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Aβ Potentiates Inflammatory Activation of Glial Cells Induced by Scavenger Receptor Ligands and Inflammatory Mediators in Culture

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Abstract Alzheimer disease (AD) is a neurodegenerative disorder characterized by the accumulation of β amyloid (Aβ) aggregates. Aβ induces the inflammatory activation of glia, inducing secretion of Interleukin 1β (IL1β), nitric oxide (NO) and superoxide radicals. The specific receptor responsible for the induction of inflammatory activation by Aβ, is still an open question. We propose that scavenger receptors (SR) participate in the activation of glia by Aβ. We assessed production of NO, synthesis of IL1β and activation of ERK, JNK and NF-κB signaling pathways by Western blot, in primary rat glial cultures exposed to SR ligands (fucoidan and Poly I), LPS + IFNγ (LI), and Aβ. Poly I but not fucoidan nor fibrillar Aβ increased threefold NO production by astrocytes in a time-dependent manner. Fucoidan and Poly I increased 5.5- and 3.5-fold NO production by microglia, and co-stimulation with Aβ increased an additional 60% NO induced by SR ligands. Potentiation by Aβ was observed later for astrocytes than for microglia. In astrocytes, co-stimulation with Aβ potentiated ERK and JNK activation in response to Fucoidan and Poly I, whereas it reduced induction of JNK activation by LI and left unaffected NF-κB activation induced by LI. Levels of pro-IL1β in astrocytes increased with Aβ, SR ligands and LI, and were potentiated by co-stimulation with Aβ. Our results suggest that SRs play a role on inflammatory activation, inducing production of NO and IL1β, and show potentiation by Aβ. Potentiation of the inflammatory response of Aβ could be meaningful for the activation of glia observed in AD.

Keywords Alzheimer disease · Astrocytes · MAPKs · Interleukin-1β · Nitric oxide · Inflammation

Introduction

Alzheimer disease (AD) is a neurodegenerative disorder, characterized by the presence of senile plaques conformed of β-amyloid (Aβ) aggregates, surrounded by activated astrocytes and microglia (Walsh and Selkoe 2004). Imbalance between production and degradation of Aβ could play a key role in AD. Astrocytes and microglia give structural support and maintain brain homeostasis. In the brain of AD patients, glial cells have been found closely associated with senile plaques, being proposed their role on recognition, internalization and degradation of Aβ. In vitro experiments confirm the interaction and phagocytosis of Aβ by glial cells (von Bernhardi and Ramírez 2001; Alarcón et al. 2005; von Bernhardi et al. 2007) and suggest that a deficit on the capacity of these cells to phagocytose Aβ could have a central role in AD (Farina et al. 2007). Aβ induces the production of inflammatory mediators such as tumor necrosis factor alpha (TNFα), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein 1 alpha (MIP-1α), interleukin 1beta (IL1β), Interleukin 6 (IL6) (Akiyama et al. 2000; Bamberger and Landreht 2001; Mrak and Griffin 2000), as well as the production of nitric oxide (NO), a classic marker of inflammatory activation (von Bernhardi and Ramírez 2001; von Bernhardi and Eugenín 2004). In astrocytes, Aβ induces production of NO and increased expression of glial fibrillary acidic protein (GFAP), an activation marker, as well as inflammatory

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cytokines, such as TNFα, IL1, and IL6, which in turn induce NO production (Jana et al. 2005). In astrocyte cell lines, Aβ can act synergistically with inflammatory cytokines, inducing the expression of iNOS (Rossi and Bianchini 2006). In AD, glial cells act in a concerted way exerting mutual regulation (von Bernhardi and Ramírez 2001), which is mediated by soluble molecules (von Bernhardi and Eugenín 2004). Activated astrocytes secrete factors that inhibit Aβ neurotoxicity, both directly (Ramírez et al. 2005) and indirectly, by attenuating microglia activation (von Bernhardi and Eugenín 2004). This modulation could be affected by aging and chronic inflammation (von Bernhardi et al. 2010) and the presence of Aβ, inducing a dysregulation of glial activation that could promote the development of AD (von Bernhardi et al. 2007).

The recognition, internalization and degradation of Aβ by glial cells and the induction of inflammatory activation by Aβ could be mediated by surface receptors present in astrocytes and microglia. Several receptors have been identified as capable of binding Aβ. However, the identity of the specific receptor mediating the response generated by Aβ and therefore responsible for the activation of intracellular signaling and the induced inflammatory answer has not been demonstrated. Some of the receptors of microglial cells capable of binding Aβ are CD14 (Fassbender et al. 2004), LRP (Marzolo et al. 2000), RAGE receptor (Yan et al. 1996), Scavenger receptors (SR): SR-BII, SR-AI/II (Husemann et al. 2002), CD36 (Coraci et al. 2002; Husemann et al. 2002) and SR-MARCO (Alarcón et al. 2005). In astrocytes, receptors that appear to bind Aβ are SR-BI, CD36 (Husemann et al. 2002) and SR-MARCO (Alarcón et al. 2005). All these receptors can bind soluble and fibrillar Aβ (Farina et al. 2007), and SR ligands such as acetylated LDL, fucoidan, mel-BSA, dextran sulfate and Poly I can block Aβ uptake (Paresce et al. 1996; Alarcón et al. 2005). Some of these ligands can induce inflammatory activation and NO production (Palkama 1991; Hsu et al. 2001; Campa et al. 2005).

IL1β is one of the major inflammatory cytokines found in AD patients and in AD animal models (Bamberger and Landreht 2001). IL1β is produced both by microglia and astrocytes, participates during early stages of inflammation and has been implicated in neurodegenerative diseases (Akiyama et al. 2000; Simi et al. 2007). Production of IL1β depends on the activation of MAP kinases and NF-κB signaling pathways (Simi et al. 2007; Kim et al. 2004). Aβ induces IL1β production through ERK, JNK and p38 MAPKs activation in microglia (Kim et al. 2004), which in turn are involved in iNOS expression by glial cells (Pyo et al. 1998; Zhang et al. 1996; Kim et al. 2004). In vitro, IL1β can induce ERK, JNK and p38 activation (Parker et al. 2002). In vivo, intracranial injection of IL1β similarly induces ERK activation in neurons, astrocytes and microglia of rodent brains (Nadjar et al. 2005). IL1β can also induce iNOS expression, resulting in an increased production of NO (Marcus et al. 2003; Nadjar et al. 2005).

Patients with advanced AD show an increased iNOS expression in cortical neurons and glial cells, with high levels of NO synthesis (Fernández-Vizarra et al. 2004). High concentrations of NO in the presence of superoxide anion are cytotoxic, generating peroxynitrite anion, a powerful cytotoxic oxidant agent (Chen et al. 1998; Contestabile et al. 2003). Therefore, ERK, JNK, p38 and NF-κB signaling pathways are key pathways that can be involved in Aβ-mediated cytotoxicity. However, the receptors responsible for the induction of these pathways are not well-defined.

SRs are pattern recognition receptors, which recognize potentially damaging molecules (Alarcón et al. 2005; Rotshenker 2009). Therefore, Aβ could activate a danger signal for cells or generate an inflammatory activation leading to the clearance of Aβ. We propose that Aβ binding to this SR family could be relevant for the understanding of the association between inflammation and production of inflammatory molecules such as NO and IL1β in AD.

Materials and Methods

Materials

Fucoidan, Poly I, LPS (O111: B4 from *E. coli*) and Poly C were purchased from Sigma (USA); PD98059 was purchased from Calbiochem (USA) and IFNγ was purchased from R&D (USA). Cell culture media, antibiotics and serum were purchased from Gibco (Life Technologies, USA). $A\beta_{1-40}$ and $A\beta_{1-42}$ were received as a generous gift from Dr. Heinz Döbeli (Hoffmann-La Roche, Switzerland). To generate fibrillar $A\beta_{1-40}$ and $A\beta_{1-42}$, Aβ was resuspended in Tris buffer and incubated in constant agitation at room temperature for 3–4 days (Jarret et al. 1993). The Aβ suspension was centrifuged at 23,000×*g*; 4°C for 1 h. Pellet corresponds to insoluble fibrillar Aβ. Samples were run in a Tris–Tricine SDS-PAGE under reducing and non-reducing conditions. Gels were transferred to a nitrocellulose membrane, blocked, and then probed with the anti-Aβ monoclonal antibody BAP-18 (kindly provided by Dr. H. Döbeli, Hoffmann-La Roche, Switzerland) as previously described (Alarcón et al. 2005). Animals were obtained from the institutional animal facility. All procedures followed the animal handling and bioethical requirements defined by the Pontificia Universidad Católica de Chile School of Medicine Ethics Committee. Animals were anesthetized before killing.

Glial Cultures

Mixed glial cell cultures containing astrocytes and microglia were obtained from cerebral cortex of 1 to 2-day-old Sprague–Dawley rats, as described by Giulian and Baker (1986). Cortices were rinsed with Ca^{2+}/Mg^{2+} free Hank's balanced salt solution (HBSS); meninges were removed, and the tissue was minced, incubated with 0.25% trypsin–EDTA in HBSS at 37°C for 10 min, and mechanically dissociated. Cells were seeded in 75 cm^2 cell culture flasks (one brain per flask) in DMEM/F12 supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cultures were incubated at 37°C in a 5% CO_2, water saturated atmosphere.

After 14 days in culture, microglial cells were obtained by shaking the mixed glial culture at 110 rpm in an orbital shaker (Unimax 1010, Heidolph, Germany) at 37°C for 15–20 min. After removal of microglia, astrocytes were purified by trypsinization. Cells were seeded for 10 min. Non-attached cells were recovered, centrifuged and counted to be used for the experiments. This procedure yields a highly enriched astrocytes culture (over 95% of cells are astrocytes) and microglia cultures over 99% pure.

Astrocytes and microglia were seeded in 96-well plates at a density of 3×10^4 cells per well for nitrite determination and at a density of 5×10^5 cells per 35-mm diameter petri dishes for Western blot assays. Cell types present in cultures were evaluated by labeling with fluorescein isothiocyanate (FITC)-conjugated lectin *Griffonnia simplicifolia* (1:200; Sigma), which recognizes microglia, and immunocytochemistry for glial fibrillary acidic protein (GFAP; 1:200; Dako, Denmark) to identify astrocytes (data not shown).

Determination of Nitrites (NO^{2-})

Nitrites (NO^{2-}), a stable downstream product of NO released by cells, was determined in the culture medium by the Griess assay (Pfeiffer et al. 1997). 3×10^4 cells were maintained at control culture conditions, or incubated with 1 µg/ml LPS + 10 ng/ml INFγ (LI) or SR ligands (100 µg/ml Fucoidan, 200 µg/ml Poly I or 200 µg/ml Poly C). For determination of NO^{2-}, 50 µl of medium was mixed with 10 µl EDTA:H2O 1:1 (0.5 M, pH 8.0) and 60 µl of freshly prepared Griess reagent (20 mg *N*-[1-naphtyl]-ethylenediamine and 0.2 g sulphanilamide dissolved in 20 ml of 5% phosphoric acid, w/v). Calibration curves were established with 1–80 µM NaNO2. Absorbency was measured at 570 nm in a microplate auto reader (ANTHOS 2010, Anthos Labtec Instrument).

Western Blot Analysis

Astrocytes (5×10^5 cell per 35 mm plaques), after being exposed to inflammatory molecules or SR ligands, were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and protease inhibitors). Protein concentration was determined by the BCA assay. Cell samples (30 µg protein) were electrophoretically separated on 12% poly-acrylamide gels and transferred to a nitrocellulose membrane. The membrane was blocked with 0.1% Tween 20, 5% milk in PBS for 1 h, and then incubated with the primary antibody in blocking buffer: goat α IL1β (1:1,000; R&D), rabbit α p-ERK/ERK (1:500; Santa Cruz Biotechnology), rabbit α p-JNK/JNK (1:500; Cell signal), rabbit α IKB (1:500; Santa Cruz Biotechnology) or mouse α βTub I + II (1:1,000; Chemicon). Primary antibodies were rinsed and membranes were incubated with the secondary antibody, horseradish peroxidase-conjugate donkey α-goat, α-rabbit or α-mouse, depending on the primary antibody previously used. Signals were detected by enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer's instructions. Densitometry was done with the ImageJ program.

Statistical Analysis

In vitro data correspond to at least 3–4 independent experiments in triplicate and was expressed as mean ± SEM. Statistical analysis was done with the Kruskal–Wallis one-way ANOVA and the Wilcoxon Rank Sum/Mann–Whitney *U* test. Relevance of Aβ for the induction of inflammatory activation was evaluated by the non-parametric Quade Test or a 2-way ANOVA. Evaluation was performed using the GB-stat statistical software (Dynamic Microsystems, Inc.). For statistical analysis, a value of $P < 0.05$ was considered significant for a single comparison. Multiple comparisons were corrected by the α value.

Results

SR Ligand Poly I but not Aβ Induced NO Production by Astrocytes

Astrocyte cultures exposed to 2 µM $A\beta_{1-42}$ or $A\beta_{1-40}$ peptide for 48 or 96 h (Fig. 1a) did not induce NO production compared with their control condition. Both fibrillar forms were equivalent in their effect. The SR ligand fucoidan (200 µg/ml) also failed in inducing NO (Fig. 1b). However, astrocytes treated with 200 µg/ml Poly I increased NO production 1.4- and 2.5-fold after 48 or 96 h, respectively. Co-stimulation with Aβ did not further induce NO production (Fig. 1c). Stimulation with LI, a well-studied inflammatory condition, induced NO production 4.5- and 4-fold at 48 or 96 h, respectively (Fig. 1d). There was no potentiation by Aβ at 48 h. In contrast, co-stimulation with LI plus Aβ increased 2.3-fold NO

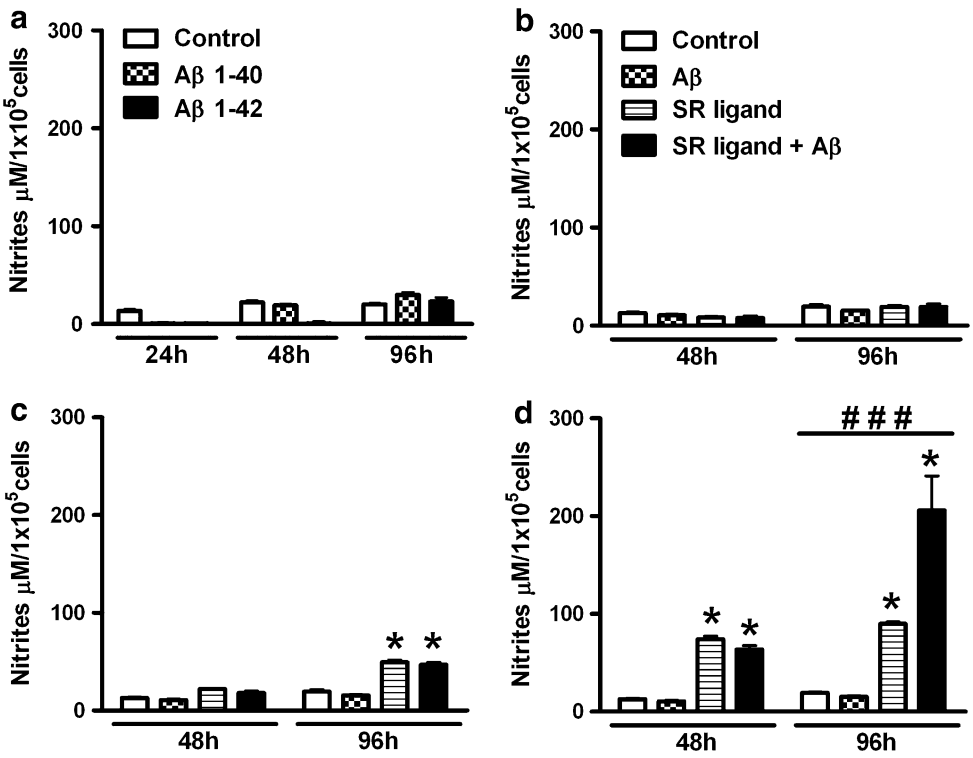

Fig. 1 NO production was not increased in astrocytes exposed to Aβ. NO production by astrocytes exposed to **a** 2 μM $A\beta_{1-40}$ or $A\beta_{1-42}$ for 24, 48 and 96 h. **b** 200 μg/ml fucoidan, with or without 2 μM Aβ. **c** 200 μg/ml Poly I, with or without 2 μM Aβ and **d** LI (1 μg/ml LPS, plus 10 ng/ml IFNγ) with or without 2 μM Aβ for 48 and 96 h. Data is expressed as mean ± SEM correspond to four independent experiments in triplicate, $^{*}P < 0.05$, $^{***}P < 0.0001$ for astrocytes stimulated with SR ligands and Aβ compared with the control condition. The Quade Test showed that co-stimulation with Aβ significantly increased NO production at 96 h. $^{\#\#\#}P < 0.0001$

production compared with NO production by LI stimulation at 96 h. Note that LI induced a 2- to 4-fold higher NO production compared with stimulation with Poly I (Fig. 1c, d). In contrast, Poly C, a negative control for SR ligands, did not induce NO production at any stimulation duration (data not shown). Our results show that astrocytes are activated only by certain SR ligands, slowly increasing production of NO.

Co-Stimulation with SR Ligands and Aβ Promotes NO Production by Microglial Cells

Whereas astrocytes exposed to Aβ and fucoidan did not show increased NO production compared with control cells (Fig. 1b), microglial cells exposed to fucoidan for 48 h showed a 4.5-fold increase on NO production. NO induction decreased to 2.4-fold by 96 h (Fig. 2a). In microglia, Poly I induced a 3- and 4-fold increase of NO production at 48 and 96 h, respectively (Fig. 2b). Co-stimulation with Aβ (fibrillar forms of $A\beta_{1-42}$ and $A\beta_{1-40}$ were equivalent in their effect) and Poly I induced a 2.8- and 5.5-fold increase on NO production in microglia compared with control cultures at 48 and 96 h, respectively (Fig. 2b). Microglia stimulated with LI showed a 6.7- and 6.0-fold induction of NO production at 48 and 96 h (Fig. 1c). Co-stimulation with Aβ and LI induced an 87% increase of NO production compared with LI treatment at 48 h (Fig. 2c). However, induction of NO production reached the same level that was observed with LI alone at 96 h (Fig. 2c). Thus, compared with astrocytes, microglia were activated by both SR ligands, NO production occurred earlier and was potentiated by co-stimulation with Aβ.

Aβ Potentiated Activation of Signaling Pathways Induced by SR Ligands in Astrocytes

For the assessment of the activation of signaling pathways, astrocytes were exposed to SR ligands with or without Aβ co-stimulation for 24 h. To assess the activation of NF-κB signaling, we evaluated the reduction of IκB levels, inhibitor of the transcriptional factor NF-κB, which is degraded via proteasome when this signaling pathway is active. Presence of p-ERK, p-JNK and IκB was detected by Western blot assay. Astrocytes exposed to Aβ did not show activation of p-ERK, p-JNK or NF-κB pathways (Fig. 3). Similarly, fucoidan did not induce the activation of any of the evaluated signaling pathways (Fig. 3). However, co-stimulation with Aβ and fucoidan induced a statistically significant increase on JNK activation (Fig. 3b).

Astrocytes exposed to Poly I showed a 2.9-fold increase on ERK phosphorylation compared with its control. Co-stimulation with Poly I plus Aβ increased ERK phosphorylation by 3.2-fold, reaching statistical significance (Fig. 3a). On the other hand, a 2.5-fold increase of JNK phosphorylation was observed in astrocytes exposed to Poly I, and co-stimulation with Aβ resulted in a 3.4-fold increase on JNK activation compared with control astrocytes (Fig. 3b).

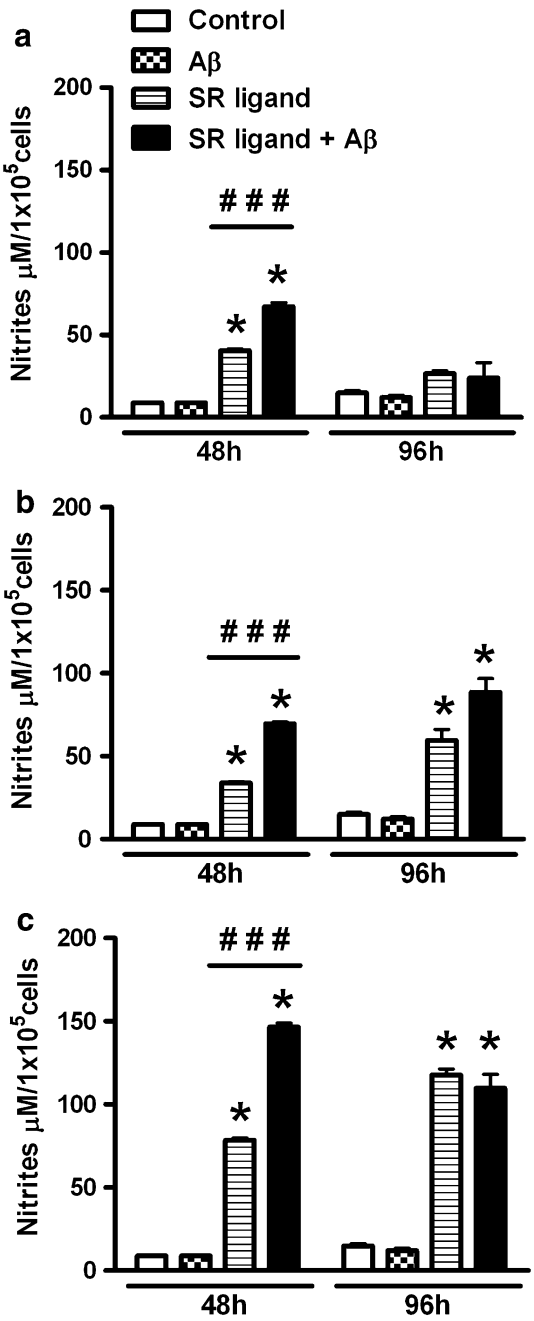

Fig. 2 Aβ potentiated NO production induced by SR ligands in microglial cells in culture. NO production by microglia exposed to **a** 200 μg/ml fucoidan and 2 μM Aβ. **b** 200 μg/ml Poly I and Aβ and **c** 1 μg/ml LPS plus 10 ng/ml INFγ (LI) plus 2 μM Aβ for 48 and 96 h. Data correspond to the of mean ± SEM of 4 independent experiments in triplicate, $^{*}P < 0.05$ for microglia stimulated with SR ligands and Aβ compared with the control condition. The Quade Test showed that co-stimulation with Aβ significantly increased NO production at 48 h, but no at 96 h. $^{###}P < 0.0001$ compares microglial cells stimulated with SR ligands with and without Aβ

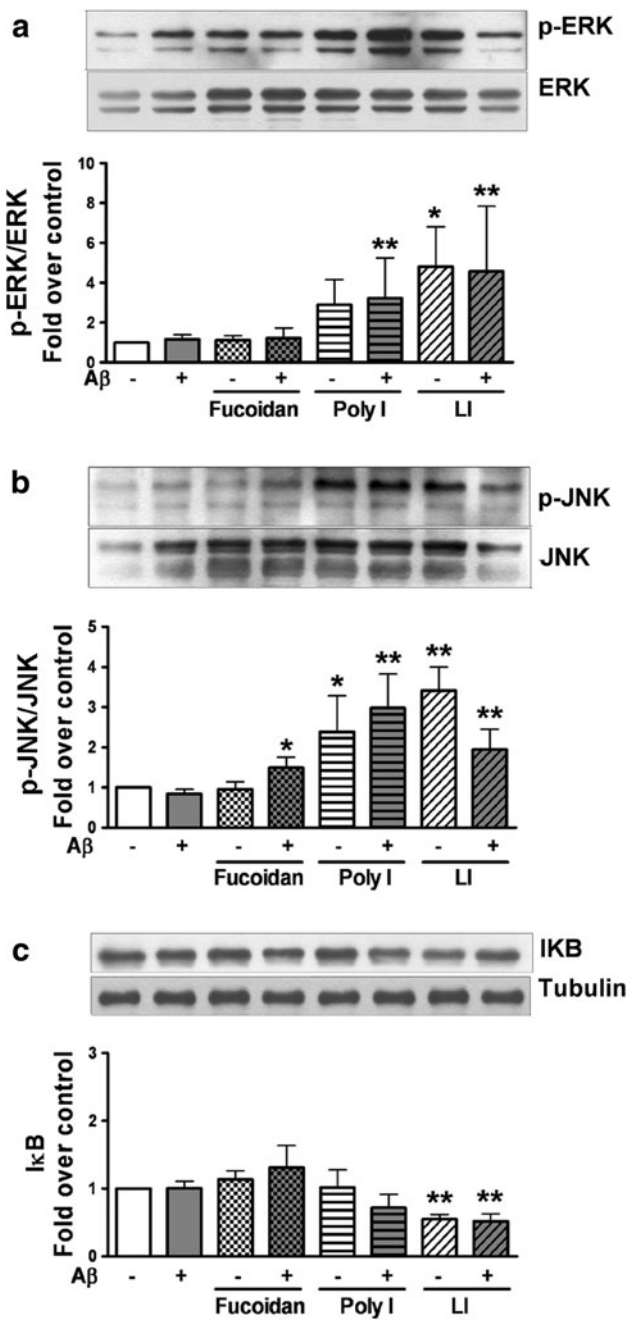

Fig. 3 Aβ had a mild effect on the activation of inflammatory signaling pathways by Poly I and LI in astrocytes. **a** Levels of phospho- and total-ERK (p-ERK and ERK). **b** phospho- and total-JNK (p-JNK and JNK) and **c** IκB of astrocytes exposed to fucoidan, Poly I and LI with or without 2 μM Aβ for 24 h. Co-stimulation with Aβ was associated with a 40% decrease of JNK activation. Data correspond to the mean ± SEM of four independent experiments in triplicate, $^{*}P < 0.05$, $^{**}P < 0.01$ for astrocytes stimulated with SR ligands and Aβ when compared with the control condition

LI induced a 4.8-fold increase of ERK and a 3.5-fold increase of JNK activation compared with the control condition (Fig. 3a, b, respectively). Co-stimulation with LI and Aβ increased 4.6-fold ERK phosphorylation compared with control astrocytes. LI plus Aβ induced a 60% decrease of JNK activation compared with astrocytes exposed to LI and a 2.2-fold increase of JNK phosphorylation compared

with control cells (Fig. 3b). Finally, LI, both with or without co-stimulation with Aβ induced a 50% decrease in IKB levels when compared with its control, whereas SR ligands did not affect this signaling pathway (Fig. 3c). Our results show that Aβ is able to specifically strengthen the activation of ERK and JNK induced by SR ligands but not by LI.

Aβ Induced and Potentiated SR Ligand-Induced Synthesis of IL1β

Astrocytes were treated with Aβ for 24 h. SR ligands and IL1β synthesis was assessed by Western blot and ELISA assays. Astrocytes exposed to Aβ showed a 3.3-fold increase in pro-IL1β when compared with non-treated astrocytes. Astrocytes exposed to fucoidan showed a 7.3-fold increase of pro-IL1β, and those treated with Poly I and LI increased pro-IL1β levels by 10.7- and 10-fold, respectively (Fig. 4a). Co-stimulation with fucoidan plus Aβ increased by tenfold level of pro-IL1β. Similarly, co-stimulation with Poly I and Aβ and LI and Aβ increased by 15.6- and 18.2-fold pro-IL1β synthesis when compared with the control condition (Fig. 4a). A two-way ANOVA showed that both SRA ligands ($P = 0.0006$) and co-stimulation with Aβ ($P = 0.0124$) significantly induced pro-IL1β production. The production of IL1β in the presence of Poly I, LI, Aβ and the combinations between Poly and LI were evaluated by ELISA (Fig. 4b). The production of IL1β increased 2.2-fold after Aβ treatment, and by 5.3- and 10.9-fold in presence of Poly I and LI compared with the control condition (Fig. 4b). Co-stimulation with Poly I or LI plus Aβ similarly increased IL1β release by 5.6- and 10.1-fold compared with the control condition (Fig. 4b). Thus, Aβ, SR ligands and LI were all able to induce production of pro-IL1β and IL1β by astrocytes. Moreover, Aβ significantly potentiated the induction of pro-IL1β.

ERK Signaling was Involved in the Activation of Astrocytes by SR Ligands

To evaluate the participation of ERK signaling on the stimulation of NO and IL1β production by SR ligands and LI, astrocytes were exposed to the ERK1/2 inhibitor, PD98059 for 1 h before stimulation with Aβ, Poly I, or LI. As control we used dimethyl sulfoxide (DMSO), the inhibitor vehicle (Fig. 5). In astrocytes pretreated with PD98059 for 1 h, induction of ERK phosphorylation after stimulation with LI was abolished (Fig. 5a) compared with astrocytes exposed to LI. In astrocytes pretreated with PD98059 and then stimulated with Poly I and LI, production of NO decreased between 50% and 2.2-fold in presence of Poly I and LI, respectively, compared with astrocytes exposed to ligands alone (Fig. 5b; $P < 0.05$).

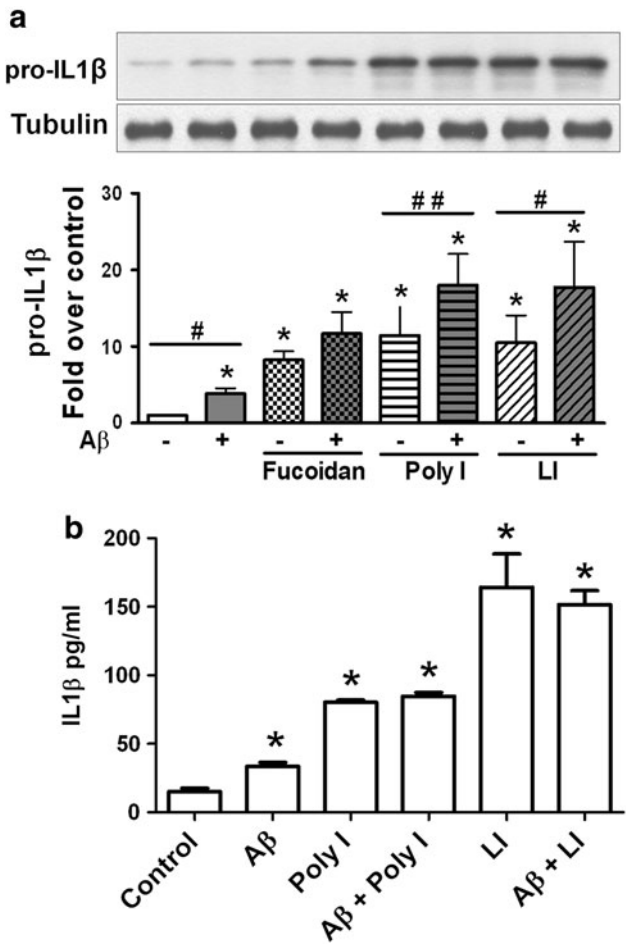

Fig. 4 Aβ and SR ligands induced synthesis and release of IL1β by astrocytes. **a** Detection of pro-IL1β by Western blot in astrocytes exposed to 2 μM Aβ, fucoidan, Poly I and LI for 24 h. **b** Detection of IL1β secreted to the media by ELISA. Astrocytes exposed to Poly I, LI, Aβ and co-stimulation with Aβ plus Poly I or LI for 96 h. Data correspond to the mean ± SEM of 4 independent experiments, $^{*}P < 0.05$ for astrocytes stimulated with SR ligands and Aβ compared with the control condition. The Quade Test showed that co-stimulation with Aβ significantly increased pro-IL1β production. $^{\#}P < 0.05$, $^{\#\#}P < 0.001$ compares cells stimulated with SR ligands with and without Aβ

Poly I- and LI-induced release of IL1β was inhibited in 35 and 50% by PD98059. In contrast, astrocytes exposed to Aβ plus the ERK inhibitor showed a 60% increase in IL1β secretion compared with astrocytes stimulated with Aβ alone (Fig. 5c). These results indicate that activation of ERK is needed for the induction of iNOS (Fig. 6).

Discussion

A large body of evidence suggests that inflammation is a key element for the progression of neurodegeneration. Glial cells are actively involved in inflammatory processes, and can participate in neuronal impairment. McGeer and

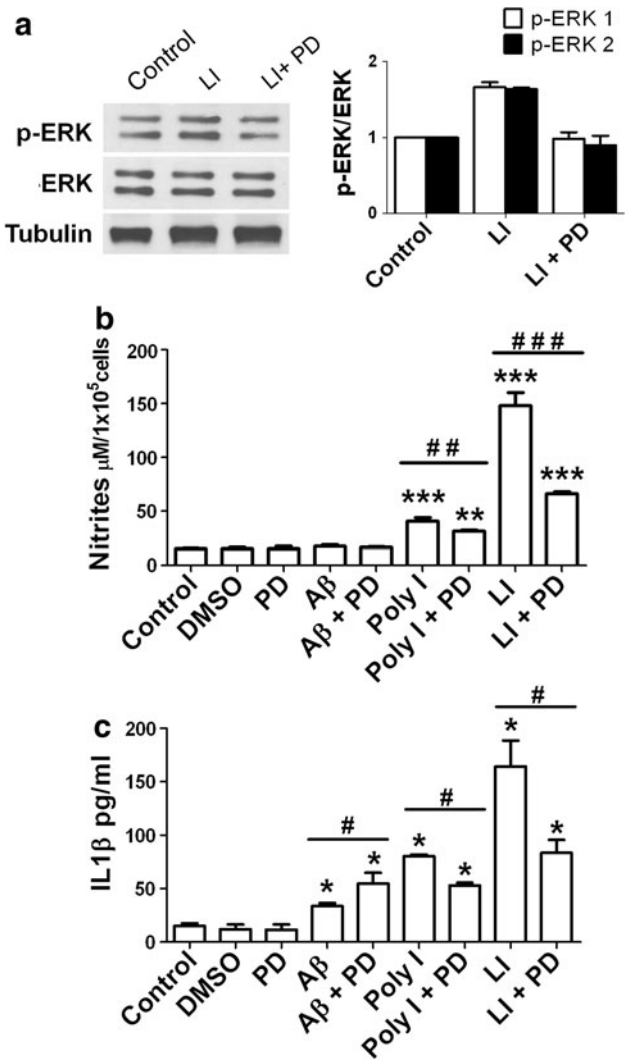

Fig. 5 ERK signaling pathway participated in the production of NO and IL1β by astrocytes exposed to SR ligands. **a** Astrocytes were exposed to 10 μM PD98059 for 1 h and then were stimulated with LI for 30 min. Levels of phospho- and total-ERK (p-ERK and ERK) were detected through Western blot analysis. Tubulin was immunodetected as internal loading control. PD98059 nearly abolished the increment of ERK activation (p-ERK/ERK). **b** NO production and **c** IL1β release were assessed on astrocytes exposed to ERK inhibitor and then stimulated for 96 h with Aβ, Poly I and LI. Data correspond to the mean ± SEM of 3 independent experiments in triplicate. $^{*}P < 0.05$ and $^{***}P < 0.0001$ for astrocytes stimulated with SR ligands and Aβ compared with the control condition. The Quade Test showed that co-stimulation with Aβ significantly increased pro-IL1β production. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.0001$ compares cells stimulated with SR ligands with and without Aβ

our group (McGeer and McGeer 1995; von Bernhardi 2007) propose that neuronal damage in AD is not caused by the accumulation of Aβ, but rather by its inflammatory response and glial activation. Sheng demonstrated that neuroinflammation induced by LPS increases intracellular accumulation of Aβ inducing neuropathological changes similar to those found in AD brains (Sheng et al. 2003). Those reports suggest that Aβ accumulation could be secondary to inflammatory processes in the nervous system. Here, we show that Aβ was unable to induce NO production by astrocytes and microglia. A possible explanation is that previous work used high concentrations of Aβ (50 μM) to stimulate cells (Johnstone et al. 1999), compared with the low concentration (2 μM) used in this work; suggesting that high concentrations of Aβ are needed to induce by itself an inflammatory response.

It has been proposed that inflammatory cytokines and other microglia-derived factors account for neurotoxicity, whereas reactive astrocytes would be neuroprotective (Giulian and Corpuz 1993; Giulian et al. 1993, 1994). However, there is also evidence that astrocytes can undergo activation in response to activated microglial cells and can even cooperate in microglial cell activation, enhancing oxidative stress (Schubert et al. 2000). Here, we show that both astrocytes and microglia were able to produce NO when stimulated with SR ligands. However, not all SR ligands evaluated were capable of inducing production of NO or activating signaling pathways involved in inflammation, such as ERK, JNK and NF-κB, in astrocytes (Akiyama et al. 2000; Brambilla et al. 2005; van Loo et al. 2006; Borsello and Forloni 2007). Differences of reactivity between astrocytes and microglia could depend on the fact that microglia present a higher reactivity for these SR ligands than astrocytes.

Being the macrophages of the central nervous system (CNS), microglia are key cells for the removal of exogenous molecules. In macrophages and microglia, fucoidan induce NO production (Nakamura et al. 2006) and activated MAPKs signaling pathways (Hsu et al. 2001). Fucoidan, for example, classically used as a SR ligand capable of activating MAPKs signaling pathways (Hsu et al. 2001) was unable to induce NO production, or activate MAPKs and NF-κB in astrocytes. In contrast, fucoidan induced a 3.5-fold increase of NO production by microglia (Nakamura et al. 2006), a difference that could be explained because of the high density of SRs present in microglia (Farina et al. 2007).

On the other hand, Poly I induced NO production by astrocytes and microglia. However, NO release by astrocytes was delayed compared with NO production by microglia, which was already observed at 48 h. In astrocytes, Poly I activated JNK signaling, but a significant activation of ERK was only observed after co-stimulation with Aβ. Similarly, only co-stimulation of fucoidan plus Aβ activated JNK. In contrast, SR ligands were unable to induce activation of NF-κB, a classically signaling pathway involved on iNOS induction (Campa et al. 2005; Nakamura et al. 2006) and in many inflammatory diseases (Lawrence 2009). LI, in turn, induced a robust induction of the production of NO in both astrocytes and microglia,

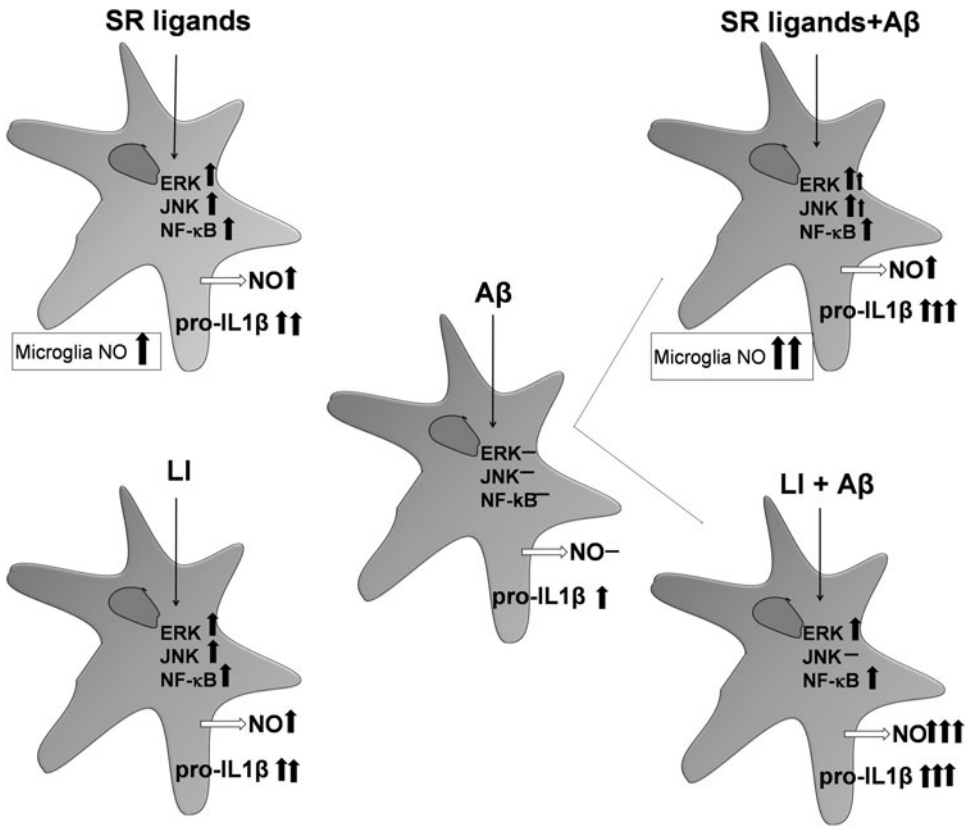

Fig. 6 Explicative model of the potentiating of co-stimulation with Aβ plus SR ligands. Astrocytes exposed to Aβ showed an increased synthesis of pro-IL1β but not NO nor activation of MAPK and NFκB signaling. Astrocytes exposed to SR ligands and LI increased synthesis of pro-IL1β and NO. Co-stimulation with LI and Aβ potentiated production of NO after long lasting stimuli (96 h) in contrast to the early potentiating of NO production observed in microglia. Synthesis of Pro-IL1β was induced by Aβ as well as by the other ligands. However, activation of inflammatory signaling was restricted to certain SR ligands and LI; and showed a discrete increase in the activation of ERK and JNK inflammatory signaling after co-stimulation with Aβ and fucoidan or Poly I. In contrast, co-stimulation with Aβ decreased activation of JNK by LI

activating MAPKs and NF-κB signaling pathways, which are probably responsible for the persistent inflammatory activation responsible for NO production. However, we consistently found a reduction of NO production at 96 h. This is hard to explain phenomena, but we propose it may depend on an increased oxidative stress observed after long inflammatory activation, oxygen radical species can oxidize NO and nitrite into nitrate, making it undetectable by the Griess assay (Koppenol et al. 1992; Nims et al. 1996).

As previously mentioned, although Aβ was unable to induce NO production by glial cells by itself, co-stimulation of astrocytes with both Poly I or LI for 96 h, or earlier co-stimulation of microglia with both fucoidan and with Poly I and LI resulted in a potentiating of NO induction compared with stimulation with SR ligands alone.

Astrocytes exposed to LI plus Aβ increased NO production. However, increased NO was delayed compared with microglia. Co-stimulation with LI and Aβ induced activation of NF-κB, whereas JNK activation decreased in presence of Aβ. There is evidence that the activation of NF-κB pathway inhibit a phosphorylation of JNK (Liu and Lin 2005), a signaling pathway involved in inhibition of apoptosis. LPS and INFγ are powerful inflammatory molecules (Ramírez et al. 2005). LPS binds to Toll like receptor-4 (TLR-4) and this receptor can activate MAPKs and NF-κB signaling pathways. Binding of INFγ to its receptor activated JAK/STAT signaling pathway also induce NO production (Dell'Albani et al. 2001). Therefore, astrocytes could prevent cell death in presence of LI plus Aβ through inhibition of JNK phosphorylation and activation of NF-κB (Cogswell et al. 1994; Saha and Pahan 2006; Liu and Lin 2005).

Synthesis of pro-IL1β is activated by ERK, JNK and NF-κB pathways (Kim et al. 2004). Astrocytes exposed to Aβ, fucoidan, Poly I and LI showed a differential activation of signaling pathways as we already discussed. There are several types of SR that appear to bind to these ligands (Neyen et al. 2011), but the cellular response they induce appears to be different depending on the ligand (Platt and Gordon 1998). Differences on the activation of signaling pathways could determine differences on the induction of iNOS or IL1β synthesis (Hsu et al. 2001). JNK activation mediates IL1β expression induced by fucoidan in macrophages (Hsu et al. 2001), whereas IL1β induction by SR ligands was not accompanied by induction of JNK phosphorylation in astrocytes. The induction of pro-IL1β by Aβ was not associated with the activation of any of the pathways assessed in this work, suggesting that other pathways are also involved in the production of IL1β. However, here we demonstrated that ERK signaling pathway participates at list in part in the release of IL1β in astrocytes exposed to SR ligands.

We propose that SRs play an active role in neuroinflammation through soluble factors like NO and IL1β. Increased levels on cytokine induction in presence of Aβ lead to impairment of glial modulation resulting in a cytotoxic activation and neurodegenerative changes.

Conclusions

- Low concentrations of Aβ (2 μM) did not induce NO production by glial cells.
- SR ligands differentially activated signaling pathways involved in the inflammatory activation of glial.
- Co-stimulation of SR ligands and Aβ potentiated NO production by microglia and induced an activation of inflammatory signaling pathways in astrocytes.
- Aβ induced synthesis of pro-IL1β and release of IL1β by itself, and potentiated SR ligand-dependent pro-IL1β synthesis by astrocytes.
- ERK signaling pathway participated in the NO and IL1β production in astrocytes exposed to SR ligands.

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Conflict of interest Authors (PM, BG and RvB) declare they have no conflict of interest.

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