MECHANISMS OF SOLUBLE \( \beta \)-AMYLOID IMPAIRMENT OF ENDOTHELIAL FUNCTION


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SUMMARY

Alzheimer’s disease (AD) has been recently associated with vascular risk factors. β-amyloid peptides (AβP), the main component of senile plaques typical of AD, circulate in soluble globular form in bloodstream. Interestingly, AβP is able to induce endothelial dysfunction and this effect may represent the link between vascular and neuronal pathophysiological factors involved in AD. We aimed to clarify the molecular mechanisms underlying globular AβP induced vascular toxicity.

Using several methodological approaches we have observed that in vascular tissues globular AβP is unable to induce oxidative stress, one of the mechanisms hypothesized involved in β-amyloid toxicity. More important, we have demonstrated that globular AβP is able to localize on vascular endothelium where it inhibits eNOS enzymatic activity. In particular, AβP enhances eNOS phosphorylation on Threonin495 and Serine116 and reduces acetylcholine-induced phosphorylation on Serine1177. Such effect depends on a PKC signaling pathway, as suggested by its phosphorylation on Serine660. In fact, selective inhibition of the calcium dependent group of PKC (cPKC) is able to rescue β-amyloid-induced alteration of eNOS phosphorylation, NO production and endothelial vasorelaxation. The activation of these Ca^{2+} dependent pathways is likely due to the ability of AβP to evoke Ca^{2+} leakage from IP3 receptors on endoplasmic reticulum.

Our data demonstrate that globular AβP induced endothelial NO dysfunction can be attributed to an alteration of intracellular Ca^{2+} homeostasis which could lead to the activation of cPKC with consequently change of eNOS phosphorylation pattern. These mechanisms could contribute to shed further light on the toxic effect of β-amyloid in vascular tissues.
Alzheimer’s disease (AD) is a progressive neurodegenerative disorder characterized by irreversible cognitive and physical deterioration. It is a major cause of death and a growing public health problem as life expectancy in the general population increases (1).

Typical features of AD are the senile plaques present in the brain, cerebral blood vessels and other tissues (2). The plaques are composed mainly of fibrillar amyloid β peptides (AβP) generated from the amyloid precursor protein (APP), a ubiquitously expressed transmembrane glycoprotein. The amyloid peptides, constituted primarily of 39 to 42 residues (AβP1-39, 1-40, 1-42), are released continuously during cellular metabolism. These peptides circulate in soluble globular form in bloodstream (3-4) and accumulate on vascular wall of AD patients (5). In the brain, soluble β-amyloid monomers are able to deposit and transform into insoluble and fibrillar aggregates, forming amyloid plaques (6-7).

Although AD has been considered for long to be of non-vascular origin, a growing body of recent studies has indicated the possibility that vascular risk factors could be involved in the pathophysiology of AD (8). In particular, patients with AD have morphological alterations of the cerebral vasculature, reduced cerebral blood flow and increased permeability of blood brain barrier, which mainly depends on endothelial function (9). Furthermore, it has been shown that the fibrillar form of AβP is able to produce a severe damage of the endothelial lining with deleterious effects on endothelial nitric oxide (NO) function (10-12). This effect of the fibrillar form of AβP has been attributed to an enhanced NO catabolism due to an increased production of reactive oxygen species (ROS) (13-14), since superoxide scavengers are able to prevent fibrillar AβP induced endothelial toxicity (11,15). Recent data indicate that also the circulating form of AβP is able to provoke endothelial NO dysfunction, but it is still unclear whether this alteration is oxidative stress-dependent.

On the other hand, a recent study suggests that the soluble form of AβP could exert its deleterious effect on endothelial NO inhibiting nitric oxide synthase enzymatic activity (16).
Thus, aim of this study was to explore whether the globular circulating form of AβP impairs endothelial NO function by enhancing oxidative stress or influencing NO production and, subsequently, clarify the molecular mechanisms involved.
EXPERIMENTAL PROCEDURES

Pure, scrambled and biotynilated AβP1-40 were obtained from Biosource. AβP1-40 stock solution (0.5 mg/ml) was prepared by dissolving the peptides in deionized water and stored as aliquots at –20°C until used.

Experimental animals.

Experiments were conducted in male Wistar Kyoto rats (Charles River Laboratory) 10-14 weeks old. All experimental procedures were in accordance with guidelines for research in animals of our institution.

Vascular reactivity studies

Vascular reactivity studies have been performed on aortas as previously described (17). Vasorelaxation to Acetylcholine (Sigma, ACh, 10⁻⁹–10⁻⁵ M) and to Sodium NitroPrusside (SNP, Sigma, 10⁻¹⁰–10⁻⁶ M) with or without AβP (10⁻⁶ M) was expressed as percent of reduction in contraction obtained with phenylephrine (Phe, 10⁻⁶ M, Sigma).

Basilar arteries were dissected and excised free of connective and adipose tissue and mounted on a micromyograph, as previously described by Mulvany et al. (18). Basilar arteries were precontracted with 10⁻⁶ mol/L phenylephrine, and the vasodilator responses to increasing doses of Substance P (10⁻⁹–10⁻⁵ mol/L), alone or after 30’ preincubation with 10⁻⁶ mol/L AβP, were tested.

Measurement of ex vivo aortic O₂ production.

Lucigenin enhanced methodology is specific for O₂ detection. Segments of thoracic aorta were isolated, placed in a modified Krebs buffer (17) added with 20 mM Na-Hepes and
allowed to equilibrate for 30 min at 37°C. After 5 min of dark adaptation, scintillation vials containing 2 ml Krebs-Hepes buffer with 5 μM lucigenin were placed into a scintillation counter switched to the out-of-coincidence mode. Chemiluminescence values were obtained at 40 s intervals over 15 min, and readings were averaged. The vessel was dried and the dry weight was measured. Lucigenin counts were expressed as counts per minutes per milligram of the dry weight of vessel. Background counts were determined by vessel-free incubations and subtracted from vessel readings.

Soon after the isolation, vessels were treated with 1 μM AβP1-40 for 1 h in Krebs buffer. Vascular rings were frozen at −20°C, and cut into 20 μm thick section in a Jung CM3000 Cryostat (Leica). Sections were placed onto polylysinated microscope slides, either incubated at room temperature for 30 min with DHE (Sigma; 10 μM) and protected from light, or blocked with PBS 0.1% BSA for 1 h at room temperature, incubated with anti-nitrotyrosine antibodies (5 μg/ml, Transduction Laboratories) overnight at 4°C and with anti-IgG TRITC conjugated for 1 h at room temperature. Images were observed under an Axiophot2 fluorescence microscope (Zeiss) equipped with a FITC-filter (excitation 450-490nm; emission 515-560nm), using a 200X magnification and an imaging system.

**Evaluation of NO production**

Aortic rings were incubated for 2 h in the darkness in aerated (95% O₂, 5% CO₂) Krebs buffer containing DAF-2 diacetate (DAF-2DA, 10 μM; Alexis). During this time aortic rings were treated with AβP (1 μM; 1 h) and/or ACh (1 μM; 5 min).

Vascular rings were frozen at −20°C and cut in 10 μm-thick sections placed onto microscope slides without any mounting medium or coverslip. Specimens were observed with fluorescence microscope. Twenty-four bit color pictures were taken at three different wavelengths (corresponding to green, red, and blue fluorescence) using a digital camera.
system coupled to imaging software (Spot, Diagnostic Instruments) under constant exposure time, gain and offset. To account for fluorescence decay, all images were taken in the first 30 seconds of light exposure. The images were then merged so that the 515nm-emitted fluorescence due to DAF appeared green above a purple all-wavelengths-emitted autofluorescence background derived from the fibers of the vessel. Green fluorescence intensity was quantified using the imaging software.

**Analysis of AβP localization**

Vascular rings were treated with 1μM biotynilated AβP or biotynilated scrambled peptide for 1h, frozen at –20°C and cut into 20μm-thick section. Sections were placed onto polylysinated microscope slides, blocked with PBS 0.1% BSA for 1h at room temperature, incubated with streptavidine-peroxidase for 15min at room temperature. Biotynilated AβP and peptide were revealed by DAB-chromogen solution (NovaRed, Vector) and slides were observed under a fluorescence microscope. Endothelium was revealed with anti von-Willebrant antibodies (1:100, Santa Cruz Biotechnologies).

HAEC were plated as 10000cells/well in a multi well chamber slides and treated with 1μM biotynilated AβP for 1h. The cells were fixed with methanol at –20°C for ten minutes and permeabilized with Triton X-100 0.1% in PBS for 30 min at room temperature. Biotynilated AβP was revealed DAB-chromogen solution (NovaRed, Vector) after incubation of slides with streptavidine-peroxidase for 15 min at room temperature. Afterwards, slides were incubated with anti-IP3 receptor (IP3R) antibodies (1:100, Santa Cruz Biotechnologies) overnight at 4°C, and then with anti-mouse IgG FITC conjugated for 1h at room temperature. Slides were dried out, mounted with an aqueous based mounting medium and observed under the microscope equipped with a FITC filter. Red and green images were merged.
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Evaluation of eNOS enzymatic activity

eNOS activity was evaluated measuring the conversion of 14C-arginine to 14C-citrulline in vitro. Aortas were treated with AβP or scrambled AβP (1µM, 1h) then rapidly frozen in liquid nitrogen and homogenated in lysis buffer (mM: 25 TRIS-HCl pH 7.4, 1 EDTA, 1 EGTA, 0.1 NaF, 0.1 Na3VO4). Protein concentration was determined by Bradford method. 50µg of protein extract were incubated at room temperature for 30min with reaction buffer (50mM TRIS-HCl pH 7.4, 1mM CaCl2, 0.3µM BH4, 1µM FAD, 1µM FMN, 0.1µM CAM, 1mM NADPH, 0.5µCi 14C-Arginine). The reaction was blocked on ice for 10min with stop buffer (20mM HEPES, 5mM EDTA). The reaction mix was applied to a 1ml column containing Dowex AG 50x8 resin that had been equilibrated with stop buffer. 14C-citrulline was eluted twice with 2ml of water and radioactivity was determined by scintillation counting.

Analysis of eNOS phosphorylation pattern

After isolation and treatment with AβP (1µM, 1h) and/or ACh (1µM, 10min) and/or Gö6976 (2µM, 15min, Calbiochem), proteins were extract as previously described. 20µg of protein extract were subjected to a 10% SDS-PAGE and electroblotted on nitrocellulose membranes, which were blocked at room temperature for 1h in blocking solution (5% milk in TBS-T), incubated overnight at 4°C with anti-phospho eNOS (Thr495) or (Ser1117) or (Ser116) (1µg/ml, Cell Signaling). Membrane was incubated with peroxidase-conjugate anti-rabbit antibodies (1:10000, Amersham). Proteins were revealed by ECL kit.

Normalization was performed with anti-NOS-III antibodies (1µg/ml, Tansduction Laboratories) for 3h at room temperature, and incubated with peroxidase-conjugate antimouse IgG (1:10000 in TBS-T). Proteins were revealed as above. The intensity of the bands was quantified by scanning densitometry using the NIH Image 1.61 software.
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Analysis of PKC phosphorylation

After isolation and treatment with AβP (1µM, 1h) and/or ACh (1µM, 10min), specimens were lysated as above. 20µg of protein extract were subjected to a 10% SDS-PAGE. Gels were electroblotted on nitrocellulose membrane (BioRad). Membrane was blocked at room temperature for 1h in blocking solution (5% milk in TBS-T), incubated overnight at 4°C with anti-phospho-Ser⁶⁶⁰-PKC (1µg/ml, Cell Signaling). Membrane was incubated with peroxidase-conjugate anti-rabbit antibodies (1:10000, Amersham). Proteins were revealed as above.

Primary and secondary antibodies were completely removed with Stripping Buffer (0.5M NaCl, 0.5M glacial acetic acid) at room temperature for 30min. Membranes were blocked in blocking solution (5% milk in TBS-T) for 1h at room temperature, incubated with anti-PKC (1µg/ml, Transduction Laboratories) for 3h at room temperature, and incubated with peroxidase-conjugate antimouse IgG (1:10000 in TBS-T). Proteins were revealed as above.

The intensity of the bands was quantified as described.

Analysis of Calcium release in endothelial cells

Intracellular calcium was imaged using a calcium sensitive dye Fluo4AM and a Pelkin-Elmer laser confocal microscope. Cells were cultured on coated glass bottom microwell dishes (MatTek Corporation). To load dye into the cells, 5µM Fluo4AM was incubated with cells for 30’ at 37°C in PBS without Ca²⁺ and Mg²⁺ and with EGTA 100µM, then washed in PBS and observed at room temperature in Calcium-free DMEM w/o serum (Life Technologies). The focal planes were set across the middle of the cell bodies. After about 5’ of observation, 1µM AβP, 1µM ACh, 1µM scrambled peptide and 10µM furanophostin alone or in combination, were added to each HAEC culture. Furanophostin was added to cells permeabilized with 1% picric acid. Some cell cultures were preincubated with 50 µM 2APB for 10 minutes before the addition of the stimuli. Images were collected at 10 seconds intervals.
The concentration of calcium was calculated according to the equation $[\text{Ca}^{2+}] = K_d \times (F - F_{\text{min}})/(F_{\text{max}} - F)$, where $K_d$ is the dissociation constant for Fluo 4-AM, $F$ is fluorescence, $F_{\text{min}}$ and $F_{\text{max}}$ are minimum and maximum fluorescence measured by adding 10µM ionomycin in presence of 10mM EGTA or 10mM CaCl$_2$, respectively.

**Statistical analysis**

All results are expressed as mean±SE. Statistical analysis was performed by *t*-test or by Two-Way analysis of variances (ANOVA) followed by *Bonferroni test*, as appropriate. A two-tailed value of $p<0.05$ was considered significant.
RESULTS

**Soluble AβP induces endothelial NO dysfunction**

Soluble AβP was able to attenuate ACh induced dose-dependent vasodilatation on rat aorta, although had no effect on basal vascular tone. In contrast, AβP did not influence vasodilatation evoked by SNP (Fig. 1A-C) indicating that globular AβP impaired endothelial NO function, but not vascular smooth muscle sensitivity to NO. Moreover, in a vessel of the cerebral district, such as basilar artery, we observed that AβP was able to attenuate the vasodilation induced by SubP, a potent nitric oxide stimulus on cerebral arteries (Fig 1B). Finally, soluble AβP was able to attenuate ACh-induced DAF-2 fluorescence while it did not alter basal DAF-2 signal (Fig 1D).

**Soluble AβP does not induce vascular oxidative stress**

To explore whether soluble AβP was able to induce endothelial NO dysfunction by enhanced production of ROS, we evaluated vascular O$_2^-$ production by three different methodologies: lucigenin-enhanced chemiluminescence, DHE dyeing and nitrotyrosine detection. All revealed that soluble AβP neither alone nor after incubation with ACh induced ROS production in vessels. Yet, our experimental setting was able to detect oxidative stress evoked by Angiotensin II (AngII), used as positive control (Fig. 2A-B). Moreover, preincubation with a ROS scavenger as super oxide dismutase (SOD) was unable to rescue ACh-induced vasodilatation altered by AβP (Fig.1A) suggesting that oxidative stress was not involved in vascular toxicity mediated by the globular form of AβP.

**Soluble AβP localizes on vascular endothelium and attenuates eNOS activity.**

Our data revealed that biotinylated AβP, but not a different biotinylated scrambled peptide (data not shown), was able to localize mainly on vascular endothelium (Fig 3A).
Furthermore, the analysis of the catalytic activity of eNOS on aortic rings showed that soluble AβP, but not scrambled AβP, significantly inhibited the enzymatic activity (Fig 3B). These results indicate that globular AβP interacts with the endothelial lining where inhibits eNOS enzymatic activity.

**Soluble AβP alters eNOS phosphorylation pattern.**

Since eNOS activity can be regulated by a coordinated control of enzyme phosphorylation on Serine and Threonine (24), we extended our analysis to these latter. As shown in Fig 4A, in control condition eNOS was phosphorylated in Thr^{495} and in Ser^{116}. The addition of ACh markedly reduced eNOS Thr^{495} phosphorylation and concomitantly evoked an increase in eNOS Ser^{1177} phosphorylation, while did not influence eNOS Ser^{116} phosphorylation. The exposure to soluble AβP alone markedly increased eNOS Thr^{495} and Ser^{116} phosphorylation. More important, in presence of soluble AβP, ACh was unable to induce the dephosphorylation of Thr^{495} and Ser^{116} and the concomitant phosphorylation of Ser^{1177}.

**Soluble AβP alters eNOS phosphorylation pattern and endothelial dysfunction through a cPKC dependent mechanism.**

It has been described that eNOS phosphorylation on Thr^{495} is mediated by a PKC signaling (27). To explore the impact of PKC on AβP-induced eNOS phosphorylation pattern we tested the effect of the globular AβP in presence of Bisindolylmaleimide (data not shown), a pan-PKC inhibitor, and of Gö6976, a selective inhibitor of the calcium dependent group of PKC (cPKC). Fig 4A shows that in presence of Gö6976 alone both Thr^{495} and Ser^{116} were phosphorylated as in control conditions. The addition of ACh together with Gö6976 evoked the reduction of Thr^{495} and Ser^{116} phosphorylation level and the increase of the Ser^{1177} one. In
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presence of AβP and Gö6976, ACh was still able to induce dephosphorylation of Thr^{495} and Ser^{116} and concomitant phosphorylation of Ser^{1177}, indicating that AβP is unable to evoke its effect on eNOS phosphorylation when the cPKC are inhibited. Finally, the exposure to Gö6976 was also able to rescue the impaired ACh-evoked vasorelaxation observed in presence of AβP (Fig 4B).

**Soluble AβP influences PKC-signaling pathway in vascular tissues.**

To further investigate the hypothesis that a PKC-dependent mechanism could be involved in AβP-induced eNOS dysfunction, we analyzed PKC Ser^{660} phosphorylation, an activating site of the enzyme, in vascular tissues. We revealed that AβP markedly increased phosphorylation at this site indicating that AβP induces PKC structural modifications necessary to the enzyme to be fully active and suggesting the involvement of PKC in AβP-induced vascular effect (Fig 5).

**Soluble AβP induces Ca^{2+} leakage in endothelial cells**

To clarify how AβP affects calcium-dependent signaling, we investigated whether AβP alters calcium homeostasis in endothelial cells. AβP induced a slow and progressive increase in intracellular calcium concentration within minutes, lasting for all the period of observation after the addition of the peptide (Fig 6A), whereas, a scrambled peptide did not induce any variation in intracellular calcium concentration. Moreover, acetylcholine evoked as expected a spike of intracellular calcium, which returned at basal levels after 10 minutes and the same occurs when AβP is added to cells at the same time of ACh. More important, we observed that, twenty min after the addition of AβP, since basal intracellular calcium concentration was drifted up, the gain of acetylcholine-evoked response was significantly reduced but the final concentration of intracellular calcium reached the same extent to that previously observed
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with Ach alone. Finally, in presence of twenty min exposure to a scrambled peptide, Ach-evoked calcium response was unaffected (Fig 6B).

**Soluble AβP localizes in the perinuclear region of endothelial cells**

To explore the mechanisms through which AβP was able to induce intracellular calcium leakage, we analyzed AβP localization in HAEC. Fig 7A shows that biotynilated AβP, evident as red fluorescence, was able to localize in perinuclear region (a) of endothelial cells suggesting a colocalization with endoplasmic reticulum. IP3 receptors (IP3R) are the main represented Ca^{2+} channels in endoplasmic reticulum of endothelial cells. For this issue we analyzed the localization of IP3R and observed that these latter localize in the perinuclear region too, as indicated by the green fluorescence in (b). The merge of the two figures showed that the two proteins colocalize (c) suggesting that AβP may influence IP3R function.

**Soluble AβP induces calcium leakage from IP3 receptors of the endoplasmic reticulum.**

In the light of the data described above, we further investigate the effect of AβP on intracellular calcium stores regulated by IP3R. In particular, the calcium release from endoplasmic reticulum evoked by furanophostin, a selective non receptorial IP3R agonist, was significantly blunted by previous exposure to beta-amyloid (Fig.7B). More important, beta-amyloid induced calcium leakage was prevented by the administration of 2APB, a selective IP3R antagonist (Fig.7C). These data indicate that beta amyloid influences IP3R function with a consequent calcium leakage from intracellular calcium stores.
DISCUSSION

Our results show that the globular form of AβP, at the dose comparable to the increased levels observed in AD patients (3), induces an endothelial NO dysfunction through the reduction of eNOS enzymatic activity. In particular, the globular form of AβP alters the pattern of eNOS phosphorylation via a cPKC-dependent signaling pathway. This cPKC activation could be due to the ability of the globular form of AβP to induce calcium leakage from intracellular stores.

Our data further demonstrate that globular AβP localizes mainly on vascular endothelium where evokes endothelial dysfunction, which is due neither to an altered vascular smooth muscle sensitivity to NO, nor to an enhanced NO catabolism by oxidative stress. Actually, the vasodilatation to exogenous NO is unaffected by the presence of soluble AβP. In addition, the evaluation of oxidative stress with different biochemical methodologies as well as the use of ROS scavengers, clearly demonstrates that soluble β-amyloid-induced endothelial dysfunction does not depend on oxidative stress. Our conclusion is consistent with results obtained in other studies, which have demonstrated that AβP is unable to increase ROS production in other cell systems (3).

Interestingly, our data demonstrate a defect in NO production in presence of the globular AβP as revealed by an impaired eNOS enzymatic activity, and a concomitant reduced presence of agonist stimulated endothelial NO in both aorta and basilar artery. The fact that basal NO production observed with DAF-2 was not influenced by AβP, and the awareness that the measurement of eNOS activity is performed in a reaction buffer containing all the factors eNOS needs to be fully active, among which the Ca^2+/calmodulin complex, suggest that AβP-induced eNOS alteration can be observed only when eNOS is stimulated.

Actually, eNOS enzymatic activity is also influenced by its phosphorylation at three or more sites; i.e. Ser^{1177}, Thr^{495} and Ser^{116} (21). Among these phosphorylation sites, the
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regulation of eNOS Ser\textsuperscript{1177} has been most extensively characterized. In fact, Ser\textsuperscript{1177} phosphorylation increases NO production by facilitating the electron transport inside the enzyme (22-26). On the contrary, Thr\textsuperscript{495} is basally phosphorylated and may be dephosphorylated in response to several stimuli. Phosphorylation at this site inhibits eNOS activity by opposing the binding of Ca\textsuperscript{2+}/calmodulin complex to the specific site on the enzyme, thus preventing its activation (27-28). Like Thr\textsuperscript{495}, Ser\textsuperscript{116} is basally phosphorylated and is dephosphorylated in response to certain stimuli in endothelial cells, even if the relative contribution of Ser\textsuperscript{116} to the regulation of NO production by eNOS has not been well established (26).

Our results confirm that acetylcholine, like the other previously observed agonists, influence eNOS phosphorylation pattern inducing dephosphorylation of the residues Ser\textsuperscript{116} and Thr\textsuperscript{495} and a concomitant phosphorylation of Ser\textsuperscript{1177}. Interestingly, the addition of β-amyloid alters per se eNOS phosphorylation status markedly increasing eNOS Thr\textsuperscript{495} and Ser\textsuperscript{116} phosphorylation. More important, in this latter condition, acetylcholine is not able to exert its action on eNOS phosphorylation pattern. Thus, we can infer that AβP affects eNOS enzymatic activity influencing the enzyme’s responsivity to certain activating stimuli such as acetylcholine.

Recent studies have shown that a PKC-dependent signaling pathway is able to increase eNOS Thr\textsuperscript{495} phosphorylation (28) and previous observations have shown that AβP is able to induce PKC activation in neuronal cells (29-30). Thus, we extended our analysis to clarify whether a PKC-dependent signaling pathway is implicated in AβP induced endothelial dysfunction. We observed that the globular AβP is able to induce PKC phosphorylation on Ser\textsuperscript{660}, a key step in the activation of the enzyme (31), thus suggesting that AβP interferes with PKC signaling pathway also in vascular tissues. The fact that Bisindolilmaleimide, a
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panPKC inhibitor, was able to abolish AβP effect on eNOS phosphorylation, confirmed an involvement of PKC in AβP-induced endothelial dysfunction.

Since it has been reported that AβP is able to elicit calcium current in several cell lines (32), we assessed the involvement of the calcium dependent group of PKC (cPKC) in AβP-induced eNOS dysfunction. In particular, we demonstrated that the use of a selective inhibitor of cPKC is able to rescue β-amyloid impact on eNOS phosphorylation, NO production and endothelial vasorelaxation, narrowing to a cPKC dependent signaling pathway the responsibility of the vascular toxicity induced by the globular form of β-amyloid.

Both eNOS and cPKC signaling tightly depend on intracellular calcium dynamics since eNOS is activated by the Ca²⁺/calmodulin complex and calcium promotes the interaction of cPKC with plasma membrane. Thus, we explored whether AβP vascular effects are associated to an altered calcium homeostasis. On this issue, we observed that AβP induced a slow and progressive increase in cytoplasmic calcium concentration in endothelial cells. This finding is in agreement with a previous report showing that soluble β-amyloid is able to induce calcium leakage in fibroblasts (32). Furthermore, the analysis of calcium dynamics during acetylcholine stimulation showed that concomitant infusion of AβP is unable to affect acetylcholine induced calcium response, ruling out a rapid effect of AβP on acetylcholine stimulated calcium signaling. Indeed, more important, when cells were preincubated with AβP, in a condition similar to that in which AβP induced vascular abnormalities, the gain of acetylcholine evoked calcium response was significantly reduced even if the total amount of calcium reached in the cytoplasm after acetylcholine stimulation was similar. Thus, these data indicate that AβP induces alteration in calcium dynamics slowly.

Our results were obtained in endothelial cells cultured in a Ca²⁺-free medium, clearly indicating that the observed leakage comes from intracellular calcium stores. The main source of intracellular calcium is the endoplasmic reticulum from which calcium can reach cytoplasm...
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through specific channels (33). In particular, on the endoplasmic reticulum of endothelial cells is mainly present a ligand-gated Ca^{2+} channel (IP3R) which allows mobilization of intracellular calcium stores in response to inositol 1,4,5-triphosphate (IP3) (34). On this issue we observed that β-amyloid localizes in the perinuclear region, and, more important, colocalizes with IP3R on endoplasmic reticulum of endothelial cells, suggesting a possible effect of AβP on these intracellular calcium channels. This hypothesis was tested using selective IP3R agonist and antagonist. Actually, after preincubation with AβP, the challenge of IP3R function by a selective non receptorial agonist showed an impairment of the calcium release through these intracellular calcium channels. More important, the concomitant administration of a selective IP3R antagonist with AβP was able to prevent the previously observed calcium leakage. All together these data demonstrate that AβP impairs IP3R function altering intracellular calcium stores. Thus, AβP alters intracellular calcium dynamics and this action could account for its effect on eNOS function.

In fact, increase in intracellular calcium concentration stimulates NO production through direct eNOS activation, but at the same time activates cPKC, which diminishes NO generation by eNOS Thr^{495}/Ser^{116} phosphorylation, the resulting eNOS enzymatic activity is the balance of these two opposite mechanisms (35). In the light of our and other studies, we can suggest that soluble circulating AβP induced intracellular calcium leakage could shift the calcium dependent balance towards a sensitization of the cPKC pathway with severe reduction of eNOS enzymatic activity. In fact, it is well characterized that cPKC signaling needs for its activation a calcium threshold (EC_{50}: 200 nM) lower than eNOS (EC_{50}: 400 nM) (13,31). Thus, in presence of β-amyloid intracellular calcium concentration reaches firstly the level that lead to an activation of cPKC signaling which phosphorylates eNOS on Thr^{495} so that, when challenged with acetylcholine, eNOS may be unable to bind Ca^{2+}/calmodulin complex and catalize NO production. On the other hand, when cPKC is inhibited, the intracellular calcium
concentration observed after acetylcholine stimulation is able to activate eNOS even in presence of AβP. Thus, the effect of AβP on calcium dynamics influences the balance of intracellular signaling pathways between cPKC and eNOS leading to an altered endothelial function.

Since soluble β-amyloid peptides are a physiological product of the cellular catabolism, in the light of our results, it is tempting to speculate that the negative impact of AβP on eNOS function could also play a pathophysiological role in other condition of vascular dysfunction. Thus, our data contribute to shed further light not only on the vascular pathophysiological mechanisms involved in the onset of AD but also on the identification of novel factors that participate to the complex network controlling vascular function.
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FIGURE LEGENDS

**Fig. 1. Soluble AβP induces endothelial NO dysfunction.** Panel A represents vascular response of aortic rings precontracted with phenylephrine to increasing doses of acetylcholine, alone (○) or after preincubation with β-amyloid (▼) or with β-amyloid and SOD (■) (*p<0.01 β-amyloid compared to control, #p<0.01 β-amyloid and SOD compared to control, n=10). Panel B represents vascular response of basilar arteries precontracted with phenylephrine to increasing doses of substance P, alone (■) or after preincubation with β-amyloid (∆) (*p<0.01 as compared to control, n=5). Panel C shows vascular response of aortic rings to increasing doses of Sodium NitroPrusside, alone (○) or after preincubation with β-amyloid (▼). Panel D represents quantification of DAF-2 fluorescence (FAU=fluorescence arbitrary unit) performed on aortic rings in control condition and after preincubation with β-amyloid and/or Acetylcholine (*p<0.01 as compared to control, #p<0.05 as compared to ACh alone, n=5).

**Fig.2. Soluble AβP does not induce vascular oxidative stress.** Panel A shows representative high power micrographs, showing nitrotyrosine detection (a-c) and DHE dyeing (d-f) on aortic rings in control conditions (a,d) and after preincubation with β-amyloid (b,e) or with Angiotensin-II (c,f), used as positive control. Panel B shows a quantification of the lucigenin-enhanced chemiluminescence in control conditions and after preincubation with β-amyloid and/or Acetylcholine or Angiotensin-II (*p<0.01 as compared to control, n=6).
Fig. 3. Soluble AβP localizes on vascular endothelium and attenuates eNOS activity. Panel A displays representative high power micrographs showing aortas incubated with biotinylated β-amyloid and revealed with anti-vW-factor antibodies to mark endothelium (n=3). In panel B is shown a quantification of the eNOS activity in aortic tissues alone and after preincubation with β-amyloid and scrambled β-amyloid (*p<0.01 as compared to control, n=5).

Fig. 4 Soluble AβP alters eNOS phosphorylation pattern and endothelial dysfunction through a cPKC dependent mechanism. Panel A shows a representative Western Blot analysis showing eNOS phosphorylation on Threonine495, Serine116 and Serine1177 after stimulation with acetylcholine, β-amyloid, and Gö6976, a selective cPKC inhibitor, alone or in combination and a quantitation of eNOS phosphorylation (black bar: Thr495; gray bar: Ser116; white bar: Ser1177 *p<0.05 vs basal, °p<0.05 vs ACh alone, #p<0.05 vs control condition, n=3). In panel B is shown vascular response of aortic rings precontracted with phenylephrine to increasing doses of acetylcholine, alone (□), or after preincubation with β-amyloid (Δ), Gö6976 (■) and with β-amyloid+Gö6976 (▼) (*p<0.01 as compared to control; n=6).

Fig. 5 Soluble AβP influences PKC-signaling pathway in vascular tissues. Representative Western Blot analysis showing PKC phosphorylation on Ser660 after incubation with β-amyloid and/or acetylcholine and a quantitation of PKC phosphorylation (*p<0.01 vs basal, n=3).
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**Fig 6** Soluble AβP induces Ca\(^{2+}\) leakage in endothelial cells. A: representative real time confocal microscope images showing intracellular calcium concentration in HAEC before and after addition of AβP. The time in seconds is indicated in each photogram. Calcium concentration is indicated by Fluo-4 AM fluorescence intensity, and expressed in a color scale ranging from blue (low concentration) to red (high concentration). B: quantitation of Ca\(^{2+}\) concentration in HAEC treated with ACh alone (□) or with AβP and ACh together (■), AβP followed after 20' by ACh (▲) and scrambled peptide (Δ). In both panel and graph, arrows indicate the time when agonists were added. Data represent the mean from 5 independent experiments.

**Fig 7** Soluble AβP localizes in the perinuclear region of endothelial cells and influence IP3 receptors function. Panel 7A displays high power micrographs showing HAEC biotynilated AβP localization (a), IP3R localization (b) and merge (c). Graph 7B shows quantitation of Ca\(^{2+}\) concentration in HAEC treated with furanophostin alone (□) or after preincubation with AβP (■); graph 7C shows quantitation of Ca\(^{2+}\) concentration in HAEC preincubated with 2APB treated with (■) or without (□) AβP. In both graphs, the arrows indicate the time when agonists were added. Data represent the mean from 4 independent experiments.

**Fig 8** Proposed action of soluble AβP peptides in endothelial cells. Soluble circulating AβP, interacting with IP3R, could determine a leakage of intracellular calcium. This alteration of cellular homeostasis could sensitize the cPKC pathway shifting the eNOS phosphorylation balance towards a pattern of impaired eNOS enzymatic activation.
Mechanisms of soluble β-amyloid impairment of endothelial function
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