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Expression of amyloid beta peptide in human platelets: Pivotal role of the phospholipase Cγ2-protein kinase C pathway in platelet activation

Ming-Yi Shen [a], George Hsiao [b], Tsorng-Han Fong [c], Duen-Suey Chou [b], Joen-Rong Sheu [a,b,*]

[a] *Graduate Institute of Medical Sciences, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan*
[b] *Graduate Institute of Pharmacology, Taipei Medical University, Taipei, Taiwan*
[c] *Department of Anatomy, College of Medicine, Taipei Medical University, Taipei, Taiwan*

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Abstract

The amyloid β peptide (Aβ), a mediator of neuronal and vascular degeneration in the pathogenesis of Alzheimer's disease and cerebral amyloid angiopathy may have peripheral actions. Platelets are enriched with Aβ and have been shown to enhance platelet actions. However, the detailed signaling pathways through which Aβ activates platelets have not been previously explored. In this study, we examined the intra-platelet Aβ distribution using a gold labeling technique and noted that Aβ was predominantly localized in the cytoplasm of resting platelets. A marked increase in Aβ-gold labeling in an open canalicular system was observed in collagen-activated platelets. Exogenous Aβ (2–10 μM) stimulated platelet aggregation accompanied by phospholipase Cγ2 (PLCγ2) phosphorylation, phosphoinositide breakdown, and $[Ca^{2+}]i$ mobilization as well as protein kinase C (PKC) activation. Ro318220, an inhibitor of PKC, suppressed Aβ-induced platelet aggregation, PKC activation, and $[Ca^{2+}]i$ mobilization in platelets, suggesting that the PLCγ2-PKC pathway is involved in Aβ-induced platelet aggregation. In the electron spin resonance study, Aβ (2 and 10 μM) markedly triggered hydroxyl radical formation in platelets. In an *in vivo* study, Aβ (2 mg/kg) significantly shortened the latency for inducing platelet plug formation in the mesenteric venules of mice. In conclusion, we are the first to demonstrate (1) the distribution of Aβ in human platelets; and that (2) Aβ activation of platelets is mediated, at least partially, by the PLCγ2-PKC pathway; and (3) Aβ triggers thrombus formation *in vivo*.

Keywords: Amyloid β; Platelet aggregation; Phospholipase C; Protein kinase C; Free radicals

1. Introduction

Amyloid β peptide (Aβ) is cytotoxic to neurons [1], cerebral endothelial cells, and vascular smooth muscle cells, and has been implicated in the pathogenesis of Alzheimer's disease and cerebral amyloid angiopathy (CAA) [2]. Aβ is derived from proteolytic cleavage of the amyloid β precursor protein (APP) of 695–770 amino acids. Aβ contains 39–43 amino acid residues with a molecular weight of ~4 kDa. The major Aβ species found in vascular plaques is $Aβ_{1–40}$, which ends at Val_{40}, while the dominant species in neuronal plaques is $Aβ_{1–42}$, which has two additional hydrophobic residues, Ile and Ala. As a result, $Aβ_{1–42}$ is more hydrophobic and has a higher aggregation potential (and thus is more amyloidogenic) [3].

The cellular sources of Aβ deposited in neuronal or vascular plaques remain to be delineated. Soluble Aβ probably originates from cells within the central nervous system as well as the peripheral circulation [4]. Indeed, human platelets contain high levels of APP, which may contribute to more than 90% of the circulating APP [5]. Platelet APP may also be the major source of Aβ detected in whole blood [6]. Aβ is also released upon platelet activation [7]. The main species of Aβ released from activated human platelets is $Aβ_{1–40}$, consistent with the contention that circulating Aβ contributes to vascular amyloid deposits dominated by $Aβ_{1–40}$ [8]. It is possible that blood Aβ may contribute to amyloid deposits in the cerebral vasculature leading to CAA. However, the physio-

* Corresponding author at: Graduate Institute of Medical Sciences, Taipei Medical University, 250 Wu-Hsing Street, Taipei 110, Taiwan.
Tel.: +886 2 27361661x3199; fax: +886 2 27390450.
E-mail address: sheujr@tmu.edu.tw (J.-R. Sheu).

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logical and pathophysiological roles of circulating Aβ are not clear.

Aβ secreted upon platelet activation may have a physiological role in normal platelet functions. Aβ subcellular localization within platelets is still unknown. Therefore, we performed immunogold labeling of Aβ on London resin (LR) white-treated sections of both resting and activated platelets. In addition, it was found that Aβ induces platelet aggregation [9]. In the present study, we further explored in greater detail the pathways through which Aβ activates platelets in both *in vitro* and *in vivo* studies.

2. Materials and methods

2.1. Materials

Aβ ($A\beta_{25–35}$ and $A\beta_{1–40}$), collagen (type I, bovine Achilles tendon), fluorescein sodium, Dowex-1 (100–200 mesh; X_8, chloride form), myoinositol, prostaglandin E_1 (PGE_1), phorbol-12, 13-dibutyrate (PDBu), apyrase, heparin, osmium tetroxide, gold-conjugated anti-mouse immunoglobulin G (IgG), phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, NaF, sodium orthovanadate, sodium pyrophosphate, anti-Aβ monoclonal antibody (mAb), 5,5-dimethyl-1 pyrroline N-oxide (DMPO), and bovine serum albumin (BSA) were purchased from Sigma Chemical (St. Louis, MO); Ro318220 was from BIAFFIN (Kassel, Germany); Fura 2-AM was from Molecular Probe (Eugene, OR); anti-phospho-(Ser/Thr) Akt substrate, anti-phospholipase Cγ2 (PLCγ2), and anti-phosphate (Tyr759) PLCγ2 mAbs were from Cell Signaling (Beverly, MA); the anti-α-tubulin mAb was from NeoMarkers (Fremont, CA); and the Hybond-P PVDF membrane, myo-2-[^{3}H] inositol, ECL Western blotting detection reagent and analysis system, horseradish peroxidase-conjugated donkey anti-rabbit IgG, and sheep anti-mouse IgG were from Amersham (Buckinghamshire, UK).

2.2. Platelet aggregation

Human platelet suspensions were prepared as previously described [10]. This study conformed to the principles outlined in the *Helsinki Declaration*, and human volunteers gave informed consent. In brief, blood was collected from healthy human volunteers who had taken no medicine during the preceding 2 weeks, and was mixed with acid/citrate/glucose (9:1, v/v). After centrifugation, the supernatant (platelet-rich plasma; PRP) was supplemented with PGE_1 (0.5 μM) and heparin (6.4 IU/ml), then incubated for 10 min and centrifuged at 500 × *g*. Washed platelets were finally suspended in Tyrode's solution containing BSA (3.5 mg/ml). The final concentration of Ca^{2+} in Tyrode's solution was 1 mM. A turbidimetric method was applied to measure platelet aggregation [10], using a Lumi-Aggregometer (Payton, Canada). Platelet suspensions (3.6×10^8 platelets/ml) were pretreated with or without Ro318220 (1 and 2 μM) for 3 min, followed by the addition of Aβ (2–10 μM) or collagen (1 μg/ml). The reaction was allowed to proceed for at least 6 min at 37 °C, and the extent of aggregation was expressed in light-transmission units (LTU).

2.3. Post-embedding gold labeling for intra-platelet Aβ

Resting and collagen (1 μg/ml)-activated human platelets were fixed with 2% paraformaldehyde and 2% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) for 30 min at 37 °C. After rinsing with cacodylate buffer, platelets were postfixed with 1% osmium tetroxide for 30 min. After dehydration in a graded series of ethanol, samples were embedded in LR white resin. Ultrathin sections were mounted on Formvar-coated nickel grids, and blocked for 20 min with 1% BSA in phosphate-buffered saline (PBS). Sections were incubated for 1 h with an anti-Aβ mAb (1:200) in PBS. After rinsing with PBS, sections were incubated for 1 h with gold-conjugated anti-mouse IgG (10 nm in size, 1:20) in PBS. Sections were examined with a Hitachi H-600 electron microscope (Hitachi, Tokyo, Japan).

2.4. Determination of lactate dehydrogenase (LDH)

In brief, washed human platelets (3.6×10^8 ml^{-1}) were preincubated with Aβ (0.5–100 μM) for 10 min at 37 °C. An aliquot of supernatant (10 μl) was deposited on a Fuji Dri-Chem slide LDH-PIII (Fuji, Tokyo, Japan), the absorbance wavelength was read at 540 nm using an ultraviolet-visible recording spectrophotometer (UV-160; Shimazu, Japan). A maximal value (MAX) of LDH was observed from sonicated platelets.

2.5. Labeling of membrane phospholipids and measurement of the production of [^{3}H]-inositol phosphates

The method was carried out as previously described [11]. Briefly, citrated PRP was centrifuged, and the pellets were suspended in Tyrode's solution containing [^{3}H]-inositol (75 μCi/ml). Platelets were incubated for 2 h at 37 °C followed by centrifugation, and were finally re-suspended in Ca^{2+}-free Tyrode's solution (5×10^8 ml^{-1}). Aβ (2 and 10 μM) and collagen (1 μg/ml) were added to trigger platelet activation. The reaction was stopped, and samples were centrifuged for 4 min. Inositol phosphates in the supernatant were separated on a Dowex-1 column (Sigma). Only [^{3}H]-inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

2.6. Measurement of platelet $[Ca^{2+}]_i$ mobilization by Fura 2-AM fluorescence

Citrated whole blood was centrifuged at 120 × *g* for 10 min to collect PRP, which was then incubated with Fura 2-AM (5 μM) for 1 h at 37 °C. Fura 2-AM-loaded platelets were isolated and then suspended in Tyrode's solution as described above with the Ca^{2+} concentration adjusted to 1 mM. $[Ca^{2+}]_i$ was monitored using a fluorescence spectrophotometer (CAF 110, Jasco, Tokyo, Japan) at excitation wavelengths of 340 and 380 nm and an emission wavelength of 500 nm.

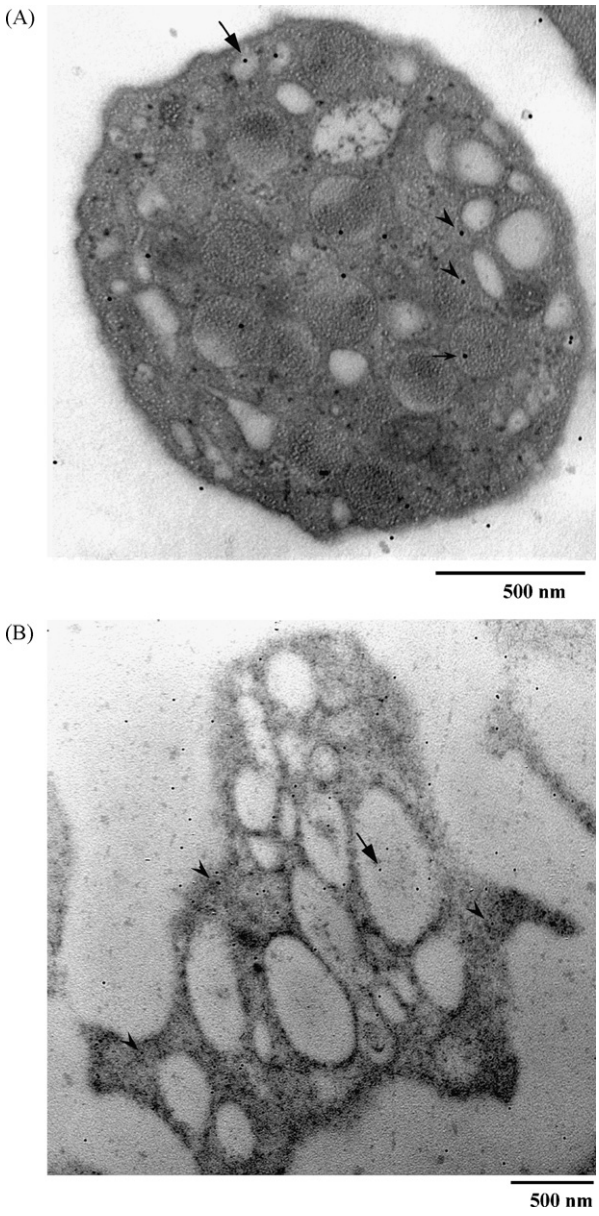

Fig. 1. Immunogold labeling of amyloid β (Aβ) on LR white-sections in resting and collagen-activated human platelets. (A) Resting and (B) collagen (1 μg/ml)-activated platelets were fixed followed by the addition of an anti-Aβ monoclonal antibody. The illustrated sections show gold particles in the cytoplasm (arrowheads), in the open canalicular system (OCS) (large arrow), and in α-granules (small arrow) in both resting and activated platelets. The figures are representative examples of four similar experiments.

2.7. Quantitative immunoblotting

Washed platelets (1.2×10^9 ml^{-1}) were stimulated with Aβ or other activating agents for 6 min at 37 °C, the reaction was stopped by the addition of EDTA (10 mM), and the suspensions were centrifuged at 3000 × *g* for 5 min and then immediately re-suspended in 200 ml of lysis buffer as described previously [12]. Collected lysates were centrifuged at 10,000 × *g* for 5 min, and the supernatant was treated with β-mercaptoethanol (5%) and bromophenol blue (0.1%). Samples containing 80 μg of protein were separated by SDS-PAGE (12%); the proteins were electrotransferred onto a Hybond-P PVDF membrane by a semidry transfer (Bio-Rad, Hercules, CA). Blots were blocked with TBST containing 5% BSA for 1 h and then probed with primary antibodies (diluted 1:1000 in TBST) specific for anti-phosphate (Ser/Thr) Akt substrates, anti-PLCγ2, and anti-phosphate (Tyr759) PLCγ2 for 2 h. Membranes were washed and then incubated with horseradish peroxidase-linked anti-mouse IgG or anti-rabbit IgG (diluted 1:3000 in TBST) for 1 h. The chemiluminescence of the immunoreactive bands was detected using the ECL-enhanced chemiluminescence system. The bar graph depicts the ratios of quantitative results obtained by scanning reactive bands and quantifying the optical density using videodensitometry (Bio-1D version 99 image software).

2.8. Measurement of free radicals by electron spin resonance spectrometry

The electron spin resonance (ESR) method used a Bruker EMX ESR spectrometer as described previously [13]. In brief, platelet suspensions (3.6×10^8 ml^{-1}) were preincubated with Tyrode's solution or Ro318220 (1 μM) for 3 min at 37 °C before the addition of Aβ (2 and 10 μM) or collagen (1 μg/ml). The reaction was allowed to proceed for 5 min, followed by the addition of DMPO (100 μM) for the ESR study.

2.9. Fluorescein sodium-induced platelet thrombi in mesenteric microvessels of mice

As described previously [10], mice were anesthetized, and an external jugular vein was cannulated with PE-10 to administer the dye and drugs. A segment of the small intestine was placed onto a transparent culture dish for microscopic observation. Venules (30–40 μm) were selected for irradiation to produce a microthrombus. Filtered light for which wavelengths below 520 nm had been eliminated was used to irradiate a microvessel. Various doses of Aβ (0.5 and 2 mg/kg) or isovolumetric Tyrode's solution (control) were administered 1 min after fluorescein sodium (15 μg/kg) had been given. The time lapse for inducing thrombus formation leading to cessation of blood flow was measured.

2.10. Statistical analysis

The experimental results are expressed as the mean ± S.E.M. with the number of experiments from different subjects. Paired Student's *t*-test was used to determine significant differences in the *in vivo* studies of platelet plug formation. Results from other experiments were assessed by the method of analysis of variance (ANOVA). If a significant difference among the group means was noted, the difference between two groups was assessed using the Newman–Keuls method. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Distribution of Aβ in resting and activated platelets

Platelets are anuclear cells, which are incapable of proliferating. Immunogold labeling of resting platelets on LR white-sections revealed that Aβ was mainly localized in the cytosol (Fig. 1A), with only a trace found in α-granules and the open canalicular system (OCS). To determine whether platelet activation might cause the redistribution of Aβ, we processed collagen-activated platelets in the same manner. Platelet activation resulted in heavy Aβ localization in the OCS. Aβ secretion was also apparent as evidenced by an increase in extracellular Aβ labeling (Fig. 1B).

3.2. Effect of Aβ on platelet aggregation and lactate dehydrogenase in human platelets

In our preliminary studies, $A\beta_{1–40}$ and $A\beta_{25–35}$ peptides showed equal potencies in inducing platelet activation (data not shown). Subsequent studies were then conducted using $A\beta_{25–35}$ to explore the mechanisms of platelet activation. Aβ (2–10 μM) triggered platelet aggregation like collagen (1 μg/ml) in both washed human platelets (Fig. 2A) and PRP (data not shown). Pretreatment with Ro318220, a PKC inhibitor [14], concentration-dependently (1 and 2 μM) inhibited platelet aggregation stimulated by Aβ (10 μM) (Fig. 2A).

The LDH study revealed that Aβ (0.5–20 μM) incubated with human platelets for 10 min did not significantly increase LDH activity except at higher concentration (100 μM) (resting platelets, 46.4 ± 8.6 U/l vs. Aβ (100 μM)-treated platelets, 137.1 ± 22.4 U/l; $p < 0.01$, $n = 8$) (new Fig. 2B). This result indicates that Aβ did not affect platelet permeability or induce platelet cytolysis under this range of concentrations (0.5–20 μM), clearly disproving the cytotoxic effect of Aβ on platelets in this study.

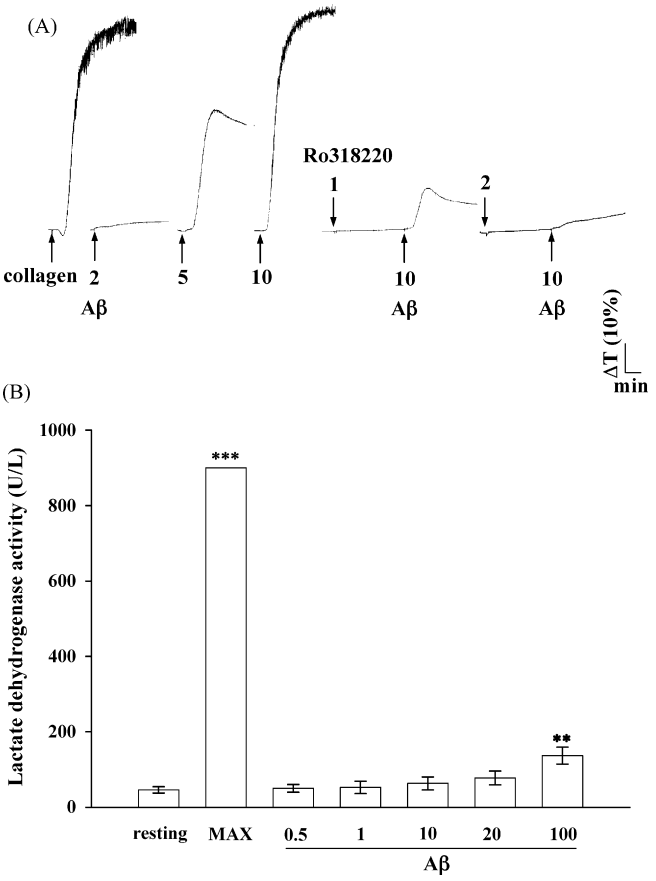

Fig. 2. Effects of amyloid β (Aβ) stimulation of platelet aggregation and lactate dehydrogenase release in human platelets. (A) Platelets (3.6×10^8 ml^{-1}) were incubated with Aβ (2–10 μM) or collagen (1 μg/ml) with or without Ro318220 (1 and 2 μM) to trigger platelet aggregation. Aggregation profiles are representative examples of four similar experiments. For the lactate dehydrogenase experiment, platelets were incubated with Aβ (0.5–100 μM) for 10 min, and a 10-μl aliquot of the supernatant was deposited on a Fuji Dri-Chem slide LDH-PIII as described in Section 2. Data are presented as the mean ± S.E.M. ($n = 4$). $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with the resting group.

3.3. Effects of Aβ on phospholipase Cγ2 (PLC γ2) and phosphoinositide breakdown in washed platelets

PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate two secondary messengers: inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) [15]. Phosphorylation is one of the key mechanisms that regulate the activity of PLC. The immunoblotting analysis revealed that treatment with collagen (1 μg/ml) or Aβ (2 and 10 μM) resulted in marked phosphorylation of PLCγ2 compared with resting platelets (Fig. 3A). In addition, phosphoinositide breakdown occurs in platelets activated by many different agonists. In this study, collagen (1 μg/ml) or Aβ (10 μM) induced rapid formation of radioactive IP, IP_2, and IP_3 in human platelets loaded with [^{3}H]-inositol (Fig. 3B). We only measured [^{3}H]-IP formation as an index of total inositol phosphate formation. As shown in Fig. 3B, the addition of collagen (1 μg/ml) and Aβ (2 and 10 μM) resulted in rises in IP formation of about 1.4-, 1.3-, and 2.1-fold, respectively, compared to that in resting platelets.

3.4. Effects of Aβ on PKC activation and [Ca^{2+}]i mobilization

Stimulation of platelets with a number of different agonists, PDBu in particular, induces activation of PKC, which then phosphorylates proteins of 40–47 kDa. In this study, phosphorylation experiments were performed to examine the role of Aβ in PKC activation in human platelets. When collagen (1 μg/ml), PDBu (0.06 μM), or Aβ (2 and 10 μM) was added to human platelets, a protein with an apparent size of 47 kDa (p47) was predominately phosphorylated compared with resting platelets (Fig. 4A). Ro318220 (1 μM) markedly inhibited this phosphorylation stimulated by Aβ (10 μM). As shown in Fig. 4B, Aβ (2, 5, and 10 μM) concentration-dependently evoked an increase in [Ca^{2+}]i, and this increase was markedly inhibited in the presence of Ro318220 (1 μM).

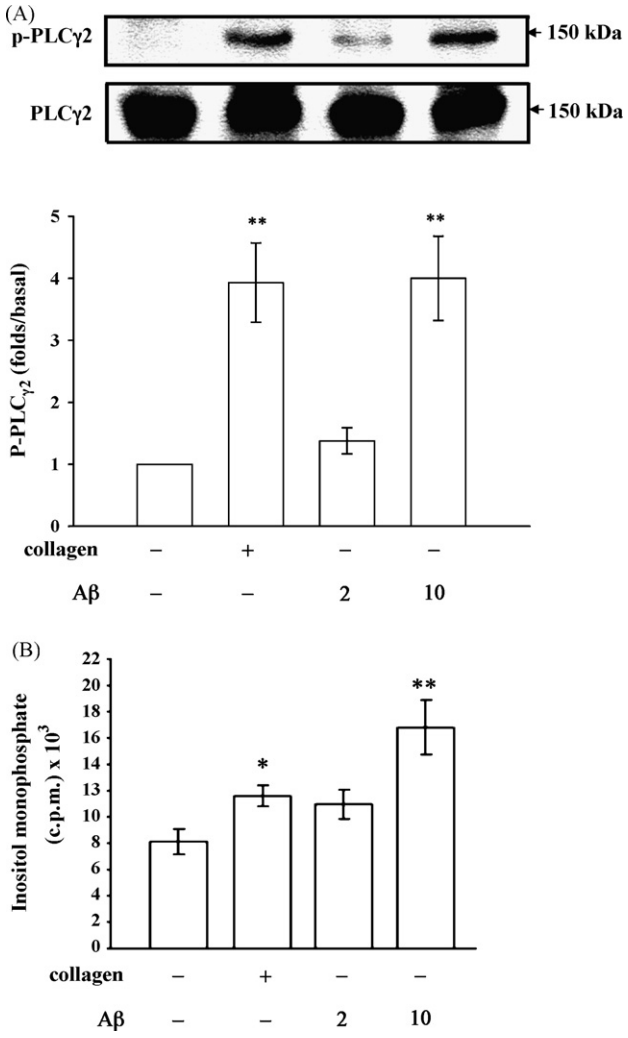

Fig. 3. Effects of (A) phospholipase Cγ2 phosphorylation and (B) phosphoinositide breakdown stimulated by amyloid β (Aβ) in human platelets. Platelets were incubated with Tyrode's solution (control), Aβ (2 and 10 μM), or collagen (1 μg/ml) to trigger platelet activation as described in Section 2. Profiles (A) are representative examples of four similar experiments. The bar graph (A) depicts the ratios of quantitative results obtained by scanning the reactive bands of anti-p-PLCγ2 against total PLCγ2 and quantifying the optical density using Bio-1D version 99 image software. Data are presented as the mean ± S.E.M. ($n = 4$). $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with the control group.

3.5. Effect of Aβ on hydroxyl radical formation in human platelets

In this study, a typical ESR signal of the hydroxyl radical ($OH^{\bullet}$) was induced by collagen (1 μg/ml) in platelets compared with resting platelets (Fig. 5A and B). Aβ (2 and 10 μM) also significantly triggered increases in hydroxyl radical formation by about 1.7- and 2.4-fold compared with resting platelets (Fig. 5C and D), respectively. Pretreatment with Ro318220 (1 μM) markedly suppressed Aβ (10 μM)-induced hydroxyl radical formation by about 63.9% (Fig. 5E). The antioxidant, catalase (1000 U/ml), markedly suppressed hydroxyl radical formation by about 76% (data not shown).

3.6. Effect of Aβ on thrombus formation in microvessels of fluorescein sodium-pretreated mice

Thrombus formation in microvessels developed in approximately 202 s following fluorescein sodium (15 μg/kg) treatment in mice. Aβ (0.5 mg/kg) failed to significantly alter the time course of thrombus formation induced by fluorescein sodium (fluorescein sodium + Tyrode's solution: 202.7 ± 9.4 s; fluorescein sodium + Aβ: 188.5 ± 6.7 s, $p > 0.05$, $n = 10$) (Fig. 6A). At a higher dose (2 mg/kg), Aβ markedly shortened the time required for fluorescein sodium to induce thrombus formation (fluorescein sodium + Tyrode's solution: 207.8 ± 15.3 s; fluorescein sodium + Aβ: 138.7 ± 9.4 s, $p < 0.01$, $n = 10$) (Fig. 6A). The typical microscopic image of a microthrombus formed

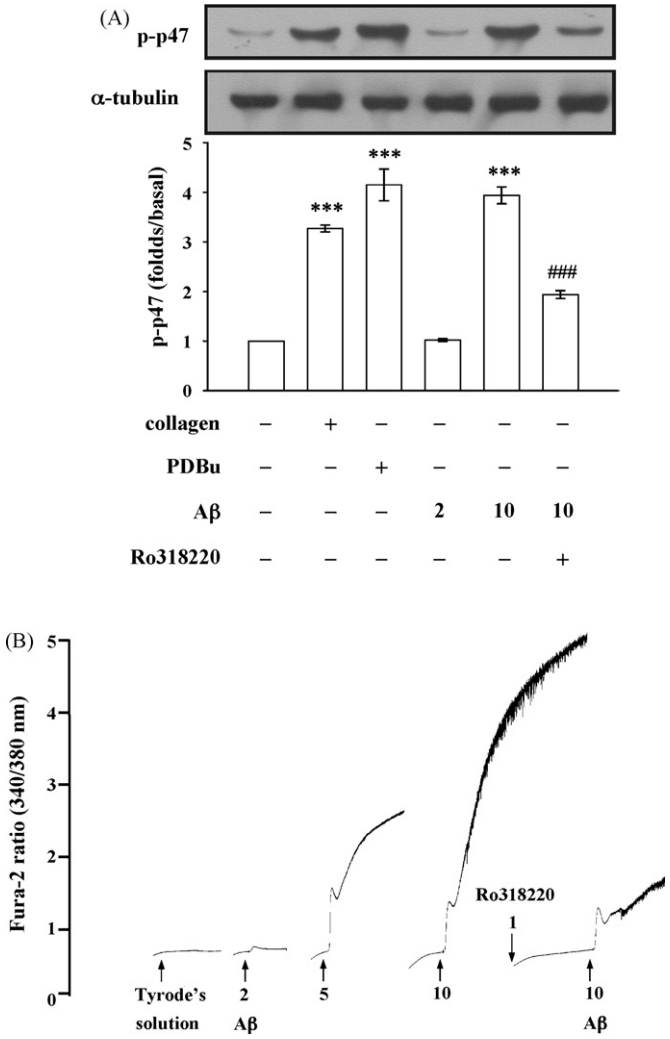

Fig. 4. Effects of (A) PKC activation and (B) $[Ca^{2+}]i$ mobilization stimulated by amyloid β (Aβ) in human platelets. Platelets were incubated with Aβ (2–10 μM), collagen (1 μg/ml), PDBu (0.06 μM), or Tyrode's solution (control) to trigger platelet activation as described in Section 2. Profiles (A and B) are representative examples of four similar experiments. The bar graph (A) depicts the ratios of quantitative results obtained by scanning the reactive bands of anti-p-p47 against anti-α-tubulin and quantifying the optical density using Bio-1D version 99 image software. Data are presented as the mean ± S.E.M. ($n = 4$). $^{***}p < 0.001$ compared with the control group; $^{###}p < 0.001$ compared with the Aβ-treated group.

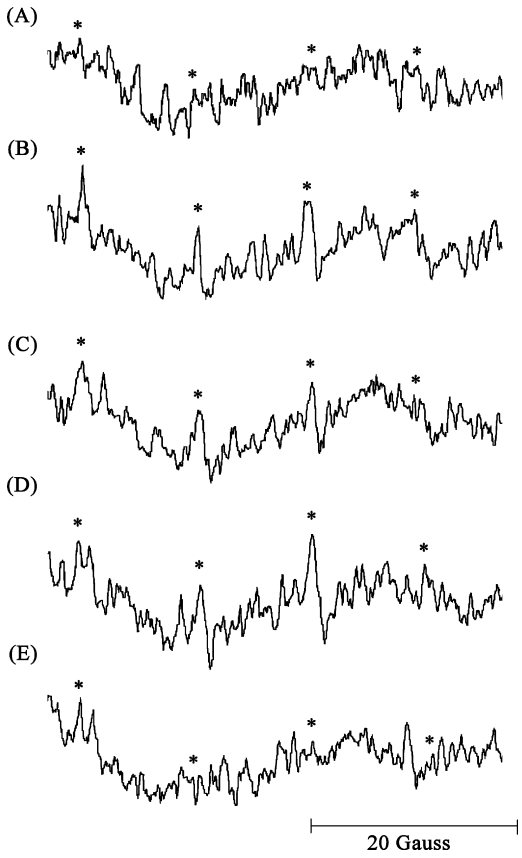

Fig. 5. Electron spin resonance (ESR) spectra of amyloid β (Aβ) in triggering hydroxyl radical formation in platelets. Platelet suspensions were incubated with (A) Tyrode's solution, (B) collagen (1 μg/ml), (C) Aβ (2 μM), and (D) Aβ (10 μM), or (E) Aβ (10 μM) in the presence of Ro318220 (1 μM). The reaction was allowed to proceed for 5 min, followed by the addition of DMPO (100 mM) for the ESR experiments. Spectra are representative examples of four similar experiments.

with fluorescein sodium treatment is shown in Fig. 6B. The thrombotic platelet plug was not observed with irradiation of mesenteric microvessels for either 5 or 134 s after fluorescein sodium treatment in Tyrode's solution-treated mice (control) (Fig. 6Ba, b). With administration of Aβ (2 mg/kg), platelet plug formation was evident at 134 s but not at 5 s after irradiation (Fig. 6Bc, d). The blood flow rate of the Aβ-treated venule was slower than that of the Tyrode's solution-treated venule, because the platelet plug had become apparent at 134 s (Fig. 6Bd).

4. Discussion

Results obtained from the present study demonstrate for the first time the localization of Aβ in both resting and activated human platelets. Using TEM, the presence of Aβ immunoreactivity was gold labeled in the cytoplasm and OCS in platelets. At 2–10 μM, Aβ itself directly activated aggregatory responses. It is conceivable that the Aβ concentration may be high at the site of vascular injury where platelet activation can lead to Aβ secretion into the extracellular space, creating a vicious cycle of platelet aggregation.

In this study, Aβ-induced platelet aggregation triggered Ca^{2+} release from intracellular Ca^{2+} storage sites (i.e., dense tubular systems or dense bodies), and this is in accord with the concept that $[Ca^{2+}]i$ release is responsible for platelet aggregation. Stimulation of platelets by agonists (i.e., collagen) results in PLC-catalyzed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), with concomitant formation of IP_3 and diacylglycerol [16]. There is strong evidence that IP_3 induces the release of Ca^{2+} from intracellular stores [17]. Diacylglycerol activates PKC, inducing protein phosphorylation (p47). The PKC activation represents a strategy adopted by the cell to allow selected responses to specific activating signals in distinct cellular compartment [18]. Phosphoinositide-specific phospholipase C (PLC) is a key enzyme in signal transduction [19]. There are six major families of PLC enzymes which consist of at least 13 PLC isoforms

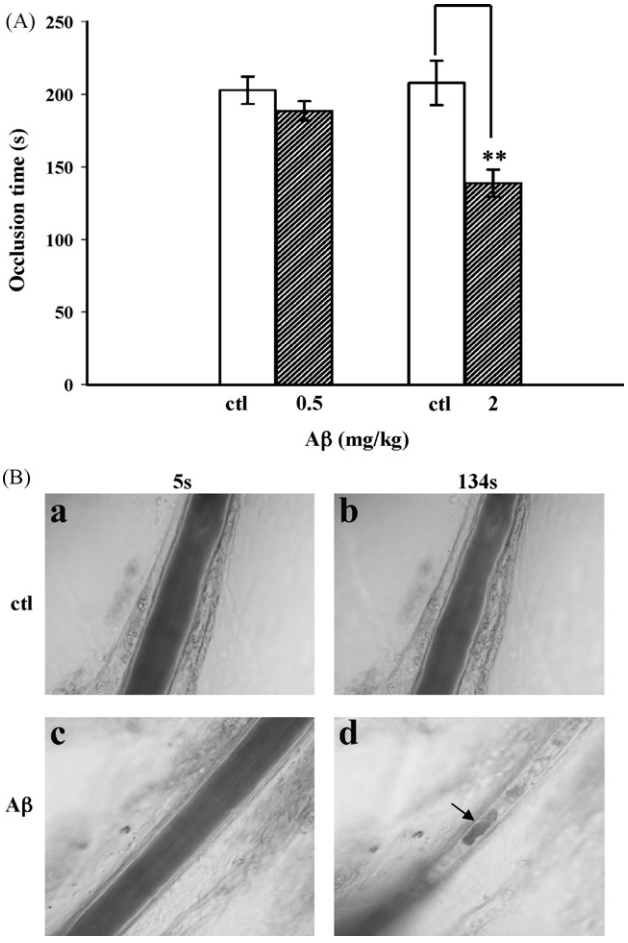

Fig. 6. Effect of amyloid β (Aβ) on shortening the occlusion times for inducing thrombus formation in mesenteric venules of mice. Mice were administered isovolumetric Tyrode's solution (ctl) or Aβ (0.5 and 2 mg/kg), and then mesenteric venules were selected for irradiation to induce microthrombus formation. The bar graphs (A) are presented as the mean ± S.E.M. of the occlusion time (s) for inducing platelet plug formation ($n = 10$). $^{**}p < 0.01$ compared with the control group. Microscopic images (B) were observed during the time courses of 5 (a and c) and 134 s (b and d) after injection of the fluorescent dye (15 μg/kg) and transillumination in Tyrode's solution-treated mice (a and b) or Aβ (2 mg/kg)-treated mice (c and d). The arrow (d) indicates platelet plug formation. Photographs are representative examples of ten similar experiments (×400).

(PLCβ(1-4), PLCγ(1 and 2), PLCδ(1, 3, and 4), PLCε(1), PLCζ(1), and PLCη(1 and 2)) [19]. PLCγ2 is involved in antigen-dependent signaling in B cells and collagen-dependent signaling in platelets [20]. In this study, PLCγ2 phosphorylation, phosphoinositide breakdown, and PKC activation were activated by Aβ, suggesting that Aβ-induced platelet aggregation involves PLCγ2 activation. Furthermore, platelet aggregation, PKC activation, and $[Ca^{2+}]i$ mobilization activated by Aβ were markedly abolished in the presence of the PKC inhibitor, Ro318220. These results further confirm that Aβ stimulation of platelet aggregation is, at least partly, mediated by the PLCγ2-PKC signal pathway.

Platelet aggregation plays a pathophysiological role in cerebrovascular disorders. We previously demonstrated that endothelial cell injury induces platelet aggregation and adhesion at the site of injury, followed by arterial thrombus formation [21]. When platelets aggregate, they release a number of substances, including free radicals (i.e., hydroxyl radicals), which can cause contraction of vascular smooth muscle cells [22]. Free radical species act as secondary messengers that increase the cytosolic Ca^{2+} during the initial phase of platelet activation processes, and PKC is involved in receptor-mediated free radical production in platelets [13,23]. It is also evident that some of the hydrogen peroxide produced by platelets is converted into hydroxyl radicals, as platelet aggregation can be inhibited by hydroxyl radical scavengers [24]. Thus, Aβ induction of platelet plug formation *in vivo* may be involved, at least in part, in the stimulation of free radical formation in platelets. Indeed, a role for Aβ in atherogenesis was indicated by studies in rat aorta which showed that Aβ caused vasoconstriction and endothelial damage via superoxide generation [25]. Hence, platelets release Aβ on activation would provide a mechanism whereby Aβ deposition in the walls of blood vessels leading to angiopathy occurs in aging and Alzheimer's disease. In the thrombotic study, the mesenteric venules were continuously irradiated by fluorescein sodium throughout the entire experimental period, thus leading to strong damage to endothelial cells as described previously [10]. Therefore, the dosage (2 mg/kg) of Aβ employed in this *in vivo* model was relatively higher than that used for the *in vitro* study (2–10 μM).

In conclusion, we demonstrate for the first time (1) the distribution of Aβ in human platelets by TEM, and that (2) Aβ activation of platelets is mediated, at least in part, by PLCγ2-PKC pathway and (3) Aβ triggers thrombus formation *in vivo*. These findings raise the possibility that Aβ activation of platelets may contribute to cerebrovascular degeneration in CAA. Understanding the underlying mechanism of Aβ activation of platelets may contribute to future development of preventive or therapeutic strategies to slow down the development of CAA.

Acknowledgements

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