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Amyloid β(1–42) and its β(25–35) fragment induce activation and membrane translocation of cytosolic phospholipase A_2 in bovine retina capillary pericytes

Carmelina Daniela Anfuso[a], Giovanna Assero[a], Gabriella Lupo[a], Ambra Nicotra[a], Giuseppe Cannavò[b], Robert P. Strosznajder[c], Piero Rapisarda[a], Ryszard Pluta[d], Mario Alberghina[a,*]

[a]*Department of Biochemistry, Faculty of Medicine, University of Catania, Viale A. Doria 6, 95125 Catania, Italy*
[b]*Department of Hygiene and Public Health, University of Messina, Via Consolare Valeria, 98125 Messina, Italy*
[c]*Department of Neurophysiology, Medical Research Center, Polish Academy of Sciences, 5 Pawinskiego Street, 02-106 Warsaw, Poland*
[d]*Department of Neurodegenerative Disorders, Medical Research Center, Polish Academy of Sciences, 5 Pawinskiego Street, 02-106 Warsaw, Poland*

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Abstract

We investigated changes in cytosolic phospholipase A_2 ($cPLA_2$) and calcium-independent PLA_2 ($iPLA_2$) activities in bovine retina capillary pericytes after stimulation with 50 μM amyloid-β (Aβ) (1–42) and its (25–35) fragment, over 24 h (mild, sublethal model of cell damage). In the presence of Aβ peptides, we found that $cPLA_2$ activity was increased and translocated from the cytosolic fraction to the membrane system, particularly in the nuclear region. Reversed-sequence Aβ(35–25) peptide did not stimulate or induce $cPLA_2$ translocation. Exposure to both Aβ peptides had no significant effect on $cPLA_2$ protein content as tested by Western immunoblot analysis. The addition of Aβs to quiescent pericytes was followed by phosphorylation of $cPLA_2$ and arachidonic acid release. Treatment with inhibitors ($AACOCF_3$, staurosporine and cycloheximide) resulted in a sharp decrease in basal and stimulated $cPLA_2$ activity. Inactivating effects of bromoenol lactone (BEL), inhibitor of $iPLA_2$, demonstrated that the stimulation of total PLA_2 activity by Aβs was mediated by both PLA_2 enzymes. Taken together with our previous observations that both Aβ peptides may induce hydrolysis of phosphatidylcholine, the present results provide evidence that this process is cooperatively mediated by $cPLA_2$ activation/translocation and $iPLA_2$ activation. The effect is very likely triggered by a mild prooxidant mechanism which was not able to divert the cell to degeneration. The data confirm the hypothesis that pericytes could be a target of potential vascular damage and reactivity during processes involving amyloid accumulation.

Keywords: Amyloid-β; Pericytes; Phosphatidylcholine; Cytosolic phospholipase A_2; Calcium-independent phospholipase A_2

1. Introduction

The mechanisms by which amyloid-β (Aβ) leads to brain and vascular disfunction in Alzheimer's disease (AD) remain to be elucidated. Most efforts have focused on the effects of Aβ on neurons and glial cells, but amyloid of senile plaques is often observed in close proximity to affected brain microvessels, suggesting a role for the vasculature in amyloid deposition and plaque formation in AD. Aβ typically accumulates in the basal lamina of the arteriolar tunica media and frequently extends into the adjacent neuropil. Another source of Aβ is thought to be in microcapillaries and venules of the neuroparenchyma lacking smooth muscle cells [1]. In fact, capillary basement membrane may be the site of Aβ accumulation, starting an amyloid microangiopathy and capillary fragmentation [2]. In parenchymal vessel endothelial cells (ECs) and pericytes of brain biopsies of patients with AD, tubuloreticular

* Corresponding author. Tel.: +39 095 7384070; fax: +39 095 7384220.
E-mail address: malber@mbox.unict.it (M. Alberghina).

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structures filled with amyloid fibrils were described [3]. In human brain obtained at autopsy, bands and spherical deposits of Aβ peptides were also found extensively associated with arterioles and capillaries leading to the distortion and occlusion of capillaries [4].

In an attempt to explain cerebrovascular amyloidosis, cultured human brain pericytes have also been used as a model system to study the mechanisms of microvascular amyloid formation in vitro and cytotoxic effects of Aβ on perivascular cells. The microvascular pericyte is a cell that, until recently, has been largely ignored in the clinical literature while its function is still being delineated. It has been shown to have contractile and phagocytic capacity, to be pluripotent and able to differentiate into fibroblasts, smooth muscle cells, and even osteogenic precursor cells [5,6]. There is also evidence that pericytes are involved in the transport across the blood–brain barrier and the regulation of vascular permeability [7]. Whatever the function, it must be particularly important in the retina microcirculation where pericytes are more numerous than at any other site. Pericytes produce and metabolize the amyloid precursor protein (APP) and are prone to cellular degeneration after treatment with Aβ [8]. Assembly of Aβ at the cell surface of brain pericytes is a crucial step in Aβ-induced cellular degeneration of these cells [9], presumably via oxidative stress.

Knowledge regarding the mechanisms by which a prooxidant, such as Aβ, influences their function is very limited [10]. The presence of amyloid deposits in cerebral microvessels (cerebral amyloid angiopathy) and the degeneration of vascular cells suggest that pericytes may directly contribute to the pathology of AD [11]. On the other hand, ECs and hippocampal neurons, treated with amyloid-β, display characteristics of necrotic or apoptotic cell death, prevented by free radical scavenging enzymes or antioxidants [12–15]. In cultured neurons and human smooth muscle cells, amyloid-β, which is considered to be a source of reactive oxygen species, induces death by oxidative stress, through the activation of SAPK/JNK kinase pathway and caspase-dependent apoptotic mechanism [16–18]. Increased oxidative stress, 40% cell death, caspase activation, nuclear DNA damage and mitochondrial disfunction have been observed in cultured cerebral endothelial cells, 48 h after treatment with either Aβ(1–40) or Aβ(25–35) at 10 μM [15].

We have previously reported that prolonged exposure of quiescent pericytes to sublethal doses of full-length and short neurotoxic Aβ(25–35) peptides modifies phosphatidylcholine turnover [19]. In addition, in the presence of Aβs, the significant increase in malondialdehyde levels and LDH release attested to the induction of oxidative changes in cell membranes due to free radical-mediated injury [19]. Expanding on this previous study, we examined the effect of Aβs on PLA_2 activities which are the putative enzymes increasing arachidonic acid release, precursor for eicosanoids being involved in the homeostasis or inflammatory diseases. Cytosolic PLA_2 is activated in a variety of cell types by growth factors, neurotransmitters and prooxidants, and plays a role in many physiological and pathological processes ranging from ischemia to AD [20]. The general hypothesis was that the presence of inflammatory mediators could affect lipid turnover and substance transport in exposed pericytes as well as ECs. These effects, in a cell culture model using a cell line of non-neuronal origin, could be caused by the inactivation of oxidation-sensitive phospholipid enzyme activities, increase in intracellular Ca^{2+} concentration, and activation of acylhydrolases.

2. Materials and methods

Reagent grade chemicals were purchased from Sigma Chemicals Co. (St. Louis, MO) or E. Merck (Darmstadt, Germany). Collagenase-dispase, fatty acid-free BSA, arachidonic acid, lipid standards, NADPH, and DTT were purchased from Sigma. $AACOCF_3$ (arachidonoyl trifluoromethyl ketone), BEL (bromoenol lactone), staurosporine, cycloheximide, PD98059, SB203580, GF109203X, caspase-3 inhibitory peptide DEVD-CHO and rabbit polyclonal antibody caspase-3 were purchased from Calbiochem (La Jolla, CA). Mouse monoclonal antibody $cPLA_2$ (4-4B-3C):*sc-454*, monoclonal antibody anti-PARP, and rabbit polyclonal antibody against Bax were from Santa Cruz Biotechnology (CA) and Oncogene RP (CA), respectively. Rabbit polyclonal antibody $iPLA_2$ was from Cayman Chemical Co. (Michigan, USA). Mouse monoclonal anti-phosphotyrosine antibody, clone 4G10, and mouse monoclonal anti-phospho-Ser/Thr-Pro, MPM-2 were from Upstate Biotechnology (New York, USA). Thin-layer chromatography plates (silica gel G) were produced by E. Merck. Fetal calf serum (FCS) was obtained from Euroclone Ltd, UK.

2.1. Cell cultures

Bovine retina microcapillaries and pericyte cultures were prepared and characterized following the procedures previously described [19]. Cell cultures were incubated in DMEM medium containing 5% FCS, with and without 50 μM Aβs for 24 h. Aβ(1–42), synthesized and purified by Misicka et al. [21], was dissolved in bidistilled water and stock solution was stored in aliquots at −20 °C. Before use, Aβ(1–42) was incubated 24 h at 37 °C for maturation. Synthetic Aβ(25–35) protein fragment or reverse-sequence Aβ(35–25) peptide (Bachem AG, Heidelberg) was dissolved in bidistilled water, and stock solution was stored as above. Exposure to Aβ(1–42) or Aβ(25–35) for 24 h did not significantly reduced the number of viable cells (trypan blue exclusion test). Morphological changes, cell viability, determined by MTT test, and LDH release were assessed as previously reported [19,22,23].

2.2. Arachidonic acid release

Cultures of pericytes (10^6/ml) were established in 60-mm dishes and incubated with 0.5 μCi/dish of [1-^{14}C]arachidonic acid (Dupont NEN) in a medium containing 2 mM glutamine and 5% FCS, for 24 h at 37 °C. Cells were washed three times, 5 min per wash, with HBSS buffer, and stimulated in serum-free DMEM, containing 0.5% BSA as a trap for the released arachidonic acid, with Aβ peptides (50 μM) for 24 h. After stimulation, the medium was removed and centrifuged for 5 min. The supernatant fraction was concentrated by lyophilization to 1 ml, and an aliquot (100 μl) taken to determine the radioactivity in total medium by liquid scintillation spectroscopy, normalizing to protein. Then, it was acidified with 1 M HCl to bring the pH to 3.0 (medium turns yellow) and extracted three times in equal volumes of ethyl acetate. The organic layer was evaporated under nitrogen and the residue was dissolved in ethanol. In some analyses, eicosanoid derivatives were separated on silica gel 60 TLC plates developed with the organic phase of ethyl acetate–isooctane–acetic acid–water mixture (11:5:2:10, by vol). The lipid zones were located with I_2 vapor or by preparing autoradiographs (Berthold DAR-signal analyzer), scraped into scintillation vials, and the radioactivity was measured by liquid scintillation spectroscopy. Identification of the lipids was based on a comparison of their TLC mobility with that of authentic unlabeled standards.

Effects of reverse Aβ(35–25), Aβ(25–35) and Aβ(1–42) peptides, dissolved in buffer, or in combination with MAPKs or PKC inhibitors (dissolved in DMSO stock solutions), were tested in pre-labeled cell cultures described above. Cells were pretreated with inhibitors for 10 min prior to a 24-h exposure to Aβ peptides in the continued presence of the PLA_2 inhibitors.

2.3. Assay of phospholipase A_2 and caspase-3 activities

The PLA_2 activity was determined using 1-palmitoyl-2-[1-^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine and 1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine (DuPont NEN) as substrates, in the presence or absence of calcium ions and DTT, as previously described [22]. The caspase activity was determined using 7-amino-4-trifluoromethyl coumarin (AFC)-conjugated peptide substrate (z-DEVD-AFC, Calbiochem), as previously described [22], and expressed as fluorescence units. Cell cultures treated with 5 μM staurosporine were taken as positive controls for caspase-3 activation.

2.4. Subcellular fractionation

Bovine pericytes were suspended in 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol and 10% of glycerol (buffer A) supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF) and homogenized by sonication three times for 5 s. After a low-speed centrifugation, 500×*g* for 10 min, the resulting supernatant was centrifuged at 100,000×*g* for 60 min in a Beckman TL100 ultracentrifuge. The 100,000×*g* pellet was washed once, resuspended in buffer A, and sonicated to obtain the membrane fraction. The cytosol and membrane samples were analyzed by immunoblotting.

2.5. Immunoprecipitation

Control cells and cells stimulated by Aβ peptides, after washing twice with ice-cold PBS, were subjected to lysis with immunoprecipitation buffer (150 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100, 50 mM Tris, pH 8.0, 1 μg/ml aprotinin, 1 μM/ml leupeptin, 1 mM PMSF) for 10 min in ice with gentle mixing. Cell suspensions were transferred to homogenization tubes and sonicated. Homogenates were then centrifuged at 12,000×*g* for 10 min at 4 °C to remove particulate matter. After quantification of the protein content, equal amounts of cell supernatants were transferred to new tubes and incubated with appropriate purified antibody, gently mixed overnight at 4 °C, followed by addition of 50 μl of 50% slurry Protein A Sepharose (Amersham). Incubation followed for an additional 3 h at 4 °C with gentle mixing. The suspensions were centrifuged at 12,000×*g* for 20 s and the pellets were saved. The beads were washed three times with lysis buffer. The final bead pellets were suspended in 30-μl sample buffer containing bromophenol blue, heated to 95 °C for 10 min, and centrifuged at 12,000×*g* for 20 s to remove the beads. The supernatants were carefully recovered and then used for Western immunoblot assays.

2.6. Western blot analyses

Cytosolic PLA_2 ($cPLA_2$) or calcium-independent PLA_2 ($iPLA_2$) immunoblots were performed as described elsewhere [23]. Antibody reaction was detected using horseradish peroxidase-conjugated sheep anti-mouse IgG (dilution 1:2000, 1 h at room temperature), recognizing phosphorylated and nonphosphorylated $cPLA_2$, and the peroxidase activity was determined using ECL chemiluminescent detection reagents (Amersham). Bax levels and proteolytic cleavage of poly(ADP-ribose) polymerase (PARP) were also detected by Western blot analysis. After drug treatment, cells were lysed in an ice-cold buffer containing 0.1% SDS, 1% Triton X-100, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml leupeptin, 1 μg/ml aprotinin and 1 μg/ml pepstatin. After 30 min at 4 °C, insoluble materials were removed by centrifugation at 10,000×*g* for 15 min at 4 °C. Protein content of the supernatant (cell extract) was quantified by Lowry's assay. Protein, 30–75 μg on each lane, was applied to 10% SDS-PAGE gel. After electrophoresis for 1 h at 170 V, transfer of proteins to nitrocellulose membranes was performed accord-

ing to standard procedures. After blocking of nonspecific sites, membranes were incubated with primary rabbit polyclonal antibody to Bax (1:1000 dilution) and mouse antibody to PARP (1:500 dilution) at room temperature for 2.5 h. The membranes were subsequently probed with appropriate peroxidase-labeled anti-mouse secondary antibody, and immunocomplexes were detected by enhanced chemiluminescence reagent (Amersham). The relative intensities of the $cPLA_2$, $iPLA_2$, Bax and PARP bands were analyzed by scanning the film and subsequent determination by Scion Image software.

2.7. Preparation of cells for confocal fluorescence microscopy

Cells were cultured on gelatine-coated multichamber Lab-Teck slides and were Aβ-treated as described in the text. At the end of the treatment periods, cell cultures were fixed by adding 4% paraformaldehyde in PBS and incubated with $cPLA_2$ mouse monoclonal IgG antibody in normal blocking serum, followed by fluorescein conjugated secondary antibody, and observed under Leica TCS SP2 scanning confocal microscope as described previously [23]. To quantify fluorescence intensity, the images were analyzed in a PC workstation using Leica 2.0 bild 0585 software. The program quantified the pixel values inside each nucleus on a scale of 0 to 50,000 pixels (arbitrary fluorescence units) [23].

3. Results

3.1. Cell viability

Amyloid-mediated cytotoxicity was determined by measuring the mitochondrial activity using MTT assay and plasma membrane permeability by LDH release test. Data demonstrated that amyloid-β(1–42) was ineffective on MTT at concentrations up to 50 μM (Fig. 1), in agreement with data in macrophage cell cultures [24]. Significant cytotoxicity, as compared to cells cultured without amyloid-β, was reached when the peptide was used at concentrations of 500 μM for 24 h. In addition, an induction of oxidative damage, as evaluated by malondialdehyde measurements, was previously seen in the same cellular model [19]. LDH levels also confirmed a certain degree of membrane injury at 50 μM, at least for Aβ(25–35) fragment, which was very pronounced at the highest Aβ peptide concentrations. Therefore, in our conditions, the exposure of pericytes to Aβ peptides did appear associated with significant toxicity in the range from 50 to 500 μM.

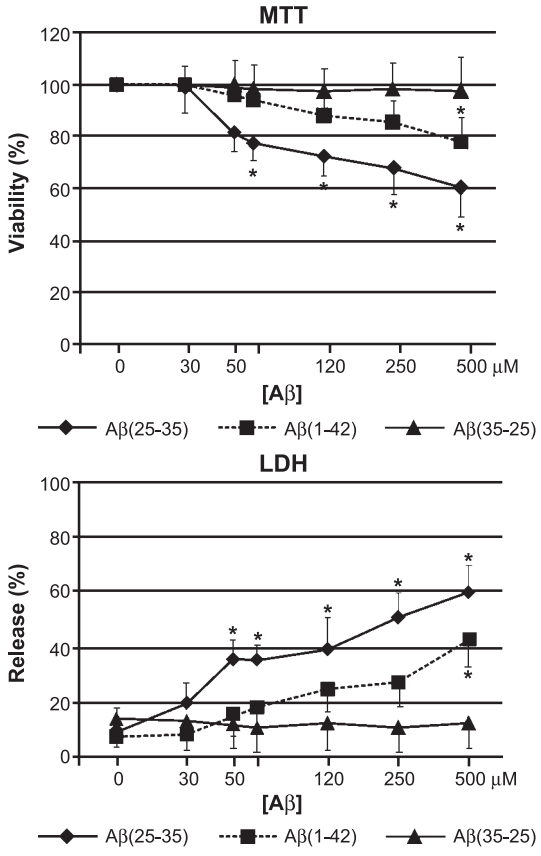

Fig. 1. Dose-dependent cytotoxicity effect was studied using MTT and LDH release assays. Bovine retina pericytes were pretreated with amyloid-β peptides for 24 h and then the cells were recovered together with their culture medium. Data are expressed as a percentage of the untreated control and represent the mean of three independent experiments (*P<0.05 vs. Aβ(35–25) reverse peptide, Student's t test).

3.2. Caspase-3 activity, and PARP and Bax levels

Caspase-3 activity was not significantly stimulated by either Aβ(25–35) or Aβ(1–42), compared to either control or reverse Aβ(35–25) peptide (Fig. 2). Measurements of the same activity showed that cell-permeable DEVD-CHO, a caspase-3-specific inhibitor, was without effect on the enzyme activity. Staurosporine (5 μM, 8 h and 1 μM, 24 h), taken as a positive control, was able to stimulate caspase-3 activity, whereas 1 and 0.1 mM H_2O_2, putative apoptosis inducer in pericytes [25], was unable to activate the enzyme activity 1 h after treatment. After cell treatment with 50 μM Aβ(25–35) or Aβ(1–42) for 24 h, Western blot analysis using a polyclonal antibody anti-caspase-3 showed no changes in caspase-3 protein level, compared to the control cells or cells treated for the same time interval with reverse Aβ(35–25) peptide (data not shown).

Degradation of PARP enzyme is an indication of caspase-3-like activity since PARP is characteristically cleaved at DEVD site, resulting in the disappearance of the 116-kDa parent protein and the specific appearance of the 85-kDa cleavage product. In our model system, Aβ peptide stimulation of pericytes did not result in a cleavage of DNA-repairing enzyme poly(ADP ribose) polymerase (PARP) (Fig. 3), confirming that Aβ treat-

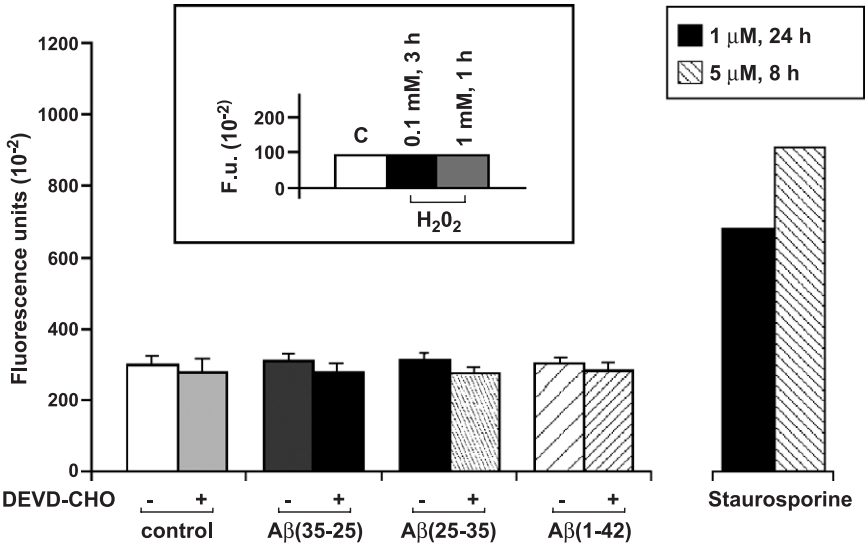

Fig. 2. Caspase activities in lysates of cells treated with Aβ peptides in the absence or presence of inhibitor. Aβ peptides (50 μM final concentration) were added to the culture medium of confluent pericytes which were incubated for 24 h with these agents. Caspase inhibitor DEVD-CHO (10 μM) was co-applied with Aβ peptides to the culture medium and maintained with the cells for the entire exposure time (24 h). Enzyme activities were measured using fluorogenic sequence-specific substrate for caspase-3, as described under Materials and methods. Values are means±S.E. of three experiments carried out in triplicate. Values for staurosporine are the means from two independent experiments. Determinations with H_2O_2, in duplicate, had their own control.

ment (50 μM for 24 h) was not seriously injurious to pericytes. As a positive control for PARP fragmentation, pericytes incubated for 24 h with two concentrations of staurosporine (0.1 and 1.0 μM) were used. At 1.0 μM concentration in the culture medium, straurosporine was able to induce PARP fragmentation, and the density of 85-kDa band increased and overcame that of 116-kDa (Fig. 3).

Having demonstrated that caspase-3 activity and PARP were unchanged, we determined the effect of Aβ peptides on Bax protein level. Western blots revealed a slight but significant increase in Bax immunoreactivity (Fig. 4), suggesting an up-regulation in terms of cellular expression of the protein at gene level 24 h after Aβ(25–35) treatment. Aβ(1–42) did not produce an increase in Bax levels (data not shown).

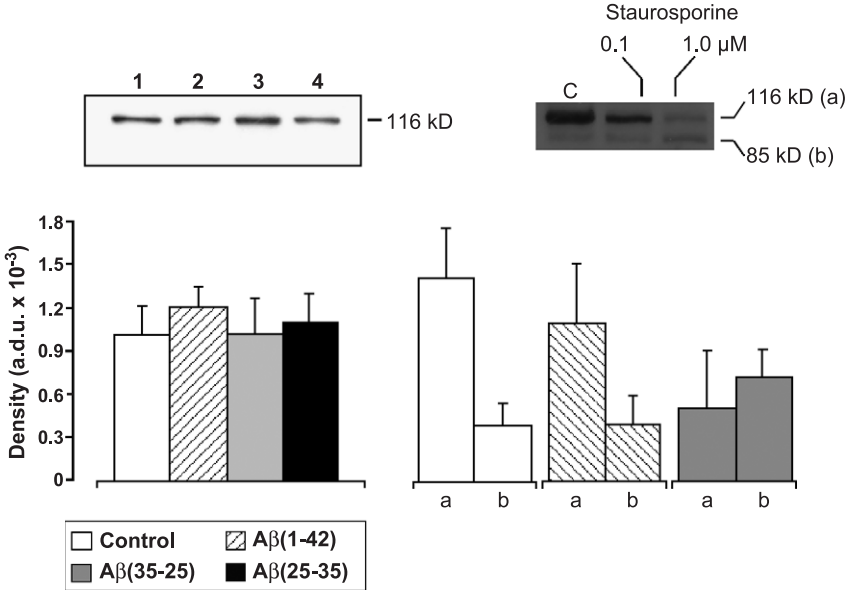

Fig. 3. Effect of Aβ peptides on poly(ADP ribose) polymerase (PARP) fragmentation in bovine retina pericytes. Supernatants of whole lysates from control and Aβ-treated cells for 24 h were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose membrane, and the blots were probed for PARP with a monoclonal antibody: 1, control; 2, 50 μM Aβ(1–42); 3, reverse Aβ(35–25); 4, Aβ(25–35). Bars below each lane represent the results of densitometric scanning (arbitrary units, a.d.u.) of the respective 116-kDa PARP bands. The results are representative of three independent experiments. As a positive control for PARP fragmentation, cells incubated for 24 h with two concentrations of staurosporine were used. Double bars below each lane represent results of densitometric scanning (arbitrary units, a.d.u.) of 116-kDa PARP (a) and 85-kDa PARP fragment bands (b), obtained from two independent experiments.

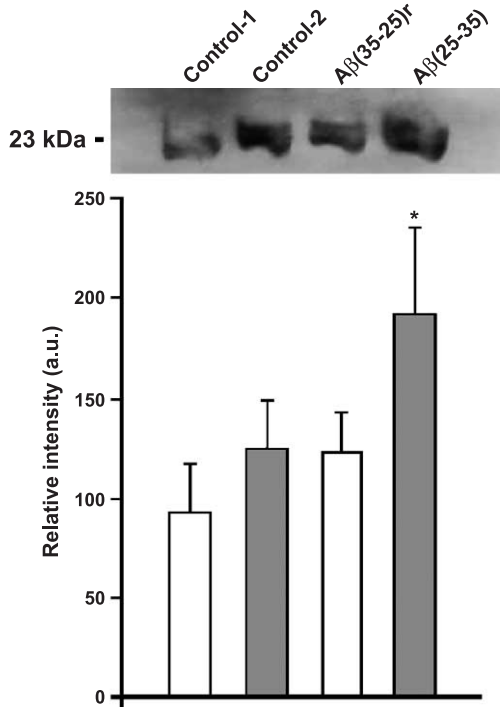

Fig. 4. Bax protein expression. Cell lysates obtained from 50 μM reverse Aβ(35–25)- or Aβ(25–35)-treated bovine retina pericytes in culture for 24 h were assessed by Western blot with Bax polyclonal antibody. Representative blot is shown from three independent experiments. Protein expression was quantified (means±S.E.) densitometrically. Control-1, pericytes grown in culture medium with serum; control-2, pericytes grown in culture medium without serum (starvation) for 24 h before adding Aβ peptides (*$P<0.05$ vs. control-1, Student's t test).

3.3. Arachidonic acid release

When cells were cultured for 24 h in the presence of 50 μM Aβ peptides, [1-^{14}C]arachidonic acid release markedly increased after treatment with either Aβ(25–35) or Aβ(1–42) compared with reverse Aβ(35–25) peptide (Fig. 5), which at this time point had no significant effect on this parameter. Co-incubation with PLA_2 inhibitors, BEL (relatively specific inhibitor of $iPLA_2$) or $AACOCF_3$ (inhibitor of both $cPLA_2$ and $iPLA_2$), for 24 h sharply decreased the AA release (58% over controls for BEL and 48% for $AACOCF_3$) compared with cells treated with Aβ(25–35) alone. Co-incubation with PLA_2 inhibitors, BEL or $AACOCF_3$, for 24 h decreased the AA release (23% over controls for BEL and 55% for $AACOCF_3$) compared with cells treated with Aβ(1–42) alone. These results may reflect inhibition of the cytosolic and calcium-independent PLA_2s, which both seem stimulated by Aβ peptides. The measured level of [^{14}C]arachidonate in the medium reflects the balance between the release of arachidonic acid (AA) from and its reincorporation into membrane phospholipids. In ethyl acetate extracts of the release medium, examined by TLC, the main spot was identified as AA, but a minor band corresponding to PGE_2 was also evident. Cells incubated for 24 h with 25 μM BEL or 50 μM $AACOCF_3$ alone did not show any morphological or survival index change.

3.4. Phospholipase A_2 activity and the effect of inhibitors

As shown in Fig. 6, compared to untreated control, Aβ(25–35) peptide fragment and Aβ(1–42) activated PLA_2 activity by 250% and 210%, respectively, whereas reverse amyloid-β(35–25) peptide had no effect. The involvement of PLA_2 in the process of pericyte response was evaluated using three pharmacological inhibitors of PLA_2, two nonspecific (staurosporine, a broad inhibitor of protein kinases, and cycloheximide) and cell-permeable agent, $AACOCF_3$. Our results demonstrated that staurosporine treatment of unstimulated or Aβ(35–25)-treated cells for 18 h resulted in, respectively, 50% and 60% decrease in PLA_2 activity (Fig. 6). This result may reflect inhibition of the basal cytosolic PLA_2 phosphorylation. In fact, several cell types contain both phosphorylated (serine residues as preferential phosphorylation substrate) and unphosphorylated active forms of $cPLA_2$, in a variable ratio depending on the cell type, differentiation and proliferation state [26–28]. This may also be the case for pericytes in which the constitutive phosphorylated form seems at about 50% level. Cycloheximide induced a striking reduction of 76% in basal enzyme activity in the control cells, and of 80% in Aβ(35–25)-treated cells.

PLA_2 activity, stimulated by Aβ peptides, was sharply suppressed by a maximum of 2.9- (staurosporine) and 6.1-fold (cycloheximide) with the Aβ(25–35) fragment. Reduction of PLA_2 activity after an 18-h exposure to cycloheximide, as expected, was probably due to full reduction of constitutive and new synthesis of 85-kDa $cPLA_2$ and $iPLA_2$ enzymes. Staurosporine, inhibitor of PKC and other kinases, is known to inhibit agonist-induced $cPLA_2$ phosphorylation and activation [26]. The inhibitory result observed using this agent supports the concept that the Aβ peptide-induced PLA_2 activation may require PKC cascade stimulation. Relative to the vehicle (0.001% DMSO in control cells), 50 μM $AACOCF_3$, incubated for 18 h, inhibited pericyte PLA_2 activity of unstimulated control cells 6.6-fold (Fig. 6). PLA_2 activity stimulated by Aβ peptides was also strongly inhibited by $AACOCF_3$, with a maximum of 10.1-fold for Aβ(1–42). A possible concern is that all three inhibitors may induce a massive cell death during the incubation period. However, in all subgroups (5 nM staurosporine, 10 μM cycloheximide or 50 μM $AACOCF_3$) following 18-h incubation, neither did absolute cell number significantly decrease nor were obvious changes in cell structures observed by morphological examination (trypan blue dye exclusion) and MTT test.

It is possible that $iPLA_2$, in addition to $cPLA_2$, might be involved in AA liberation in bovine retina pericytes. This participation in stimulus-induced AA liberation (Fig. 5) remains to be elucidated. In the present study, to clarify the role of group VI $iPLA_2$ upon Aβ peptide stimulation in

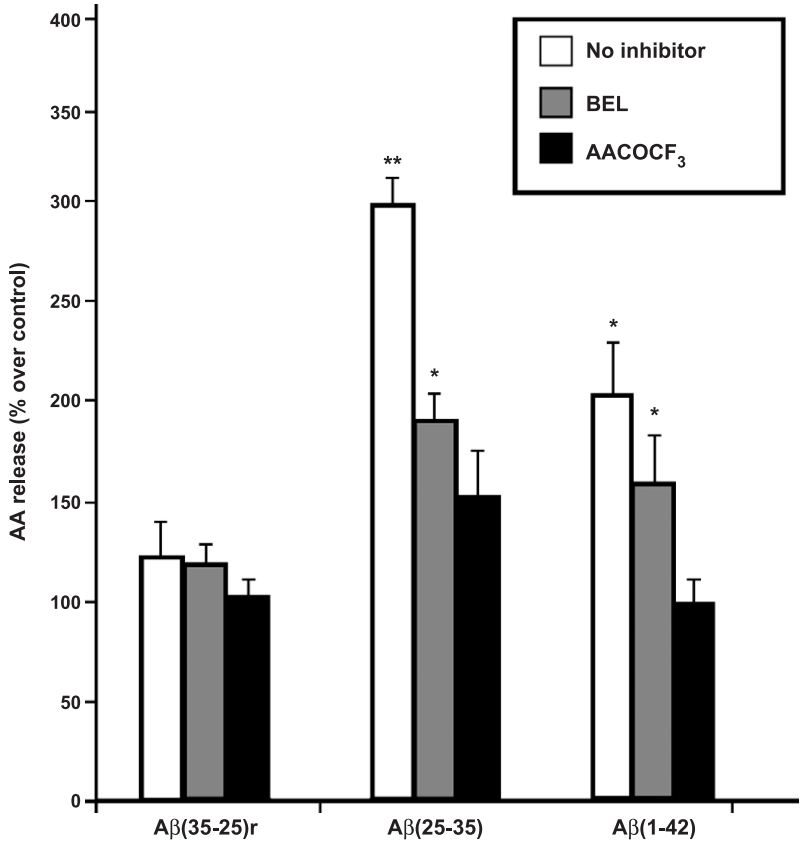

Fig. 5. Effect of PLA_2 inhibitors on amyloid-β-induced [1-^{14}C]arachidonate release in bovine retina pericytes. Cells were prelabeled with 0.5 μCi/dish of [1-^{14}C]arachidonate in DMEM containing 2 mM glutamine and 5% FCS, for 24 h and washed 3× with HBSS medium containing 0.5% BSA prior to challenge with Aβ peptides (50 μM) alone or in combination with BEL (25 μM), $AACOCF_3$ (50 μM) for 24 h in DMEM containing 5% FCS. Pericyte control cultures were also incubated without Aβ peptides or inhibitors. Labeled cells were pretreated for 1 h with the inhibitors; thereafter, the cells were stimulated with Aβ peptides without removing the medium. Arachidonate release into the culture medium was determined as described in Materials and methods. Values represents means±S.E. from five separate experiments. Statistically significant differences (*P<0.05 or **P<0.01) are indicated by one or two asterisks for cells treated with inhibitors for 24 h, versus control cell cultures (no addition, incubated over the same time interval). No significant difference was observed for control values (no addition) versus reverse Aβ(35–25)-treated cell cultures.

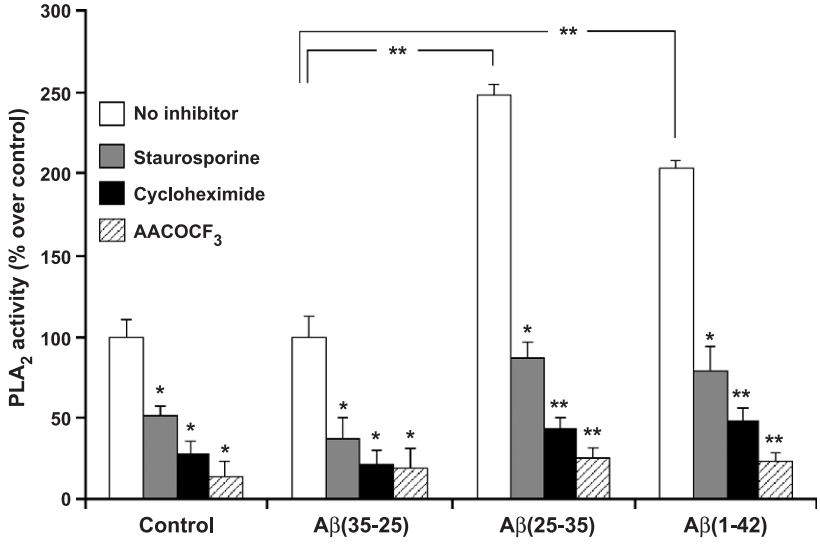

Fig. 6. Effect of inhibitors on PLA_2 activity in bovine retina pericytes in culture. Cells were co-incubated 18 h with 50 μM Aβ peptides in the presence or absence of straurosporine (5 nM), cycloheximide (10 μM) or $AACOCF_3$ (50 μM). Cells were then recovered, and the enzyme activity was measured in the lysates. Results are expressed as percentage of appropriate control and presented as means±S.E. (n=3). *P<0.01 or **P<0.005 vs. control (no inhibitor addition, incubated over the same time interval). (ANOVA with Scheffé's contrast).

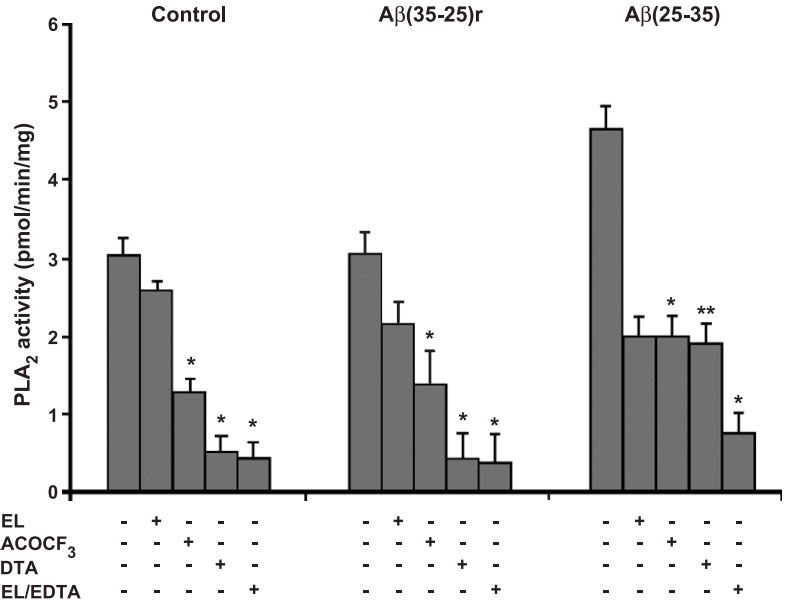

Fig. 7. Effect of inhibitors on PLA_2 activity in control and Aβ peptide-stimulated retina pericytes. Cells were challenged with 50 μM reverse Aβ (35–25) or Aβ(25–35) for 24 h in DMEM containing 5% FCS. Following Aβ peptide treatment, cell lysates were centrifuged and the enzyme activity was determined in the supernatant of 10,000×*g*. Total PLA_2 activity (Ca^{2+}-dependent and Ca^{2+}-independent PLA_2) was measured in the presence of DTT; BEL-sensitive PLA_2 activity was measured in the presence of DTT and 25 μM inhibitor; $AACOCF_3$-sensitive PLA_2 activity was measured in the presence of DTT and 50 μM inhibitor; Ca^{2+}-independent PLA_2 activity was measured in the presence of DTT and 5 mM EDTA. Enzyme activity was measured using l-palmitoyl-2-[1-^{14}C]arachidonoylphosphatidylcholine as substrate as described in Materials and methods. Values are the means±S.E. of triplicate determinations from a typical experiment that was repeated twice. *P<0.05; **P<0.01, as compared with their respective control.

pericytes, we explore the possible involvement of $iPLA_2$ by evaluating the effect of inhibitors such as BEL, $AACOCF_3$ and EDTA. The enzyme activity insensitive to BEL represents the Ca^{2+}-dependent PLA_2, whereas that insensitive to EDTA represents Ca^{2+}-independent PLA_2. As shown in Fig. 7, BEL, $AACOCF_3$ and EDTA were able to significantly inhibit total basal PLA_2 activity measured in the homogenates of control and reverse Aβ(35–25) peptide-treated pericytes, where $iPLA_2$ activity seemed to be very low. In addition, BEL quantitatively suppressed the Aβ(25–35)-enhanced PLA_2 activity by 48%, whereas $AACOCF_3$ was able to decrease Aβ peptide-stimulated PLA_2 activity by the same percentage. EDTA suppressed the Aβ(25–35)-enhanced PLA_2 activity by 52%. Co-presence of BEL (25 μM) and EDTA (5 mM) reduced Aβ(25–35)-stimulated PLA_2 activity to residual 18%. The results demonstrate that total Aβ-stimulated PLA_2 activity was mediated by both PLA_2 enzymes, even if $cPLA_2$ activation may be the slightly predominant fraction. Similar results were obtained when a different PLA_2 substrate, 1-palmitoyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphocholine, was used in the assays (data not shown).

3.5. Translocation of $cPLA_2$ to membranes

We also examined the effect of Aβ peptides on subcellular distribution of $cPLA_2$. When the $cPLA_2$ activity was compared in both cytosol and membrane fractions from pericytes, the majority of the activity was found in the cytosolic fraction of the control cells (Table 1). About 75%

Table 1
Effect of amyloid-β peptide treatment on PLA_2 activity in subcellular fractions of bovine retina pericytes

	Homogenate		Cytosol			Pellet		
	Total activity	Specific activity	Total activity	Specific activity	RSA	Total activity	Specific activity	RSA
Control	7.6±1.5	2.7±0.27	5.5±0.8	7.3±2.2	2.7	1.9±0.5	1.5±0.6	0.6
Aβ(35–25)	6.9±0.6	2.3±0.20	5.1±0.7	6.6±1.5	2.9	1.7±0.3	1.6±0.4	0.7
Aβ(25–35)	11.1±3.1*	4.1±0.31*	2.5±0.2*	3.3±0.9*	0.8	6.2±0.8*	4.8±0.9*	1.1
Aβ(1–42)	10.9±1.9*	3.9±0.16*	3.2±0.7	4.2±1.3	1.1	5.6±0.4*	4.7±0.3*	1.2

Aβ peptides were added to the culture medium of confluent pericytes incubated with these agents for 24 h. Cells were lysed by sonication, and the PLA_2 activities from homogenate (nonnuclear homogenate, supernatant 500×*g*) and subcellular fractions were measured (see Ref. [22]). Results are expressed in pmol/min (total activity), in pmol/min/mg proteins (specific activity), and presented as means±S.E., with n=3 performed in duplicate. RSA (degree of enrichment in the fraction compared to homogenate) is specific activity of fraction/specific activity of homogenate. In control cells (6.5×10^6 cells), the distribution of protein was: nonnuclear homogenate, 2.81±0.5 mg; cytosol, 0.76±0.1 mg; pellet 1.26±0.3 mg. Variation of protein recovery in subcellular preparations between control and Aβ-treated pericytes was in the range of 20%.

* P<0.05 versus untreated control.

of total activity occurred in the supernatant, and this fraction had also the highest relative specific activity, approximately 4-fold that of pellet. When the cells were incubated with Aβ(25–35) fragment and Aβ(1–42) for 24 h, $cPLA_2$ total activity was higher in these cells compared to untreated control or cells treated with the reverse Aβ(35–25) peptide. Furthermore, $cPLA_2$ activity shifted to the membrane fraction where it was found to be higher than in the cytosol. Approximately 60–65% of activity seemed to migrate to membrane fraction in cells exposed to 50 μM Aβ(25–35) compared to control or Aβ(35–25)-treated cells. Aβ(1–42) fragment at 50 μM induced $cPLA_2$ translocation to almost the same degree. However, PLA_2 activity measured in the membrane fractions might also reflect the contribution of stimulated $iPLA_2$ activity which is very low in unstimulated, control homogenates (Figs. 7 and 8).

Exposure to 50 μM Aβ peptides had no significant effect on $cPLA_2$ protein content as tested by Western immunoblot analysis, i.e., the level of the band with apparent molecular mass of 85 kDa was no different in Aβ peptide-treated (24 h) versus untreated pericytes (Fig. 8). Western blot analysis of subcellular fractions obtained from cell homogenates showed a shift in the distribution of $cPLA_2$ immunoprotein, which was more pronounced in the pellet fraction of cells stimulated with Aβs, particularly with Aβ(25–35), compared to untreated or treated with reverse Aβ(35–25) peptide.

Following subcellular fractionation, we used Western blot analysis to detect the possible subcellular distribution of $iPLA_2$ in pericytes by a specific antibody. Interestingly, Fig. 9 demonstrates that the enzyme protein was mainly present in the particulate fraction, whereas the cytosolic fraction did contain a marginal percentage of the total 85-kDa $iPLA_2$ protein. The location specificity resembles that found in rat vascular smooth muscle cells [29]. This strongly suggested that the observed $iPLA_2$ activity could be ascribed to the membrane-associated $iPLA_2$.

Fig. 9. Western blot analysis of $iPLA_2$ in subcellular fractions of retina pericytes. An aliquot of each fraction (30 μg of protein) was subjected to SDS/PAGE followed by Western blot analysis using rabbit polyclonal anti (mouse) $iPLA_2$. Data are from one representative experiment of two.

3.6. Confocal fluorescence microscopy

The distribution of $cPLA_2$ in pericytes by confocal immunofluorescence microscopy is shown in Fig. 10. In control and Aβ(35–25)-treated cells, the enzyme appeared diffusely distributed in the cytoplasm. In 50 μM Aβ(1–42)- and Aβ(25–35)-treated cells (24 h), fluorescence was seen to move to the nuclear membrane, with intense staining associated with the nuclear compartment. The fluorescence intensity (scale of 0 to 6×10^4 pixels) of the regions of interest was measured for each image, inside each cell and each nucleus, and the graph (Fig. 10, panel E) shows an intensity increase in the nucleus of Aβ peptide-treated cells.

3.7. Phosphorylation of $cPLA_2$

As arachidonic acid release was blocked by inhibitors of PLA_2, it is likely that Aβ peptides activate pre-existing PLA_2 enzymes in pericytes. Based on the findings that the $cPLA_2$ enzyme is activated by several agents through

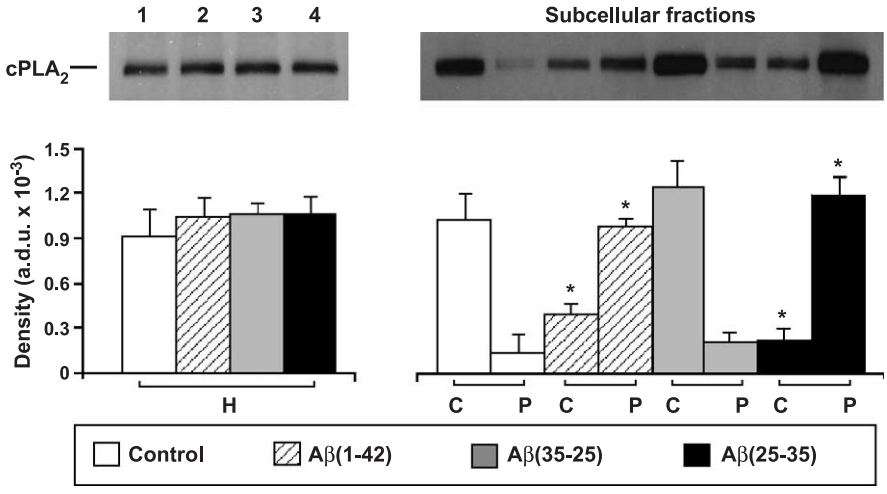

Fig. 8. Effect of Aβ treatment on $cPLA_2$ expression of pericytes in culture. Cells were challenged without Aβ peptides (control) (1) or with 50 μM Aβ(1–42) (2), reverse Aβ(35–25) (3) or Aβ(25–35) (4) for 24 h. After the incubation period, cells were sonicated and fractionated by centrifugation (see Materials and methods). Cytosol (C) and membrane fraction (pellet that includes total cell membranes, P) were separated from the cell homogenate (H). The particulate fraction, containing the membrane fraction, was washed twice and resuspended in 50 μl of buffer. The supernatant fraction was used as such. Equal protein samples of cell fractions were loaded in different wells and separated by SDS-PAGE. The blots were scanned to calculate fold increase in the antigen level. The values, expressed as arbitrary densitometric units (a.d.u.), were obtained by the reading of autoradiographs, and are the mean±S.E. of three experiments performed in triplicate (n=3); *P<0.05 vs. corresponding control subcellular fraction. Representative blots are shown.

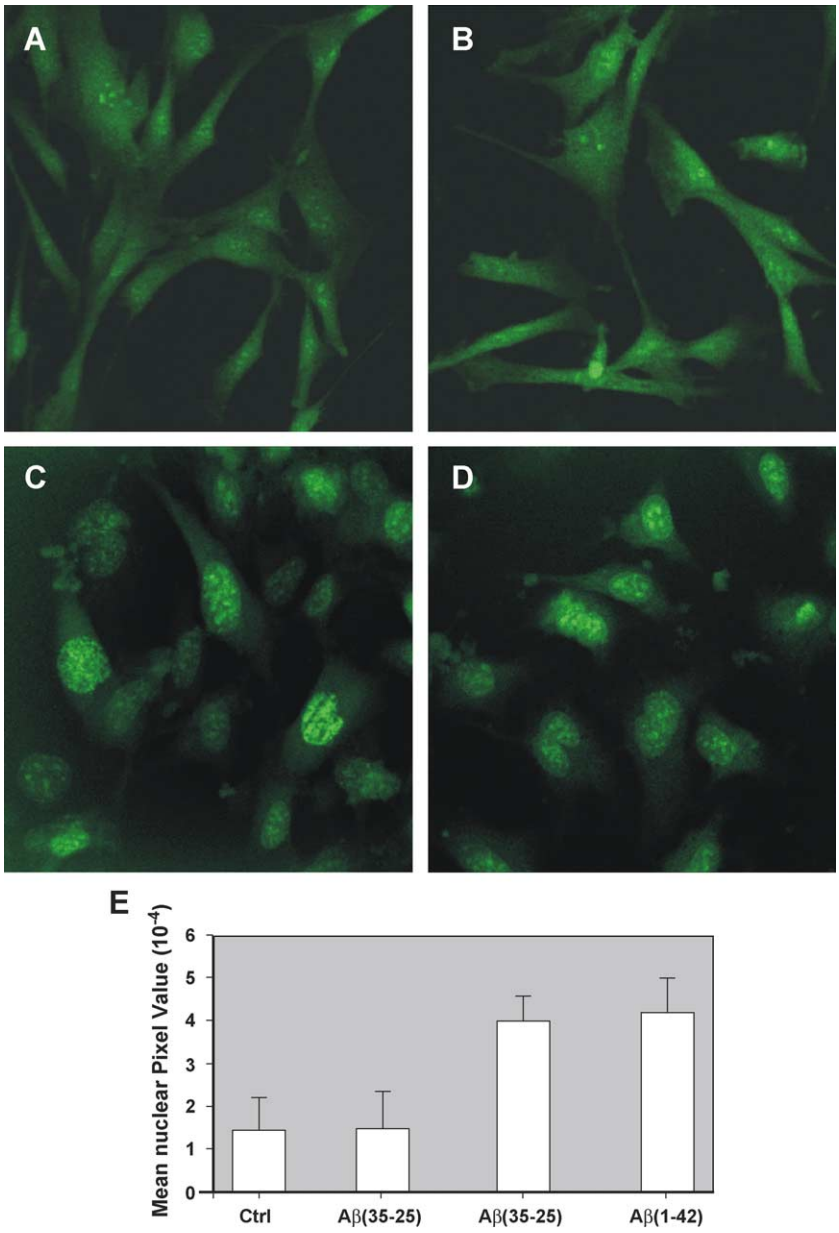

Fig. 10. Confocal micrographs of subconfluent pericytes. Cells were stained with anti-cPLA$_2$ (4-4B-3C) antibody (magnification ×400), followed by FITC conjugated anti mouse IgG. Control cells (A) and cells treated for 24 h with Aβ(35–25) (B) show a diffuse cytoplasmic staining. Pericytes treated with Aβ(25–35) show cPLA$_2$ localization to nuclear membrane (C). Intense nuclear staining is also present in cells exposed to Aβ(1–42) (D). The cells reveal staining of nuclear envelope in addition to intranuclear region. Representative fields are shown. Similar results were obtained in two independent experiments. The graph below (E) represents the quantification of mean nuclear pixel values of all nuclei in panels (A) through (D) ±S.E.

enzyme phosphorylation in other cells, we then examined the direct effect of Aβ(25–35) peptide on cPLA$_2$ phosphorylation.

Aβ(25–35) induced, in fact, phosphorylation of pericyte cPLA$_2$ (Fig. 11). Immunoblot analysis of immunoprecipitated cPLA$_2$ revealed the presence of a phosphorylated protein even in the control, indicating that a consistent amount of the enzyme was already in the phosphorylated form in unstimulated, quiescent cells. The intensity of this band was sharply increased in cell samples after Aβ(25–35) treatment for 24 h, thereby providing evidence that the observed enhancement of enzyme activity and arachidonic acid release in pericytes is accompanied by activation of phosphorylation of cPLA$_2$. To investigate the possible signaling mechanisms by which Aβ(25–35) peptide may mediate phosphorylation of cPLA$_2$, we used inhibitors of MAP kinase phosphorylation or protein kinase C (PKC) activity [30]. Pretreatment for 10 min before addition of Aβ(25–35) peptide to culture medium with PD98059, known to be selective inhibitor of the upstream kinase MEK1 involved in ERK1/2 phosphorylation, SB203580, a selective inhibitor of stress kinase p38, and GF109203X, a selective inhibitor of PKC, clearly reduced the phospho-

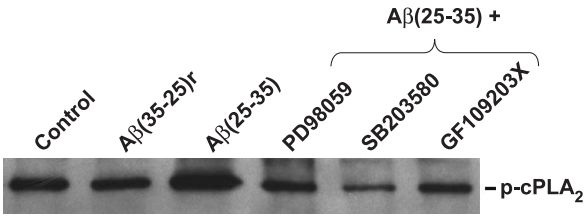

Fig. 11. Effect of Aβ(25–35) on $cPLA_2$ phosphorylation in cultured pericytes. Cells made quiescent by serum starvation for 24 h were preincubated for 10 min with vehicle (control) or inhibitors (25 μM PD98059, 10 μM SB203580, 5 μM GF109203X) and then stimulated with 50 μM Aβ(25–35) for additional 24 h. Inhibitors were present in culture medium throughout the incubation period. Cell lysates were immunoprecipitated with anti-$cPLA_2$ mouse monoclonal antibody and, after centrifugation and washing, immunoprecipitates were fractionated by SDS-PAGE, electroblotted and reacted with a mixture of anti-phosphotyrosine and anti-phosphoserine/threonine-proline antibodies, as described in Materials and methods. Immunoreactive bands were visualized by chemiluminescence (ECL system of Amersham). All blots were controlled for equal loading. The procedure allows the appearance of phosphorylated form of $cPLA_2$. Slight variations between different experiments in a total of three were observed.

rylation signal in the gel blots. Interestingly, PKC inhibitor, GF109203X, even at 5 μM concentration, was particularly effective in almost completely inhibiting constitutive as well as stimulated $cPLA_2$ phosphorylation. This result agrees with data reported in Fig. 5; staurosporine, another PKC inhibitor, attenuated the activation of both control and Aβ-stimulated PLA_2 activities. PKC is involved in the regulation of intracellular PLA_2 in several cell types, and the strong effect of its inhibitor that we observed may be due to the pivotal function of the family of PKC isoforms in the control by basal phosphorylation of many target regulatory proteins, also including phospholipase A_2, throughout cell compartments, in both early (contraction and metabolism, for instance) and late (differentiation, proliferation and apoptosis) cellular events. These findings, therefore, demonstrate that PKC and MAPK pathways may drive and control the Aβ-stimulated phosphorylation of $cPLA_2$.

4. Discussion

Data in literature are accumulating on Aβ toxicity to cerebral vascular endothelium and pericytes, besides the toxic effect on neuronal cells. With this in mind, in a model system of cultured bovine retina pericytes, one of the constituent cells of the blood–brain barrier, the objective of our study was to examine the capacity or inability of Aβ peptides to induce cell damage and the biochemical and signaling cascade events possibly related to a pre-apoptotic cellular paradigm of short-term protection from amyloidosis.

Having established in the present study that (i) MTT test was positive for cell viability, even if pericyte cultures showed a mild, but significant, oxidative damage; (ii) caspase-3 activity was unchanged after stimulation with both Aβ peptides; (iii) PARP cleavage was not accelerated under similar conditions, we confirmed (see Ref. [19]) that Aβ peptides, at concentration up to 50 μM, did not induce massive cell death by apoptosis in resting pericytes stimulated for 24 h. This last model constitutes a good paradigm for the study of sublethal oxidative injury to pericytes which have still a defense reservoir to eliminate Aβ toxic effect and may maintain significantly undamaged redox activity.

We also measured Bax protein level by Western blot analysis. Bax significantly increased in pericytes after 24-h treatment with 50 μM Aβ(25–35) peptide (Fig. 4), apparently suggesting that cells are initiated in the programmed death. Several studies support the concept that proapoptotic Bax, when overexpressed in the cell in the early phase of death, translocates to mitochondria and triggers the cytochrome *c*–caspase-3 pathway leading to programmed cell death. However, in pericytes exposed to the same experimental conditions and showing no significant loss of cell viability (Fig. 1) and cell detachment from gelatine substrate, neither decrease in caspase-3 activity nor PARP fragmentation was found. Thus, we hypothesized that our stress model associated with the Aβ action may represent a pre-apoptotic paradigm of cell injury which interestingly may have clinical relevance. In fact, in various cellular systems some pathological conditions have been associated with pre-apoptotic cellular status. For instance, it was seen that in primary cultured rat hippocampus neurons, Aβ peptides reduce neuronal Cl^--ATPase activity, probably because they may induce a pre-apoptotic status [31]. Furthermore, in pericytes from human diabetic retinas, overexpression of a member of interleukin-1β converting enzyme (ICE) family, CPP32, a death protease, decreased levels of glutathione reductase and Cu,Zn-SOD, indicating that the cells are in a "pre-programmed death" state [32]. In addition, human diabetes selectively alters the expression of Bax in the retina and retinal vascular pericytes of postmortem donors at the same time as it causes increased rates of apoptosis [33]. In the same study, bovine retinal pericytes, exposed in vitro to high glucose levels for 5 weeks, showed elevated levels of Bax and increased frequency of annexin V binding, indicative of early apoptosis. Finally, irradiated aortic endothelial cells showed a time-dependent sequence of cellular modifications supported by an early increased level of Bax, 6 h after radiation, with no evidence for an apoptotic process which, instead, started later, at 12–16 h. At this time after radiation, activation of caspases and PARP/DNA fragmentation were seen [34].

Our study was based on the premise that Aβ(1–42) and (1–40) may be transferred in vivo from the blood (nM concentrations) to the retinal and nervous tissues [35,36], and that endothelial and pericytal disfunctions are associated with vascular degenerative diseases. In addition, it is taken for granted that amyloid-β precursor protein (APP)-mRNA

is normally expressed in brain pericytes where it can provide an important contribution of locally derived Aβ (secretory pool) to cerebrovascular amyloidosis [37]. The low blood concentration of monomeric Aβ is in dynamic equilibrium with that in brain and cerebrospinal fluid, and the formation of plaques in the neuropil or amyloid deposits in the vessels is presumably due (vascular theory of AD) to an extracellular increase in Aβ (excessive sequestration in brain parenchyma and/or perivascular matrix following transport through the blood brain barrier) by as much as 1000-fold (μM concentration). Therefore, the focal concentration around the neurons, astrocytes, endothelial cells and pericytes, which determines the toxicity and the formation of precipitated fibrillary deposits, is not apparently depending on that of blood. In addition, soluble Aβ peptides may be locally secreted by a variety of cells (cell theory of AD) in CNS and exported to the blood where they are associated with lipoproteins and diluted to nanomolar concentration. Whether this route of amyloid deposit has a pathogenic role remains a controversial issue.

Thomas et al. [38] demonstrated that exogenous amyloid-β interacts with ECs on blood vessels to produce an excess of superoxide radicals, with attendant alterations in endothelial structure and function. Endothelial cell disfunction via oxidative injury is also induced in response to intracellular overexpression of APP [39]. ECs contain both APP and Aβ, and may contribute to the perivascular amyloid deposition seen in AD. The receptor for advanced glycation end products (RAGE), a signal transduction receptor for β-sheet fibril, is one of many target receptors of Aβ on neurons, microglia and vascular cells [40]. Oxidative properties of amyloid-β are redox-active metal-enhanced (Cu^{2+} and Fe^{2+} primarily) and oxygen-dependent [37,41,42], but pathways involved in Aβ-mediated oxidative injury, proof of which we have given previously [19], are only partially understood.

In the present study, we explored the possible involvement of $iPLA_2$ in stimulus-induced AA liberation using Aβ-peptide stimulated bovine retina pericytes, which weakly express, in resting conditions, this enzyme activity. In response to stimuli, $iPLA_2$ activity increases and it is expressed, participating in AA liberation. Of considerable interest are our preliminary observations of increased expression (over the undetectable steady-state level) of $iPLA_2$ mRNA after Aβ peptide stimulation of pericytes for 24 h (data not shown).

The stimulated group VI $iPLA_2$ activity has been shown to be inhibited by BEL, and under the conditions where BEL actually suppressed $iPLA_2$ activity and AA liberation, $cPLA_2$ activity was not suppressed (Figs. 6 and 7), suggesting the involvement of group IV $cPLA_2$ in the response to Aβ-peptide. The up-regulation of $iPLA_2$ and phosphorylation of $cPLA_2$ after cell stimulation suggested that these PLA_2 isotypes may be associated with the enhanced AA release. Both Aβ(1–42) and Aβ(25–35) significantly activated $cPLA_2$ and $iPLA_2$. By using specific inhibitors, our data suggest that activation process was blocked by inhibiting synthesis, phosphorylation or the active site of the acylhydrolytic enzymes. The marked decrease in $cPLA_2$ activity observed with cycloheximide indicates a rapid turnover of this enzyme during 24 h. On the other hand, the suppression of $cPLA_2$ activity found with staurosporine, reaching levels of activity of unstimulated cells, suggests that a full phosphorylation process of 85-kDa PLA_2, as expected [43–47], takes place during activation of the enzyme activity by Aβ(1–42) and Aβ(25–35) peptides. In view of the important interactions of the MAP kinase cascade, our findings obtained with the MEK inhibitor PD98059 as well as with SB203580, which inhibits p38 MAP kinase activity, suggest the implication of both kinases in signaling pathway leading to AA release, although characterization of the actual kinase(s) involved in the $cPLA_2$ phosphorylation and $iPLA_2$ activation, probably through a PKC-dependent mechanism, whether sequential or differential, requires additional studies. It has been previously suggested that PKC activation plays a role in PLA_2 stimulation in several cells types [25,45,48].

In agreement with our observations are data showing that, in human neuroblastoma LA-N-2 cells, short-time exposure (up to 60 min) to freshly prepared or "aged" toxic, short amyloid peptide Aβ(25–25) stimulated an undefined phospholipase A, and phospholipase C and D [49].

Our data underline that, after Aβ stimulation, PLA_2 in 100,000×*g* pellet, representing the membrane fraction, was considerably higher than that measured in the supernatant. Since $iPLA_2$ activity is mainly located in the membranes (Fig. 10), the decrease in soluble hydrolytic enzyme activity is at least partially due to a shift of $cPLA_2$ activity from soluble to insoluble fraction of cell lysates. This concept was reinforced by immunoblot analysis of subcellular fractions from control cells and Aβ(1–42)- and Aβ(25–35)-treated cells shown in Fig. 3. There is a decrease in $cPLA_2$ protein in the cytosolic fraction of Aβ peptide-treated cells compared to control or Aβ(35–25)-treated cells. In contrast, there is an increase in total $cPLA_2$ mass in the insoluble fraction from Aβ peptide-treated cells compared to control cells. This result is not surprising because it is well known that $cPLA_2$ translocates to nuclear membrane upon activation of other cells by some agonists [47,50–54].

To further determine whether $cPLA_2$ translocated to endoplasmic reticulum, perinuclear membrane or nuclei of Aβ peptide-treated cells, pericytes were fixed, immunostained with $cPLA_2$ antibody and observed by confocal microscope. Whereas $cPLA_2$ was found predominantly in the cytosol of control or Aβ(35–25)-treated cells, in our experiments the enzyme was massively present in the nuclei after stimulation with Aβ(1–42) and Aβ(25–35) peptides. Neither significant enzyme enrichment in plasma membrane nor evident staining of the endoplasmic reticulum was observed in response to the long-duration Aβ peptide treatment (24 h). These findings support the

view that cells, in response to increasing intracellular calcium, utilize translocation as a regulatory mechanism of phospholipase A_2 by bringing the enzyme to its natural substrate, nuclear membrane phospholipids [55]. Moreover, activation of $cPLA_2$ protein by direct interaction of intracellularly transferred amyloid-β peptide with the cytosolic enzyme seems unlikely because the nuclear or perinuclear translocation phenomenon mimics more an oxidative stress-mediated event. Indeed, of the several suggested mechanisms (perturbations in membrane fluidity or formation of ion channels) by which Aβ–membrane interactions result in cytotoxicity, free-radical production and lipid–protein peroxidation remains, in our opinion, the most plausible [19].

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