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Role of Aβ-RAGE interaction in oxidative stress and cPLA$_2$ activation in astrocytes and cerebral endothelial cells

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Abstract

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1. Introduction

Alzheimer's disease (AD) is a chronic neurodegenerative disorder, which affects approximately 10% of the population at age 65 and older. The pathology of AD is characterized by an increased deposition of amyloid-β peptide (Aβ) in the brain, and a progressive impairment of cognition and memory of affected individuals. Blood Brain Barrier (BBB) dysfunction is observed in all of the stages of AD, and may even precede neuron degeneration in AD brains (Hofman et al., 1997, Iadecola, 2003, Ruitenberg et al., 2005, de la Torre, 2006, Girouard and Iadecola, 2006, Deane and Zlokovic, 2007, Zlokovic, 2008, Bell and Zlokovic, 2009). During the early stages of AD, microvasculature deficiencies and hypertrophy of astrocytes are commonly observed (Farkas and Luiten, 2001, Borroni et al., 2002). Numerous *in vivo* and*in vitro* studies have demonstrated that the vascular deposition of Aβ induces oxidative stress in cerebral vasculature and astrocytes (Cai et al., 2003, Abramov and Duchen, 2005). Aβ-induced oxidative stress in cells, in turn, initiates a cascade of redox reactions leading to apoptosis and neurovascular inflammation (Emmanuelle et al., 1997, Suo et al., 1998, Tan et al., 1999, Xu et al., 2001, Yin et al., 2002, Hsu et al., 2007, Vukic et al., 2009)

Aβ-induced oxidative stress is associated with overproduction of reactive oxygen species (ROS) (Park et al., 2005, Girouard and Iadecola, 2006, Callaghan et al., 2008, Park et al., 2008). ROS can be generated by several enzymatic systems, but there is evidence that the superoxide-producing enzyme, NADPH oxidase, is a major source of ROS in CECs and astrocytes (Cai et al., 2003, Abramov and Duchen, 2005, Park et al., 2005, Qing et al., 2005, Park et al., 2008, Zhu et al., 2009). Although these studies demonstrate that Aβ mediates oxidative damage to astrocytes and CECs mainly through the activation of NADPH oxidase, how Aβ activates NADPH oxidase has yet to be elucidated.

Aβ-induced cytotoxic effects are also associated with the activation of MAPK/ERK1/2 cascade and the phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$) (Stephenson et al., 1996, McDonald et al., 1998,Dineley et al., 2001, Moses et al., 2006, Zhu et al., 2006, Shelat et al., 2008, Young et al., 2009). The ERKs (extracellular-signal-regulated kinases) are widely expressed protein kinases, and part of a signal transduction system through which extracellular stimuli are transduced. Activation of ERKs occurs in response to growth factor stimulation, cytokines, virus infection, transforming agents, carcinogens, and after the activation of high-affinity IgG receptors (McDonald et al., 1998). Phospholipases A_2 (PLA_2s) are ubiquitously distributed enzymes that catalyze the hydrolysis at the *sn*-2 position of phospholipids to produce lysophospholipids and release arachidonic acid (Murakami and Kudo, 2002, Sun et al., 2004). PLA_2s are classified into three major families: calcium-dependent cytosolic PLA_2 ($cPLA_2$), secretory PLA_2 ($sPLA_2$) and calcium-independent PLA_2 ($iPLA_2$). $cPLA_2$ has been implicated in diverse cellular responses such as mitogenesis, differentiation, inflammation and cytotoxicity, and overproduction of this enzyme is involved in many neurodegenerative diseases, including AD (Stephenson et al., 1996, Sun et al., 2007).

Recent studies have indicated that the receptor for advanced glycation endproducts (RAGE) is a binding site for Aβ (Yan et al., 1996, Lue et al., 2001, Sasaki et al., 2001, Arancio et al., 2004, Chaney et al., 2005). RAGE is a multi-ligand cell surface receptor which is normally expressed in brain endothelium and, at low levels, in microglia and neurons (Lue et al., 2001, Sasaki et al., 2001, Zlokovic, 2008). However, in AD brains, RAGE expression is increased by several-fold in cerebral endothelial cells, astrocytes, microglia, and neurons (Lue et al., 2001, Sasaki et al., 2001). Aβ binding to RAGE has been demonstrated to regulate Aβ transport across BBB, upregulate pro-inflammatory cytokines and adhesion molecules in CECs, and contribute to the transport of Aβ from the cell surface into the intracellular space in cortical neurons (Giri et al., 2000, Lue et al., 2001, Takuma et al., 2009). Since RAGE has been postulated to function as a signal transducing cell surface receptor for Aβ, it is reasonable to hypothesize that binding of $Aβ_{1–42}$ oligomers ($Aβ_{42}$) to surface RAGE results in activating of NADPH oxidase to induce ROS generation, and activate downstream pathways, including phosphorylation of ERK1/2 and $cPLA_2$.

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2. Experimental Procedures

2.1 Cell cultures and treatment

Mouse bEnd3 line of cerebral endothelial cells (CECs) was purchased from Fisher Scientific. Rat primary cortical astrocytes were purchased from Invitrogen (Carlsbad, CA). Purity of astrocyte culture was verified by double immunostaining with astrocytic marker (primary mouse monoclonal antibody to S100, Abcam Inc, Cambridge, MA) and microglia marker (primary rabbit polyclonal to antibody Coronin 1a, Neuromics, Edina, MN). The purity of astrocytes obtained from Invitrogen is 100%.

$Aβ_{42}$ and scrambled sequence peptide ($Aβ_{42–1}$) from American Peptide (Sunnyvale, CA) were prepared by diluting 5mM Aβ in DMSO to 100 μM in ice-cold culture Ham's medium, and sonicated. The oligomeric form of $Aβ_{42}$ in cell culture was verified by Western blot analysis. Menadione (Sigma, St. Louis, MO) was dissolved in DMSO (1mg/ml) and then diluted in cell culture medium to final concentration of 50 μM; rabbit polyclonal antibody against RAGE (Ab_{RAGE}, Abcam Inc), the specific NADPH oxidase inhibitor, gp91ds-tat (AnaSpec, Fremont,

CA), and ROS scavenger, Tiron (Sigma), were diluted in DMEM. Experimental groups: control (cells without any treatment); cells treated with 2 and 5 μM of $A\beta_{42}$ for 1 and 2 hr; cells treated with Ab_{RAGE} for 4 hr; cells treated with Ab_{RAGE} followed by $A\beta_{42}$ (5 μM for 2 hr) treatment; cells treated with gp91ds-tat (1 μM for 1 hr) and tiron (5 mM for 1 hr) followed by $A\beta_{42}$. Cells treated with menadione (50 μM for 30 min) and $A\beta_{42-1}$ (5 μM for 2 hr) served as a positive and negative controls.

2.2 Immunofluorescent staining for RAGE

Cells were grown on cover slips until confluence. After treatment with $A\beta_{42}$ (2 and 5 μM for 1 hr), cells were fixed immediately using 3.7% paraformaldehyde solution for 30 min. To block non-specific binding, 5% sheep serum in PBS was applied to the cells for 1 hr. RAGE at the cell surface was labeled with its primary antibody (Abcam) without permeabilization at 4°C overnight, followed by sheep secondary antibody (Abcam) labeling at 25°C for 1 hr. To confirm the specificity of the primary antibodies, cells were labeled by secondary antibodies alone. Secondary antibodies did not show immunostaining in the absence of the primary antibody.

2.3 Measurement of ROS production

DHE (dihydroethidium) staining was applied to determine superoxide anion production (Zanetti et al., 2005). DHE reacts with $O^{\bullet-}_2$ to produce oxyethidium (oxy-E), a highly fluorescent product, which binds to DNA and causes an increased fluorescent intensity in cell nuclei. For ROS measurements, cells were starved for 4 hr, rinsed twice with warm phenol free DMEM, and incubated with DHE (20μM) for 2 hr.

2.4 Quantitative Immunofluorescence microscopy (QIM)

Bright-field illumination and fluorescence microscopy were performed with Nikon TE-2000 U fluorescence microscope and 40X, NA 0.95 objective. Images were acquired using a cooled CCD camera controlled with a computer and uses MetaView imaging software. The typical exposure time for fluorescence image acquisition was 400 msec. Background was subtracted for all images prior to analysis. Relative expression of RAGE was quantified by calculating the intensity of secondary antibody fluorescence per cell. The intensity was then normalized by the control cells (without any treatment). A similar approach was applied to quantify the oxy-E fluorescence.

2.5 Double immunofluorescent labeling of gp91-phox and p47-phox

Cells were grown on cover slips until confluence. After treatment cells were fixed immediately using 3.7% paraformaldehyde solution for 30 min. To block non-specific binding, 5% mixture of sheep and donkey serum in PBS was applied to the cells for 1 hr. Plasma membrane gp91-phox was labeled with its primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) without permeabilization at 4°C overnight. For p47-phox labeling, cells were permeabilzed by 0.1% Triton X-100 in PBS for 5 min and labeled with its primary antibody (Santa Cruz Biotechnology) at 4°C overnight, followed by sheep and donkey polyclonal secondary antibodies (Abcam) labeling at room temperature for 1 hr. The emission spectra of the secondary antibodies were 528 nm (for gp91-phox) and 620 nm (for p47-phox). To confirm the specificity of the primary antibodies, cells were labeled by secondary antibodies alone. Secondary antibodies did not show immunostaining in the absence of the primary antibody.

2.6 Confocal immunofluorescence microscopy

Confocal immunofluorescence microscopy was performed with Olympus FV1000 confocal microscope. Confocal images were acquired with a 60 X, numerical aperture 1.2 oil immersion objective lens for colocalization studies of the cellular surface gp91-phox and p47-phox. The colocalization images were obtained by suppressing all colors, except yellow, in superimposed images using Matlab and Adobe Photoshop. A z-series at 1 μm intervals were captured to determine the spatial co-localization characteristics of gp91-phox or p47-phox staining within individual cells. The colocalization (the area of coincident intensity) between two channels was quantified by normalizing the coincident intensity (yellow) to the total intensity of the corresponding channel.

2.7 Western blot analysis

Following the treatments, primary astrocytes and CECs 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. Lysates were collected, sonicated, and equivalent amounts of each sample (40 μL) were resolved in 11.55% 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 either rabbit anti-phospho-$cPLA_2$ (1:600; Cell Signaling Technology, Boston, MA) or rabbit anti-$cPLA_2$ (1:600; Cell Signaling Technology) or mouse anti-phospho-ERK1/2 (1:4000, Cell Signaling Technology) and rabbit anti-ERK1/2 (1:500; Cell Signaling Technology) overnight at 4°C. After washing with TBS-T, they were incubated with goat anti-rabbit and goat anti-mouse 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). Phospho-ERK1/2 and ERK1/2 were detected at 42/44 kDa, and phospho-$cPLA_2$ and $cPLA_2$ were detected at 110 kDa. Ratios of phospho- ERK1/2 to ERK1/2 and phospho-$cPLA_2$ to $cPLA_2$ were calculated for each sample and normalized to the control.

2.8 Statistical analysis

Data from at least three independent experiments are reported as mean ± SD. Mean differences between experimental groups were tested with unpaired t-test. Values were considered significantly different at the $p \leq 0.05$ level. Statistical analyses were performed on the Sigma Plot 8.0 software.

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3. Results

3.1 Oligomeric $A\beta_{42}$ interacts with the RAGE

It has been reported that the binding of soluble Aβ to soluble RAGE inhibits further aggregation of Aβ peptides, while membrane bound RAGE-Aβ interactions elicit activation of the NF-κB transcription factor and promote sustained chronic neuroinflamation (Chaney et al., 2005). To further provide evidence of the $A\beta_{42}$-RAGE binding, we examined with the immunofluorescence microscopy of RAGE for primary astrocytes and CECs pretreated with 2 and 5 μM of oligomeric

$A\beta_{42}$ at 37°C and 4°C (in which condition the internalization of surface receptors is suppressed). A lower fluorescent intensity of labeled RAGE was observed for cells pretreated with oligomeric $A\beta_{42}$ in both temperature conditions (Fig. 1), suggesting that $A\beta_{42}$ oligomers compete with Ab_{RAGE} to bind to RAGE at the surface of the astrocytes as well as CECs.

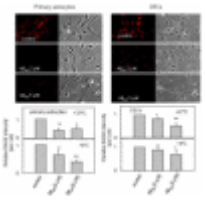

Fig.1

Oligomeric $A\beta_{42}$ reduced the fluorescent intensity of RAGE staining at the primary astrocytes and CECs surface

3.2 Polyclonal antibody to RAGE and NADPH inhibitor suppress $A\beta_{42}$-induced ROS production in astrocytes and CECs

To investigate ROS production in cells, we applied fluorescent microscopy of DHE, which reacts with $O^{\bullet-}_2$to produce oxyethidium (oxy-E) with a higher quantum yield. Fig. 2 shows the images of DHE-stained CECs (A) and astrocytes (B) treated with $A\beta_{42}$ oligomers, $A\beta_{42\text{-}1}$, Ab_{RAGE}, ROS scavenger (tiron), and NADPH oxidase inhibitor (gp91ds-tat). Quantitative analysis was accomplished by integration of fluorescent intensity for each cell. Fig. 2 C, D show that 5 μM of $A\beta_{42}$ increased DHE fluorescence in both primary astrocytes as well as in CECs by ~ 75% as compared to the control. Since menadione has been reported previously to induce ROS generation in astrocytes (Zhu et al., 2009), results from the treatment of menadione served as a positive control (Fig. 2A, C). As a negative control, reversed $A\beta_{42\text{-}1}$ did not increase ROS generation. At the same time, $A\beta_{42}$ stimulated ROS production in CECs and astrocytes was attenuated by blocking the cell surface RAGE with its antibody (Fig. A, C), or by the pretreatment with gp91ds-tat, the specific NADPH oxidase inhibitor (Fig. B, D). $Ab_{RAGE,}$ or the inhibitor alone had no effect on DHE intensity. As a control, scrambled sequence peptide sr-gp91ds-tat (sr-gp) did not suppress $A\beta_{42}$-induced ROS overproduction. To verify this technique of measurement for superoxide anions, we demonstrated that ROS scavenger suppressed an $A\beta_{42}$-mediated increase in DHE intensity. This data suggest that $A\beta_{42}$ oligomers induce ROS production through their binding to RAGE leading to NADPH oxidase activation.

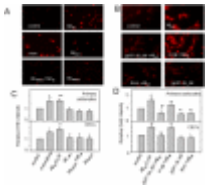

Fig.2

Representative images of dihydroethidium (DHE)-stained CECs (A) and astrocytes (B) treated with $A\beta_{42}$ oligomers, polyclonal antibody to RAGE (Ab_{RAGE}), ROS scavenger (tiron), and NADPH oxidase inhibitor (gp91 ds_tat). Relative DHE fluorescent intensity ...

3.3 Polyclonal antibody to RAGE suppresses $A\beta_{42}$-induced colocalization of cytosolic subunit p47-phox of NADPH oxidase with its membrane subunits gp91-phox

Ab_{RAGE} as well as NADPH oxidase inhibitor suppressed $A\beta_{42}$-induced ROS production in CECs and astrocytes (Fig. 2C, D). NADPH oxidase is a membrane-bound enzyme that catalyzes the production of ROS from oxygen and NADPH. NADPH oxidase is a complex system consisting

of two membrane-bound elements (gp91-phox and p22-phox), three cytosolic components (p67-phox, p47-phox and p40-phox), and a low-molecular-weight G protein (Babior, 1999). Activation of NADPH oxidase is associated with the migration of the cytosolic components to the cell membrane and assembling with its membrane subunits. To confirm the role of $A\beta_{42}$-RAGE interactions in NADPH oxidase activation and subsequent ROS generation, we quantified the effect of $A\beta_{42}$ and Ab_{RAGE} on the colocalization of p47-phox with gp91-phox by analyzing confocal images of double immunofluorescent-labeled gp91-phox and p47-phox in astrocytes and CECs (Fig. 3 A,B). Our results indicate that $A\beta_{42}$ significantly increased the colocalization of cytosolic subunit p47-phox of NADPH oxidase with its membrane subunits gp91-phox, suggesting that $A\beta_{42}$ enhances NADPH oxidase complex assembling. At the same time, pre-treatment with Ab_{RAGE}significantly suppressed the colocalization of p47-phox with gp91-phox induced by $A\beta_{42}$. To validate the fluorescent confocal microscopy method for measurement of the colocalization between these two subunits, we demonstrated that NADPH oxidase inhibitor (gp91ds-tat) suppressed $A\beta_{42}$-mediated increase in colocalization (Fig. 3 A,B). The inhibitor alone, as well as $Ab_{RAGE,}$ had no effect on the colocalization. This data indicated that $A\beta_{42}$ oligomers induced colocalization of cytosolic subunit p47-phox of NADPH oxidase with its membrane subunits gp91-phox and subsequent ROS generation through binding to RAGE.

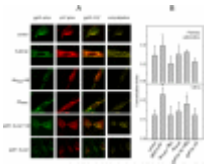

Fig.3

Polyclonal antibody to RAGE and NADPH oxidase inhibitor suppressed $A\beta_{42}$ induced colocalization of p47-phox to gp91-phox

3.4 Polyclonal antibody to RAGE inhibit $A\beta_{42}$-induced phosphorylation ERK1/2 and $cPLA_2$ in primary astrocytes and CECs

$A\beta_{42}$ has been shown to induce MAPK/ERK downstream signaling pathways, including the activation of extracellular-signal-regulated kinases (ERK), which further leads to phosphorylation of $cPLA_2$(Emmanuelle et al., 1997, Xu et al., 2001, Yin et al., 2002, Hsu et al., 2007, Shelat et al., 2008). Here, we tested if $A\beta_{42}$ binding to RAGE induced ERK1/2 activation and phosphorylation of $cPLA_2$ in CEC and astrocytes. Fig. 4 shows that $A\beta_{42}$ significantly increased phosphorylation of ERK1/2 and $cPLA_2$ in astrocytes and CECs, which were suppressed by the pretreatment with Ab_{RAGE}. Additionally, Ab_{RAGE}alone had no effect on phosphorylation of ERK1/2 and $cPLA_2$ in both cell types. Our data suggested that $A\beta_{42}$ binding to RAGE is required for the activation of ERK1/2 and further phosphorylation of $cPLA_2$induced by $A\beta_{42}$.

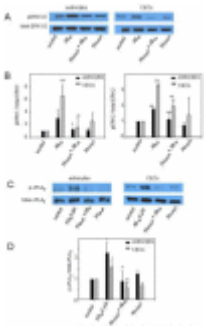

Fig.4

Polyclonal antibody to RAGE inhibits ERK1/2 and cPLA2α phosphorylation in primary astrocytes and CECs

3.5 Effects of NADPH oxidase inhibitor and ROS scavenger on ERK1/2 and $cPLA_2$ phosphorylation in CECs and primary astrocytes

ROS from NADPH oxidase has been shown to induce the activation of ERK1/2 and phosphorylation of $cPLA_2$ in primary neurons (Shelat et al., 2008). Our data suggest that $A\beta_{42}$ oligomers induce ROS production through binding to RAGE which activates NADPH oxidase, and thus blocking cell surface RAGE with its antibody suppresses Aβ-induced phosphorylation of ERK1/2 and $cPLA_2$ in CECs and primary astrocytes. Therefore, we hypothesized that $A\beta_{42}$ binding to RAGE induces phosphorylation of ERK1/2 and $cPLA_2$ through activation of NADPH oxidase and ROS production.

In support of our hypothesis, Fig. 5 B,C,E,F show that pretreatment of CECs with NADPH oxidase inhibitor or ROS scavenger could significantly suppress $A\beta_{42}$-induced phosphorylation of ERK1/2 and $cPLA_2$. Surprisingly, only Ab_{RAGE}, but neither NADPH oxidase inhibitor nor ROS scavenger, inhibited $A\beta_{42}$-induced ERK1/2 and $cPLA_2$ phosphorylation in primary astrocytes (Fig. 4 A,B and Fig.5 A,C,D,F). NADPH oxidase inhibitor alone, as well as ROS scavenger, had no effect on phosphorylation of ERK1/2 and $cPLA_2$ in both cell types (Fig. 3.5 A–F).

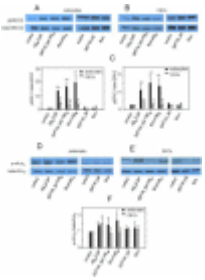

Fig.5

Effects of NADPH oxidase inhibitor and ROS scavenger on ERK1/2 and cPLA2α phosphorylation in primary astrocytes and CECs

These data suggest a presence of two different downstream pathways in the CECs and astrocytes. Although $A\beta_{42}$ binding to RAGE is required to induce phosphorylation of ERK1/2 and $cPLA_2$ in both CECs and primary astrocytes, NADPH oxidase inhibition does not suppress phosphorylation of ERK1/2 and $cPLA_2$in primary astrocytes.

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4. Discussion

Results from this study provided support for the involvement of RAGE in $A\beta_{42}$-mediated oxidative stress and downstream MAPK/ERK pathway in CECs and astrocytes. Specifically, we demonstrated that $A\beta_{42}$oligomers directly bind to RAGE at the surface of the cells to induce colocalization between NADPH oxidase subunits gp91-phox and p47-phox, and subsequently leading to generation of ROS, and phosphorylation of ERK1/2 and $cPLA_2$ in CECs. Interestingly, our data also showed that activation of NADPH oxidase and increased ROS production were not required for Aβ-induced phosphorylation of ERK1/2 and $cPLA_2$ in astrocytes.

In agreement with impairments of BBB structure and functions in AD, many studies have indicated a decrease in cerebral blood flow, reduced microvascular density, and low

immunoreactivity of endothelial markers CD34 and CD31 in AD brains (Kalaria and Pax, 1995, Luc et al., 1997, Berzin et al., 2000, Bailey et al., 2004, Ervin et al., 2004). Although there is evidence that oxidative stress is a major factor leading to a BBB cells dysfunction in AD, the mechanism for production of Aβ-induced ROS has not been clearly elucidated (Smith et al., 1991, Behl et al., 1994, Mecocci et al., 1994, Cini and Moretti, 1995, Simonian and Coyle, 1996, Behl and Holsboer, 1998, Heneka and O'Banion, 2007). Recently, RAGE has been postulated to function as a signal transducing cell surface acceptor for Aβ (Yan et al., 1996, Lue et al., 2001, Sasaki et al., 2001, Arancio et al., 2004, Chaney et al., 2005). In fact, the possible links between RAGE and oxidative stress has been reported (Yan et al., 1996, Lue et al., 2001, Sasaki et al., 2001,Arancio et al., 2004, Chaney et al., 2005). ROS has been demonstrated to be generated by the advanced glycation end products (AGE)-RAGE interaction in human endothelial cells (Wautier et al., 2001). Inhibition studies have indicated that anti-RAGE IgG significantly suppressed oxidative stress induced by Aβ both in vascular and neuronal cell (Yan et al., 1996); however, the relationship between Aβ-RAGE interactions and NADPH oxidase has yet to be elucidated.

It is suggested that NADPH oxidase is the primary source of superoxide in astrocytes and CECs (Cai et al., 2003, Abramov and Duchen, 2005, Park et al., 2005, Qing et al., 2005, Park et al., 2008, Zhu et al., 2009). We have previously reported that $A\beta_{42}$ oligomers induce ROS generation through activation of NADPH oxidase in primary astrocytes (Zhu et al., 2006). It has been shown that inhibition of NADPH oxidase significantly suppressed ROS released in rat astrocytes (Qing et al., 2005). Aβ-induced ROS overproduction and mitochondrial depolarization were absent in astrocytes cultured from gp91phox knockout transgenic mice (Abramov and Duchen, 2005). In a model of AD, inhibition of NADPH oxidase has been found to abrogate Aβ-induced ROS production and alteration of cerebrovascular functions (Park et al., 2005). APP transgenic mice lacking the NADPH oxidase subunits gp91-phox or phagocytic NADPH oxidase (Nox2) did not develop oxidative stress, cerebrovascular dysfunction, and behavioral deficits (Park et al., 2005, Park et al., 2008). Our results suggest that $A\beta_{42}$ oligomers induce ROS production through binding to RAGE and activation of NADPH oxidase (Fig. 2, ,33).

Aβ-induced ROS generation, in turn, triggers the downstream pathway of $cPLA_2$ (Shelat et al., 2008). It has been reported that the immunoreactivity of $cPLA_2$ (group IVA) increased in reactive astrocytes in severe AD brains (Stephenson et al., 1996, 1999). *In vitro*, treatment of astrocytes with Aβ increased ROS production from NADPH oxidase and activated $cPLA_2$ (Zhu et al., 2006, Hicks et al., 2008). Furthermore, $cPLA_2$ was shown to cause a decrease in mitochondrial membrane potential ($\Delta\psi_m$) and resulted in more ROS production (Zhu et al., 2006). In primary rat cortical astrocytes, menadione induced ROS production mediated by NADPH oxidase (Zhu et al., 2009). In neurons, Aβ-induced ROS generation led to phosphorylation of $cPLA_2$ and arachidonic acid (AA) release (Shelat et al., 2008). In turn, hydrolytic products of $cPLA_2$ was shown to enhance NADPH oxidase activity, forming a viscous cycle (Cherny et al., 2001, Levy, 2006). Our data showed that $A\beta_{42}$ induced phosporylation of $cPLA_2$ was totally suppressed by Ab_{RAGE} (Fig.4), suggesting the involvement of RAGE in this signaling pathway.

There are several cell-specific mechanisms identified for activation of $cPLA_2$. In primary astrocytes, phosphorylation of cytosolic $cPLA_2$ and the subsequent release of AA can be activated by G-protein-coupled receptor agonists (Yin et al., 2002, Sun et al., 2007). In cortical neurons, protein kinase C (PKC), extracellular signal-regulated kinases (ERK1/2), and p38 mitogen-activated protein kinase have been involved in activation of $cPLA_2$ and ROS production

(Sun et al., 2007). In turn, there is a link between Aβ, RAGE, and MAPKs. Several studies have indicated that Aβ induced apoptosis signal-regulating kinase 1 (ASK1) dephosphorylation and p38 MAPK activation in CECs (Emmanuelle et al., 1997, Xu et al., 2001,Yin et al., 2002, Hsu et al., 2007). Similarly, stimulation of RAGE has also been demonstrated to mediate alterations in the phosphorylation state of MAPKs (Origlia et al., 2009). Our data provided a link between these studies and demonstrated that binding of $A\beta_{42}$ to RAGE mediated alterations in the phosphorylation state of ERK1/2 and $cPLA_2$ both in primary astrocytes as well as in CECs (Fig.4).

Although $A\beta_{42}$ binding to RAGE is required to induce phosphorylation of ERK1/2 and $cPLA_2$ in both cell types, we found the existence of two slightly different downstream pathways for CECs and astrocytes. In CECs, NADPH oxidase inhibitor gp91ds-tat and ROS scavenger Tiron attenuated $A\beta_{42}$-induced ROS production, ERK1/2 activation, and $cPLA_2$ phosphorylation (Fig. 5 B,C,E,F). In primary astrocytes, inhibition of NADPH oxidase and ROS production did not suppress $A\beta_{42}$-induced ERK1/2 and $cPLA_2$phosphorylation (Fig.5 A,C,D,F). Apparently, results here are not in agreement with our previous studies showing $A\beta_{42}$-induced ERK1/2 and $cPLA_2$ phosphorylation in astrocytes through NADPH oxidase (Zhu et al., 2006, Zhu et al., 2009). One possible explanation is differences in use of NADPH oxidase inhibitors. In the study by Zhu et al., apocynin was used as NADPH oxidase inhibitor. Several studies indicate that apocynin can inhibit NADPH oxidase only in blood derived cells (leukocytes, microglia, etc) (Heumuller et al., 2008), and in other cell types, this compound can act as an antioxidant (Heumuller et al., 2008). In fact, in other cases, apocynin can even exert as an oxidant (Riganti et al., 2006). These results suggest that effects of apocynin may different depending on experimental conditions, and thus should be interpreted with caution. In our study, we used gp91ds-tat which is a specific NADPH oxidase inhibitor. Differences in effects of NADPH oxidase inhibition on ERK1/2 and $cPLA_2$ phosphorylation could also result from the preparation of astrocytes and the purity of the cell culture. Therefore, more investigations are required to unravel the mechanism(s) underlying the Aβ-RAGE interaction resulting in activations of ERK1/2 and $cPLA_2$ in astrocytes.

In summary, this study demonstrates the important role of $A\beta_{42}$-RAGE interaction for NADPH oxidase complex assembling, resulting in subsequent ROS generation, activation of MAPK/ERK pathway, and the phosphorylation of $cPLA_2$ in CECs and primary astrocytes. Our results also revealed two possibly different RAGE-dependent signal transducing pathways in CECs and astrocytes. In CEC, it is possible to demonstrate requirement for NADPH oxidase-mediated generation of ROS to activate ERK1/2 and $cPLA_2$, whereas, astrocytes may activate ERK1/2 and cPLA2 independent of NADPH oxidase. Understanding the precise molecular mechanisms underlying Aβ-mediated oxidative stress should prove to provide new insights into the development of preventive and treatment strategies for AD.

*Highlights

- > Aβ binding to RAGE to activate NADPH oxidase in endothelial cells and astrocytes.
- > Aβ binding to RAGE to activate $cPLA_2$ in endothelial cells and astrocytes.
- > NADPH oxidase activation is not needed for Aβ to activate $cPLA_2$ in astrocytes.

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Acknowledgements

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Footnotes

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