

Involvement of the receptor for advanced glycation-end products (RAGE) in β -amyloid-induced toxic effects in rat cerebromicrovascular endothelial cells cultured *in vitro*

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Abstract. To ascertain whether the potential biological effects of β amyloid (β A) on the endothelium are partly mediated by the receptor for advanced glycation-end products (RAGE), we performed a series of experiments which analyzed the effects of the β A₍₁₋₄₂₎ peptide on *in vitro* cerebro-microvascular endothelial cells (CECs). Our results suggest that RAGE is directly responsible for β A₍₁₋₄₂₎ actions on CECs, such as its toxic effect on cell survival, viability and angiogenic capability. We observed that a 6-h incubation period exposing CECs to β A₍₁₋₄₂₎ increased the extracellular levels of nitrite. Furthermore, the presence of a nitric oxide synthase inhibitor, L-NAME, was able to enhance CEC survival and viability. Immunocytochemical analyses demonstrated that the peptide induced expression of the inducible form of NOS, iNOS, typically synthesized in response to immune/inflammatory stimuli. Upon blocking the interaction of β A₍₁₋₄₂₎ and RAGE, we observed significantly decreased levels of NO and suppression of iNOS immunoreactivity. In conclusion, our data suggest the involvement of RAGE, at least partly, in mediating the effects of β A₍₁₋₄₂₎ on CECs. In particular, the decrease of *in vitro* cell viability and functionality and nitrosative stress activation was inhibited by blocking β A₍₁₋₄₂₎-RAGE interaction.

Introduction

Several studies have demonstrated that Alzheimer disease (AD) is often accompanied by cerebrovascular pathology, structural microvascular abnormalities (such as loss or thinning of endothelium, basement membrane thickening) and

hypoperfusion (1,2), suggesting that vascular deficits might play a causative role in the pathogenic mechanism of AD (3).

β amyloid (β A), the major component of senile plaque, is considered to have a causal role in the development and progression of AD, and it has been demonstrated that it may accumulate not only in the brain parenchyma but also in the cerebral blood vessels (4). Recent evidence indicates that β A deposited in the brain is linked to β A within the intravascular space, and studies in AD animal models demonstrated that a significant amount of β A is transported from blood to brain (5). This evidence highlights the importance of the transport of circulating β A as a source for brain β A through the blood-brain barrier (BBB). The peptide concentration within the intravascular space seems, in fact, to be linked to β A deposited in the brain suggesting that peptide transport across the BBB regulates β A brain levels (6). However, BBB does not allow free exchanges of polar solutes, such as β A, between blood and brain, due to the presence of a continuous cellular monolayer of tightly junctioned endothelial cells (7). Thus, specialized transporters for the peptide must exist in brain endothelium to shuttle circulating β A into the CNS. Several investigations to identify cellular binding site(s) for β A converged on the receptor for advanced glycation end-products (RAGE) and demonstrated that RAGE seems to mediate β A effects on microglia, BBB and neurons through activating different signaling pathways (8). Moreover, increased RAGE immunoreactivity has been found to be associated with vessels displaying amyloid deposition, and an increase in RAGE expression in affected cerebral vessels has been demonstrated, suggesting the potential involvement of this receptor in the associated perturbation of vascular abnormalities in amyloid-laden vessels (9).

β A-mediated cytotoxicity either in vasculature (10) or in CECs (11) seems to be correlated with oxidative stress and in particular with the involvement of nitrosative stress. There is, in fact, evidence for the involvement of nitric oxide (NO) in the pathological processes of AD (12); increased levels of nitrotyrosine-modified proteins have been found in astrocytes, blood vessels and neuronal cytoplasm in AD (13-15) suggesting that NO could play a role in the disease process. It has been demonstrated that β A, interacting with neuronal and glial membranes, induced free radical production, with consequent destabilization of cellular membranes (16,17).

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Moreover, $\beta A_{(1-42)}$ increased NO levels in microglia and astrocytes by means of inducible nitric oxide synthase (iNOS) activation (18). iNOS is expressed in response to inflammatory stimuli and, producing nanomolar amounts of NO, it is an important mediator of cytotoxicity in the brain (19). Previous studies showed up-regulated expression of iNOS in AD brains and at a cerebral microcirculation level, supporting the hypothesis that aberrant levels of iNOS increase nitrotyrosine levels, which might serve as a marker of oxidative stress in the progression of the neurodegenerative process in AD (15).

Most of these studies have been conducted on neurons, astrocytes and brain parenchyma, and considering the importance of microvasculature disorders in AD, using our *in vitro* model we studied the effect of βA on the involvement of RAGE in mediating cytotoxic effects of βA and, in particular, in the activation of the nitrosative system in microvascular endothelial cells. In fact, using rat cerebro-microvascular endothelial cells (CECs) as an *in vitro* model, we previously found that $\beta A_{(1-42)}$ induced CEC death, within a short incubation time, demonstrating peptide toxicity in a time- and concentration-dependent manner (20).

Materials and methods

Materials. Sprague-Dawley male rats (350–400 g body weight) were purchased from Charles-River (Como, Italy), and the experimental protocol was approved by the local Ethics Committee for Animal Studies. $\beta A_{(1-42)}$ was obtained from Calbiochem (Darmstadt, Germany). EC growth medium MV2 was provided by PromoCell (Heidelberg, Germany) and the Nitrate/Nitrite Colorimetric Assay Kit was from Cayman Chemical Co. (Ann Arbor, MI, USA). Mouse anti-NOS2 monoclonal IgG antibody and goat anti-RAGE polyclonal IgG were obtained from Santa Cruz (Santa Cruz, CA, USA) and the Peroxidase Substrate Kit DAB was from Vector Laboratories (Burlingame, CA, USA). ECL Advance Western blotting system for chemiluminescence detection was obtained from GE Healthcare (Chalfont St. Giles, UK).

All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Sample preparation. Cells at ~80% confluence were treated with or without either peptide $\beta A_{(1-42)}$, trypsin solution in order to cleavage membrane protein, goat anti-RAGE polyclonal IgG (anti-RAGE) in order to inhibit βA -RAGE binding, or N_{ω} -nitro-L-arginine methyl ester hydrochloride (L-NAME) in order to inhibit nitric oxide synthase (NOS).

$\beta A_{(1-42)}$ was dissolved in double-distilled deionised water at a concentration of 1 mM, stored at -20°C and used at the final concentration of 100 nM. To remove protein surface interactions, cells were pre-treated with 100 ng/ml trypsin (9590 units/mg) for 10 min, followed by addition of three molar equivalents of soybean trypsin inhibitor (SBTI 10000 units/mg) for 5 min, then the medium was exchanged and the cells were treated with βA . Anti-RAGE was added to the cultures 30 min before βA treatment at the concentration of 10 ng/ml. L-NAME was dissolved, just before use, in double-distilled deionised water and added to the medium (500 μM) with the βA peptide.

CEC culture and treatment. Rats were decapitated, and the brain was promptly removed. Cerebro-microvascular endothelial cells (CECs) were isolated and cultured according to the method of Abbot *et al* (21) with few modifications (20,22). CECs from the 3rd and 4th passages were plated on fibronectin-coated Petri dishes and cultured in MV2 medium, as previously described (23). After 24 h of culture, the medium was replaced with a fresh one containing factors, and CECs were treated for a 6-h incubation period.

Cell survival. To determine the effects of trypsin solution or anti-RAGE pre-treatment on $\beta A_{(1-42)}$ -induced toxicity, cell survival was evaluated by trypan blue exclusion assay by counting CECs which did not internalize the dye.

Western blotting. At the end of the incubations, cells were harvested in lysis buffer (50 mM Tris-HCl, 2 mM EDTA, aprotinin 10 $\mu\text{g}/\text{ml}$, leupeptin 10 $\mu\text{g}/\text{ml}$, phenylmethylsulfonylfluoride 1 mM). The protein concentration was measured by using BCA Protein Assay Kit. Total proteins (30 μg) were loaded onto 12% SDS-acrylamide gels. At the end of the run, proteins were transferred to a polyvinylidene-fluoride membrane and incubated with goat anti-RAGE polyclonal IgG (1:200,000) overnight, and then with the peroxidase-conjugated secondary antibodies for 1 h. Proteins were detected by chemiluminescence.

Cell redox activity. To determine the effects of anti-RAGE pre-treatment on $\beta A_{(1-42)}$ -induced toxicity, cell redox activity was monitored by the colorimetric MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] (CellTiter 96 Aqueous Assay, Promega). Metabolically active cells react with a tetrazolium salt in the MTS reagent to produce a soluble formazan dye that can be observed at a wavelength of 490 nm. After the incubation period, cells were rinsed with PBS (phosphate buffer solution) in order to wash out unattached cells, followed by incubation with 20% MTS reagent in culture medium for 90 min. Thereafter, aliquots were pipetted into 96-well plates, and the samples were read at 490 nm in a Microplate Autoreader EL13 (Bio-Tek Instruments, Winooski, VT, USA).

In vitro angiogenesis. Matrigel was thawed on ice overnight, and spread evenly over each well (50 μl) of a 24-well plate. The plates were incubated for 30 min at 37°C to allow Matrigel to gel. CECs were seeded (2.5×10^4 cells/ cm^2), pre-treated with anti-RAGE and subsequently with the βA peptide. After 24 h of incubation at 37°C , cultures were photographed (5 fields for each well: the four quadrants and the center) at a magnification of $\times 5$. Phase contrast images were recorded using a digital camera (DG 200; Leica Imaging System, Cambridge, UK) connected to a Laborlux S Microscope (Leitz, Wetzlar, Germany) and saved as TIFF files. Image analysis was carried out using the Qwin image analysis software (Leica Imaging System), as previously detailed (23,24), and the following parameters were estimated: percent area covered by CECs, total length of CEC network per field, number of meshes and branching points per fields.

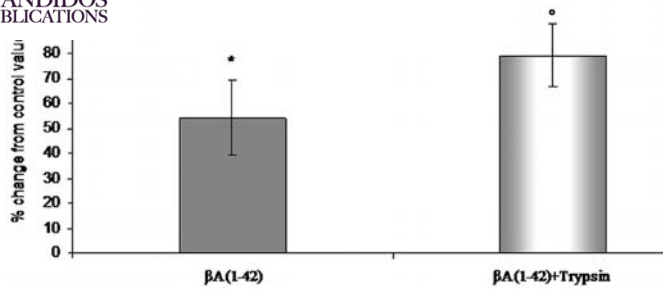


Figure 1. Effects of the $\beta A_{(1-42)}$ peptide without or with trypsin pre-treatment on the survival of cultured rat CECs, as measured by trypan blue exclusion assay. Bars are means \pm SEM of four separate experiments. * $p < 0.05$ compared with the control value; * $p < 0.05$ compared with $\beta A_{(1-42)}$ treatment.

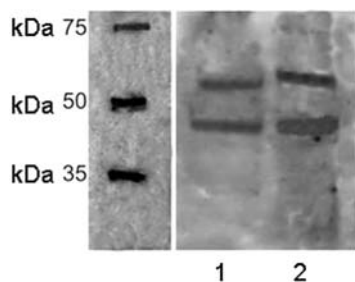


Figure 2. Western blot analysis showing RAGE expression in cultured rat CECs in control cultures (lane 1) and treated cultures (lane 2).

Measurements of nitric oxide production. The NO levels in the culture supernatants were measured by determining the concentration of nitrite using a colorimetric assay kit (Nitrate/Nitrite Colorimetric Assay Kit) based on Griess reaction. Supernatants (80 μ l) from each culture well (control and treatments) were mixed with 20 μ l of nitrate reductase for conversion of nitrate to nitrite, followed by 100 μ l of the Griess reagent (1% sulphanilamide and 0.1% naphthylene-diamine dihydrochloride in 2% phosphoric acid). The levels of nitrite in the samples were determined by measuring the absorbance of the color product at a 540 nm wavelength with the Microplate Autoreader EL-13.

Immunocytochemistry. iNOS expression was examined by immunocytochemistry. Cultures were fixed for 10 min at 4°C in ice-cold acetone, rinsed in PBS, and treated with 1% Triton X-100 in PBS for 5 min at room temperature followed by the blocking of unspecific sites with 0.5% BSA in PBS for 15-20 min. Next, cells were incubated with mouse anti-NOS2 monoclonal IgG antibody (1:100) for 60 min. Then, cells were incubated with the secondary peroxidase-conjugated anti-rabbit IgG (1:150) for 30 min. Each incubation was followed by 3 washes with PBS. The reaction was developed with the avidin-biotin-amplified immunoperoxidase method, using the Vectastain Elite ABC Kit and Peroxidase Substrate Kit DAB. Finally, samples were stained with hematoxylin. Negative controls were carried out by similarly treating cultures and omitting the primary antibody.

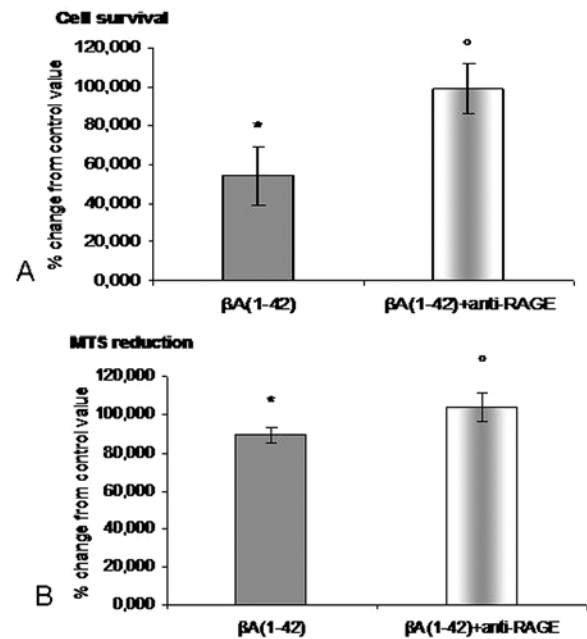


Figure 3. Effects of the $\beta A_{(1-42)}$ peptide without or with anti-RAGE pre-treatment on rat CEC (A) survival, as measured by trypan blue exclusion assay and (B) redox activity, as measured by MTS assay. Bars are means \pm SEM of four separate experiments. * $p < 0.05$ compared with the control value; * $p < 0.05$ compared with $\beta A_{(1-42)}$ treatment.

Statistical analysis. Statistical analysis was performed using the SPSS statistical software package. Results were expressed as percent change from control value, and were the mean \pm SEM of four separate experiments. Statistical analysis was performed by ANOVA followed by the t-test of Student-Newman-Keuls as a *post-hoc* test.

Results

Trypsin pre-treatment. As previously demonstrated, $\beta A_{(1-42)}$ treatment induced, after a 6-h incubation period, a significant ($p < 0.05$) decrease in cell survival in respect to the control cultures, as evaluated by trypan blue exclusion. In contrast, trypsin pre-treatment induced a significant ($p < 0.05$) increase in cell survival compared to the peptide alone incubation (Fig. 1), suggesting that CEC binding sites are likely membrane proteins.

RAGE involvement in mediating $\beta A_{(1-42)}$ effects on CECs. RAGE presence in CEC cultures was evaluated by means of immunoblotting. Western blot assay, performed on cell lysates, detected two bands with molecular sizes of 55 and 45 kDa, representing the RAGE intermembranal and C-truncated isoform, respectively. As shown in Fig. 2, both bands were present in $\beta A_{(1-42)}$ -treated and control samples.

Pre-treatment with anti-RAGE IgG induced a significant ($p < 0.05$) increase in cell survival (Fig. 3A) and redox activity (Fig. 3B) compared to the peptide alone incubation, as evaluated by trypan blue exclusion and MTS-reduction assay, respectively. CECs, cultured on Matrigel, spread and aligned with each other to form branching anastomosing tubes that gave rise within 24 h to a meshwork of capillary-

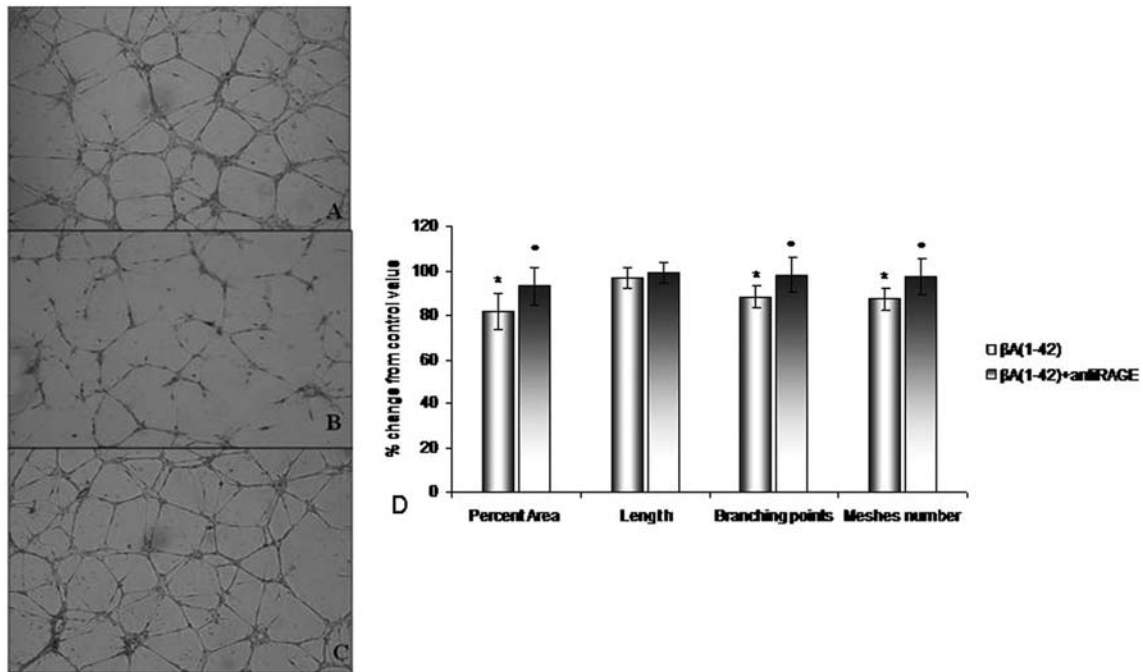


Figure 4. Phase contrast micrographs illustrating the arrangement of rat CECs into a meshwork of capillary-like tubules when cultured on (A) Matrigel for 24 h. (B) $\beta A_{(1-42)}$ peptide prevents and (C) anti-RAGE IgG pre-treatment increases the formation of the meshwork (x50). (D) Quantitative analysis of the effects of the $\beta A_{(1-42)}$ peptide without or with anti-RAGE pre-treatment on the dimensional [percent area covered by CECs and total length per field (length)] and topological parameters [number of branching points per field (branching) and number of meshes per field (number)] of CEC capillary-like meshwork. Bars are means \pm SEM of four separate experiments. * $p < 0.05$ compared with the control value; $^{\circ}p < 0.05$ compared with $\beta A_{(1-42)}$ treatment.

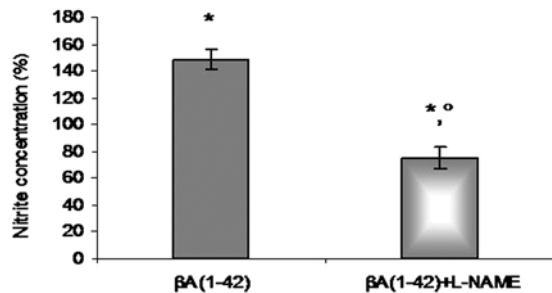


Figure 5. Effects of the $\beta A_{(1-42)}$ peptide without or with L-NAME co-treatment on nitrite release from CECs. Bars are means \pm SEM of four separate experiments. * $p < 0.05$ compared with the control value, $^{\circ}p < 0.05$ compared with $\beta A_{(1-42)}$ treatment.

like structures (Fig. 4A). Incubation with $\beta A_{(1-42)}$ prevented network formation (Fig. 4B), while, pre-treatment with anti-RAGE inhibited the anti-angiogenic effect of the peptide (Fig. 4C). Image analysis confirmed these observations, showing that the $\beta A_{(1-42)}$ peptide significantly prevented ($p < 0.05$), with respect to the control, dimensional (percent area covered by CECs) and topological parameters (number of meshes and branching points per field) of the capillary-like network. In contrast, anti-RAGE IgG pre-treatment induced a significant ($p < 0.05$) increase, with respect to the peptide incubation, of these parameters (Fig. 4D).

$\beta A_{(1-42)}$ activation of the nitrergic system and RAGE involvement. The nitrite levels in the supernatants of the

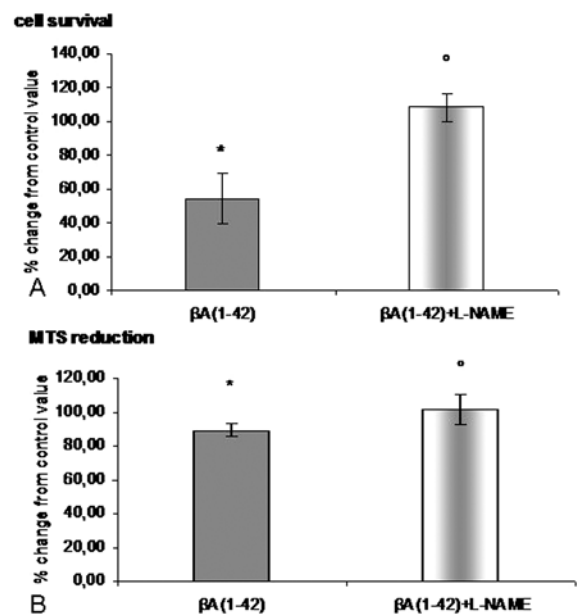


Figure 6. Effects of $\beta A_{(1-42)}$ without or with L-NAME co-treatment on rat CEC (A) survival, as measured by trypan blue exclusion assay and (B) redox activity, as measured by MTS assay. Bars are means \pm SEM of four separate experiments. * $p < 0.05$ compared with the control value; $^{\circ}p < 0.05$ compared with $\beta A_{(1-42)}$ treatment.

medium in which CECs were incubated for 6 h with the $\beta A_{(1-42)}$ peptide alone or $\beta A_{(1-42)}$ with L-NAME were quantified. The nitrite levels from CECs significantly ($p < 0.05$) increased

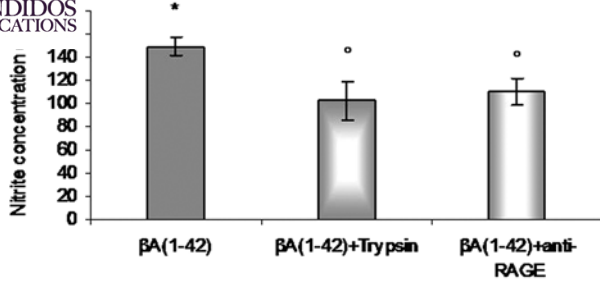
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Figure 7. Effects of the $\beta A_{(1-42)}$ peptide without or with trypsin and anti-RAGE IgG pre-treatment on nitrite release from CECs. Bars are means \pm SEM of four separate experiments. * $p < 0.05$ compared with the control value, ° $p < 0.05$ compared with $\beta A_{(1-42)}$ treatment.

by nearly 1.5-fold, compared to the control and significantly ($p < 0.05$) decreased in respect to the control and to peptide alone treatment with L-NAME co-treatment (Fig. 5). Moreover, co-treatment with L-NAME induced a significant ($p < 0.05$) increase in cell survival (Fig. 6A) and redox activity (Fig. 6B) compared to the peptide alone incubation, as evaluated by trypan blue exclusion and MTS-reduction assay, respectively.

The nitrite levels in the supernatants of the medium in which CECs were incubated for 6 h with $\beta A_{(1-42)}$ after pre-treatment with trypsin solution or with anti-RAGE IgG were evaluated. As shown in Fig. 7, both pre-treatments induced a significant ($p < 0.05$) decrease in nitrite levels compared to the peptide alone incubation.

Immunoreactivity to inducible nitric oxide synthase (iNOS) of CECs by $\beta A_{(1-42)}$ treatment is shown in Fig. 8B, which was suppressed by pre-treatment with anti-RAGE IgG (Fig. 8C).

In all experiments, neither trypsin nor anti-RAGE IgG nor L-NAME *per se* had any significant effect (data not shown).

Discussion

It has been already established that the βA peptide, and principally the $\beta A_{(1-42)}$ form, plays a major role in the development and progression of Alzheimer disease (AD), inducing neuronal, astrocyte and cerebral vessel damage (18). At the cerebral vascular endothelial level, it has been demonstrated that βA is able to induce vasoconstriction, blood flow decrease and vasoconstriction resistance consequence increase (25). Moreover, Thomas *et al* (26) demonstrated that the peptide inhibits endothelial cell replication and angiogenic capability. Historically, it has been proposed that cerebral βA has a neuronal origin; however, growing evidence suggests that the peptide may stem from several sources, one of which may be receptor-mediated transport across the blood-brain barrier (BBB) (27). Amyloid from the systemic circulation could be, hence, a major contributor to the brain amyloid burden in severe AD (28). One cell membrane receptor candidate for βA linking seems to be the receptor for advanced glycosylation-end products, RAGE, which is expressed in numerous cellular types such as neurons, microglia and endothelial cells. It has been shown that RAGE binds βA and

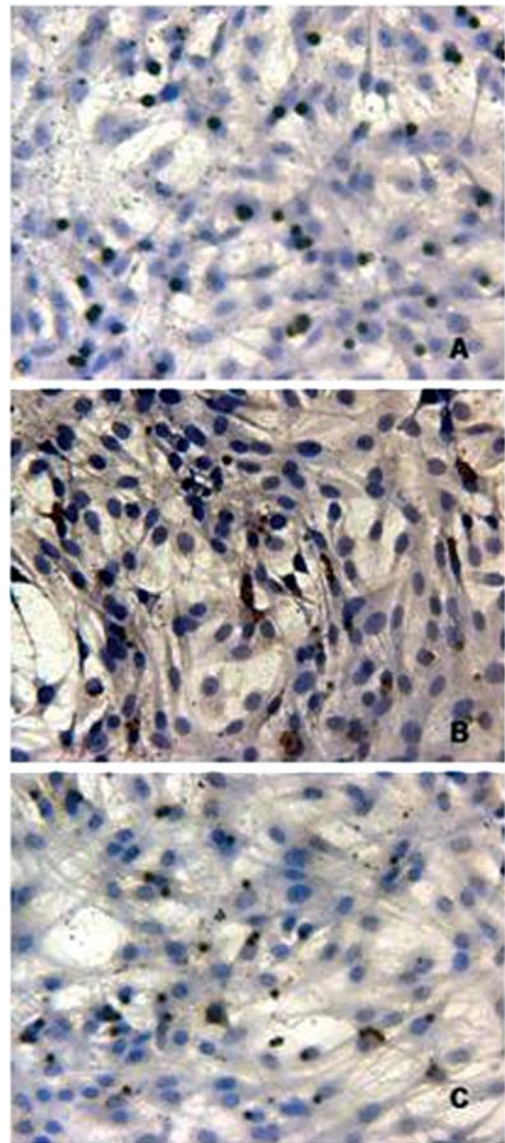


Figure 8. Immunocytochemical assay for iNOS in (A) control CEC culture, (B) after $\beta A_{(1-42)}$ treatment without or (C) with anti-RAGE IgG pre-treatment (x250).

mediates pathophysiologically relevant cellular responses (29,30), such as βA transport from blood to brain (28). It has been hypothesized that, in pathological conditions such as AD, RAGE could act as a co-factor able to increase βA brain levels, with consequent cellular dysfunction, stress oxidative induction, loss of cellular vitality and apoptosis (31). Hence, the interaction of βA with this membrane receptor could be important in the evolution of cellular dysfunction which is prominent in AD pathology.

To ascertain whether the potential biological effects of βA on microvascular endothelium are, almost in part, mediated by a proteic membrane receptor, RAGE in particular, we performed a series of experiments on *in vitro* rat cerebro-microvascular endothelial cell cultures.

First, we observed that trypsin digestion pre-treatment inhibited the toxic effects of βA on CECs, indicating that endothelial cell binding sites are likely membrane proteins, as suggested by Bateman *et al* (32) on neuronal cells.

Western blot analysis confirmed the presence of RAGE on CECs, not only the full-length type but also the C-truncated isoform, the soluble isoform, either in control and treated cultures.

In order to examine whether RAGE specifically blocks $\beta A_{(1-42)}$ peptide cytotoxicity on CECs, we used antibody anti-RAGE IgG pre-treatment. We found that anti-RAGE IgG pre-treatment markedly increased cerebrovascular endothelial cell survival, viability and angiogenic capability, confirming the role of RAGE in playing a part in $\beta A_{(1-42)}$ -induced toxic effects.

In Alzheimer disease (AD), nitric oxide (NO) has been implicated in the neurodegeneration process and neuronal cell death, through NO-mediated neurotoxicity (33). Multiple AD pathological factors have been, in fact, shown to affect nitric oxide synthase (NOS) expression, suggesting that NO involvement appears to be significantly related to oxidative damage in this pathology. Several studies have shown that βA stimulates microglial and astrocytic NO production probably throughout the activation of inflammatory mechanisms by means of an iNOS-mediated release (18,34,35). Moreover, similar mechanisms, iNOS activation and consequent NO high levels of induction, seem to be operative in brain microvessels isolated from AD patients which are amyloid burdened (36).

Our data showed that exposure of *in vitro* CEC cultures to the $\beta A_{(1-42)}$ peptide increased the extracellular nitrite levels, and the presence of L-NAME, a nitric oxide synthase inhibitor, enhanced CEC survival and viability, suggesting that NO plays a role in the $\beta A_{(1-42)}$ -induced toxicity on cerebral endothelial cells. Furthermore, our immunocytochemical data support the hypothesis that $\beta A_{(1-42)}$ in CECs induces expression of the inducible isoform of nitric oxide synthase (iNOS), typically synthesized in response to immune/inflammatory stimuli. Previous studies have shown that iNOS levels are increased in neurons and glial cells in AD (35). Moreover, Luth *et al* (14) demonstrated that NOS-reactive astrocytes in AD were associated with primitive, but not with 'burned out' plaques suggesting that this tissue response could be restricted to early stages of plaque formation (14). Our data seem to be consistent with these results demonstrating that $\beta A_{(1-42)}$ induces iNOS expression in CEC cultures.

Moreover, our data suggest that RAGE mediates the activation of the $\beta A_{(1-42)}$ -induced nitroergic system on CECs. In fact, with trypsin pre-treatment and by blocking the interaction to RAGE, we observed significant decreased levels of NO and iNOS immunoreactivity suppression. Currently, to our knowledge, there are no literature data related to RAGE involvement in βA nitroergic system activation; however, it has been demonstrated that RAGE linkage with other ligands, as advanced glycation-end products (AGE), stimulated iNOS expression in mouse macrophages (37), in murine endothelial cells (38), and in murine macrophages (39). AGE stimulation led to nitrite accumulation and iNOS protein expression, which were partially downregulated by preincubation with anti-RAGE antibody, suggesting the role of RAGE in AGE-induced nitrosative stress activation (39,40).

In conclusion, our data suggest RAGE involvement, at least partly, in mediating $\beta A_{(1-42)}$ effects on CECs. In


particular, the decrease of *in vitro* cell viability and functionality and nitrosative stress activation was inhibited by preventing $\beta A_{(1-42)}$ -RAGE interaction. These results suggest the potential utility of specifically blocking this receptor as a future therapeutic approach to decreasing βA transport from blood to brain.

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