβ42 promoted actin polymerization which peaked after 10 min of Aβ42 treatment, while no significant increase of F-actin intensity was observed when cells were pretreated with NAC. H_2O_2 was able to mimic Aβ42 induced oxidative stress, causing increased actin polymerization with similar timing.

DISCUSSION

The results of this study have demonstrated that, in bEnd3 cells, Aβ42 oligomers induce F-actin polymerization, promotes the translocation of P-selectin in a time dependent manner and in parallel to direct stilumation with exogeneous ROS, and these effects can be attenuated by pretreatment with the antioxidant NAC. P-selectin is the largest of the known selectins at 140 kDa. It contains nine consensus repeats (CR) and

extends approximately 40 nm from the endothelial surface. P-selectin is expressed in α-granules of activated platelets and in Weibel-Palade bodies (WPb) of endothelial cells. It has been reported previously that in normal conditions P-selectin is not presented on the surface of endothelial cells, while thrombin or histamine promotes rapid mobilization of P-selectin to the cell surface, peaking after ten minutes. Within 30 min almost all the receptors are eliminated by clathrin-dependent endocytosis.[14] Additional synthesis of P-selectin is brought about within two hours by cytokines such as interleukin-1 (IL-1) or tumor necrosis factor (TNF-α). In our experiment, the duration of Aβ treatment was 10 to 60 min and didn't lead to a noticeable change of P-selectin expression at the translational level. However, quantitative immunofluorescence microscopy of P-selectin has demonstrated that Aβ42 induces translocation of P-selectin to the bEnd3 cells surface within 10 min of exposure. Moreover, we observed time dependent accumulation of P-selectin on the cells surface.

One of the possible regulatory mechanisms of P-selectin release in bEnd3 cells is Aβ-induced ROS overproduction. There is evidence that in endothelial cells of human umbilical vein ROS play an active role in thrombin activated P-selectin expression.[25] This research has demonstrated dose dependent inhibition of P-selectin expression and ROS generation in response to administration of antioxidants and NADPH oxidase or xanthine oxidase inhibitors. In the present study we have demonstrated that Aβ42 induced time-dependent accumulation of ROS and cell surface P-selectin expression. We also observed similar dynamics of P-selectin fluorescence upon exposure of the bEnd3 cells by H_2O_2. In turn, the ROS scavenger NAC reduced both ROS production and P-selectin expression. These finding lead us to a conclusion that Aβ42-induced upregulation of P-selectin in bEnd3 cells is mediated, at least in part, by ROS.

ROS can be generated by several enzymatic systems, but there is strong evidence that the superoxide-producing enzyme, NADPH oxidase, is a major source of ROS in CECs and astrocytes.[26-33] We suggest that Aβ42 binding to bEnd3 cells is resulted in NADPH oxidase activation and subsequent ROS generation, which, in turn, triggers the downstream pathway leading to rapid translocation of P-selectin.

As mentioned before, P-selectin is stored in WPb inside the endothelial cells.[14,15] Recent research has shown a direct and active role of ROS in the different stages of WPb release.[25,34-37] It was shown that WPb exocytosis in endothelial cells is a rac1-dependent ROS regulating mechanism.[38] One of the mechanisms controlling exocytosis of WPb is the regulation of N-ethylmaleimide-sensitive factor (NSF).[39] NSF is the cytosolic ATPase playing a key role in fusion of WPb with plasma membrane. There is evidence that S-nitrosylation of this protein by NO inhibits activity of NSF.[40] In turn, ROS can block NO action through modifying target proteins of this messenger or by direct interaction with NO[41,42], and it may lead to activation of WPb exocytosis.

Actin filaments are involved in the later stages of WPb exocytosis as well. Some part of cellular WPbs is associated with actin cortex, and, as suggested, this pool of vesicles can be rapidly released upon stimulation.[43-45] Furthermore, in activated endothelium actin-based cytoskeleton reorganizes into stress fibers leading to reduction of the interendothelial contacts, induction of vascular leak and increases transendothelial leukocyte trafficking.[46] Several studies have shown that Aβ oligomers can alter actin polymerization within neurons and cerebral endothelial cells.[17,47,48] Our data are in good agreement with previously reported results and demonstrate that Aβ42 promote F-actin formation in bEnd3 cells. Moreover, our data confirm the hypothesis that ROS are a regulatory mediator of actin remodeling.[49-51] According to previously reported data ROS affects the speed of actin polymerization through the regulation of barbed end uncovering or by directly controlling the activity of actin depolymerizing agents and decreasing filaments depolymerization.[49,50] In our study we have demonstrated that Aβ42 as well as H_2O_2 increased F-fibers formation similarly, with a peak after 10 min of treatment, while Aβ42-induced actin polymerization could be counteracted by antioxidants. These findings lead us to conclusion that Aβ42 induces actin polymerization through ROS dependent pathways, which, in turn, may also represent one of the regulatory mechanisms of P-selectin release and transendothelial trafficking of the white blood cells.

In conclusion, the results of our study have indicated that Aβ42 induced accumulation of P-selectin on the surface of bEnd3 cells and promoted actin polymerization, and all these events were correlated with ROS generation. The rapid posttranslational cell signaling response mediated by ROS may well represent an important physiological trigger of the microvascular inflammatory response in AD and requires further investigations.

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