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ORIGINAL ARTICLE

Fibrillar amyloid peptides promote platelet aggregation through the coordinated action of ITAM- and ROS-dependent pathways

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Abstract

Background: Amyloid peptides Aβ40 and Aβ42, whose deposition in brain correlates with Alzheimer disease, are also present in platelets and have prothrombotic activities.

Objective: In this study, we analyze the ability of Aβ peptides to form fibrils and to induce platelet activation and aggregation.

Methods: Aβ40, Aβ42, and their scrambled peptides were diluted in phosphate buffered saline and fibrillogenesis was investigated by ThioflavinT and Congo Red. Aggregation, protein phosphorylation, and reactive oxygen species (ROS) production were analyzed.

Results: Aβ40 and Aβ42, but not scrambled peptides, were able to form fibrils when diluted in phosphate buffered saline. Fibrillogenesis of Aβ42 was very rapid, whereas fibril formation by Aβ40 was completed only after 48 hours of incubation. Fibrillar Aβ40 and Aβ42 promoted dose-dependent aggregation of washed platelets in the presence of extracellular $CaCl_2$. Cleavage of GPIbα by mocarhagin or blockade of the ITAM-containing FcγRIIA prevented platelet aggregation induced by fibrillary Aβ40 and Aβ42. Fibrillar Aβ peptides stimulated the phosphorylation of FcγRIIA, resulting in the downstream stimulation of PLC, protein kinase C, and phosphoinositide 3-kinases, whose activity was necessary for full aggregation of platelets. Fibrillar Aβ peptides also induced ROS generation, and NOX inhibitors, as well as ROS scavengers, prevented platelet aggregation. However, Aβ peptide-induced ROS production did not require binding to GPIbα or activation of FcγRIIA, but was initiated by CD36, which provided an important contribution to full platelet aggregation.

Conclusion: These results suggest that fibrillar amyloid Aβ40 and Aβ42 induce platelet aggregation through the recruitment of GPIb-IX-V and CD36, which requires the convergence of ITAM- and ROS-dependent pathways.

KEYWORDS

amyloid, blood platelets, platelet aggregation, protein-tyrosine kinases, reactive oxygen species

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1 | INTRODUCTION

Amyloid β (Aβ) peptides are heterogeneous peptides produced by proteolytic cleavage of the membrane-bound amyloid precursor protein (APP). The most frequently generated Aβ peptides contain 40 or 42 amino acids (Aβ40 and Aβ42, respectively) and their accumulation in brain parenchyma is correlated with the onset of Alzheimer disease (AD).

As other amyloidogenic proteins, Aβ peptides in aqueous solution spontaneously convert their soluble state into insoluble, β-sheet rich, fibrillar aggregates, termed amyloid fibrils. Fibrillogenesis does not occur in a linear fashion but evolves from the initial transient formation of oligomers, which may then give rise to fibrils.[1] Toxicity of amyloid proteins is often ascribed to their misfolded conformation and, for instance, accumulation of fibrils of Aβ42 is considered to be directly correlated to neurodegeneration.

Aβ peptides are not exclusively generated in the brain, but they are also present in plasma. Blood platelets express high amount of APP and are considered a major source of peripheral Aβ peptides.[2] Importantly, Aβ peptides are also stored in platelet α-granules and released in the bloodstream during platelet activation.[3] Under pathological conditions, platelet-derived Aβ peptides deposit on the cerebral vessel walls causing cerebral amyloid angiopathy (CAA), a frequent comorbidity in AD.[4] Moreover, Aβ peptides have been found in atherosclerotic plaques and accumulate in the growing thrombus in pathological conditions.[5]

Aβ peptides in the circulation exacerbate activation of vascular cells because they stimulate activation of leukocytes, promoting oxidative burst[6] and the formation of platelet-leukocyte aggregates, support endothelial cell activation and the expression of inflammatory markers and adhesion molecules.[7,8] These events are eventually responsible for the generation of a chronic pro-inflammatory state.

Aβ peptides also behave as platelet agonists and promote or reinforce platelet adhesion, activation, secretion, and aggregation.[9-13] Moreover, in line with their ability to stimulate oxidative burst in neutrophils, Aβ peptides trigger the generation of reactive oxygen species (ROS) in platelets in a NOX-dependent manner.[14-16]

The mechanism of platelet activation by Aβ peptides is controversial. Some studies have indicated that Aβ peptides interact with different membrane receptors, including APP itself, PAR1, CD36, GPIb-IX-V complex, or integrin αIIbβ3, but their involvement in the initiation of platelet activation is uncertain.[13,17-19] Two major methodological limitations have hampered the development of a clear and univocal model for Aβ-induced platelet activation. Most of the studies focused on the intracellular signaling pathways have been performed using a short, 11 amino-acid-long peptide, Aβ25-35, that maintains the neurotoxic properties of genuine Aβ peptides. Because of the ability of Aβ25-35 to generate pores in the plasma membrane,[20] it is likely to initiate platelet activation by a receptor-independent mechanism. The few studies that have used full, synthetic Aβ peptides (Aβ40 or Aβ42) have generally payed insufficient attention at the chemical form of the reagents, whose properties and potency as platelets agonists may differ significantly depending on their monomeric, oligomeric, or fibrillar form. This has generated inconsistent results on the ability of Aβ peptides to promote platelet aggregation and discrepancies on the relative efficiency of Aβ40 vs Aβ42.

To overcome these limitations, in the present work, we have undertaken an accurate, precise analysis of platelet activation and aggregation using Aβ40 and Aβ42 peptides in a controlled and comparable fibrillar form. The results indicate that fibrillar Aβ40 and Aβ42 are equally potent in stimulating platelet aggregation and that they operate through a receptor-mediated mechanism that integrates ITAM-dependent activation of tyrosine kinases and CD36-dependent ROS generation.

Essentials

- Aβ40 and Aβ42 form fibrils when diluted in PBS for 48 hours.
- Fibrillar Aβ40 and Aβ42 promote platelet aggregation via GPIb-FcγRIIA-induced-tyrosine kinases pathway.
- Fibrillar Aβ40 and Aβ42 activate CD36 and promote ROS formation.
- Tyrosine kinases and ROS pathway converge to promote full platelet activation.

2 | MATERIALS AND METHODS

2.1 | Materials

Synthetic amyloid Aβ40, Aβ42, and the respective scrambled peptides was purchased from Lifetein. The sequences of the peptides are as follows:

Aβ25-35	(GSNKGAIIGLM)
Aβ40 [Aβ1-40]	(DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV)
Aβ42 [Aβ1-42]	(DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA)
Aβ40Sc [Aβ1-40 scrambled]	(KVKGLIDGAHIGDLVYEFMASNSFREGVGAGHVHVAQVEF)
Aβ42Sc [Aβ1-42 scrambled]	(KVKGLIDGAHIGDLVYEFMDSNSAIFREGVGAGHVHVAQVEF)

PP2, PP3, U73122, U73343, Ro-318220, wortmannin, and the redox-sensitive fluorescent dye H_2DCFDA were from Calbiochem. ApyraseVII, prostaglandin E_1, ThioflavinT, Congo Red, NAC, VAS 2870, protein A-sepharose, BAPTA-AM, losartan, and sulfo-N-succinimidyl oleato (SSO) were from Sigma Aldrich. Crude venom of the snake *Mocambique mocambique* was from Latoxan (Valence, France) and mocarhagin was purified as previously described.[21] The monoclonal antibody (mAb) IV.3 against FcγRIIA was obtained from Medarex.

Anti-phosphotyrosine antibody (4G10) was purchased from Millipore. Anti-CD32 (C17) and anti-PLCγ2 (Q20) were from Santa Cruz Biotechnology; anti-PKC phosphosubstrates, anti P-Akt (S473), anti-P-GSK3 α/β (S21/9) were from Cell Signaling Technology. Anti-pleckstrin was from Abcam.

2.2 | Preparation and analysis of fibrillation of Aβ40, Aβ42, and scrambled peptides

Synthetic amyloid Aβ40, Aβ42, and respective scrambled peptides were purchased as lyophilized powder, dissolved in 5 mmol/L sterile dimethylsulfoxide (DMSO) and stored at −20°C until use.

Peptide fibrillation was initiated by diluting amyloid peptides in phosphate buffered saline (PBS, pH 7.4) at a final concentration of 0.25 mmol/L at 37°C; fibrillation was monitored using Thioflavin T (ThT) and Congo Red staining.

A total of 100 μL amyloid peptides (0.25 mmol/L in PBS, pH 7.4) containing 10 μmol/L ThT was incubated at 37°C in Costar 96-well black-wall plates sealed with clear sealing and subjected to 600 rpm double orbital shaking. Bottom fluorescence was recorded for 72 hours (BMG LABTECH FLUOstar Omega). Aliquots of the final ThT positive material were stained with alkaline alcoholic Congo Red and viewed under high-intensity cross-polarized light.[22] The concentration of fibrillar Aβ peptides was calculated based on the initial concentration of monomeric peptides, assuming a complete conversion into fibrils when ThT fluorescence reached a plateau.

2.3 | Human platelet preparation

Washed human platelets were prepared from buffy-coat bags collected the same day of the experiment, a previously described.[23] Briefly, the buffy-coat was diluted with one-fourth of its initial volume using a 9:1 solution of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mmol/L HEPES, 137 mmol/L NaCl, 2.9 mmol/L KCl, 12 mmol/L NaHCO3, pH 7.4) and citric acid/citrate/dextrose (152 mmol/L sodium citrate, 130 mmol/L citric acid, and 112 mmol/L glucose) and centrifuged at 120*g* for 15 minutes. A total of 1 μmol/L prostaglandin E_1 and 0.2 U/mL apyrase were added to the collected platelet rich plasma and centrifuged at 720*g* for 15 minutes. Platelets were washed with PIPES buffer (20 mmol/L PIPES, 137 mmol/L NaCl, pH 6.5) and, then gently resuspended in HEPES buffer containing 5.5 mmol/L glucose at the final concentration of 1×10^9 platelets/mL.

2.4 | Analysis of platelet activation

Platelet aggregation was evaluated in a light transmission aggregometer (Chrono-Log Corporation), essentially as previously described.[10] Platelet samples (0.3 mL at 0.3×10^9 platelets/mL) in the presence of 1 mmol/L $CaCl_2$, left untreated or preincubated with different inhibitors as indicated, were stimulated with fibrillar amyloid peptides under constant stirring at 37°C and aggregation was monitored continuously for 5 minutes.

For the analysis of protein phosphorylation, platelet samples (1×10^9 platelets/mL, 0.2 mL) were lysed with 2% SDS and heated at 95°C for 3 minutes. Aliquots of platelet lysates (containing 10 μg of proteins) were separated by SDS/PAGE on 5% to 15% acrylamide gradient gels and transferred to poly(vinylidene difluoride) membrane. Immunoblotting analysis was performed using the following specific antibodies and dilutions: anti-PKC phospho-substrates (1:2000); anti P-Akt (S473), anti-P-GSK3 α/β (S21/9), anti-PLCγ2, anti-pleckstrin, and anti-phosphotyrosine (1:1000). Images of reactive bands were acquired using a Chemidoc XRS apparatus (Bio-Rad). Immunoblots are representative of at least three different independent experiments.

2.5 | Immunoprecipitation

Washed platelets (0.3 mL samples, 1×10^9 platelets/mL) were lysed with equal volume of ice-cold immunoprecipitation buffer 2× (100 mmol/L Tris/HCl, pH 7.4, 200 mmol/L NaCl, 2 mmol/L EGTA, 20 μg/mL leupeptin, 20 μg/mL aprotinin, 2 mmol/L PMSF, 2 mmol/L Na3VO4, 2 mmol/L NaF, 2% Nonidet P-40, 0.5% sodium deoxycholate). Platelet lysates were centrifuged to remove insoluble material and precleared for 1 hour at 4°C with 90 μL of protein A-Sepharose (50 mg/mL stock solution). The cleared supernatants were incubated with 2 μg of anti-FcγRIIA (IV.3) or anti PLCγ2 antibodies for 2 hours at 4°C, and the immunocomplexes were precipitated by addition of 90 μL of protein A-Sepharose for 1 hour. After brief centrifugation, immunocomplexes were washed three times with 1 mL of immunoprecipitation buffer 1×, and finally resuspended in 25 μL SDS-sample buffer (25 mmol/L Tris, 192 mmol/L glycine, 2% SDS, 0.5% DTT, 10% glycerol, 0.01% bromophenol blue, pH 8.3).

2.6 | Measurement of ROS production

Platelets were preloaded with 10 μmol/L 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) for 20 minutes at 37°C, as fluorescent probe for intracellular ROS.[24] Platelets (2.5×10^6 platelets/tube) were stimulated with Aβ40, Aβ42, or with the corresponding scrambled peptide, and intracellular ROS produced were measured by flow cytometry using a BD FACSLyric instrument.

2.7 | Statistical analysis

The reported figures are representative of at least four different experiments. Statistical analysis was performed using Prism, version 4, software (GraphPad), and data were compared by unpaired *t* test. Data are reported as mean ± standard error of the mean (SEM).

3 | RESULTS

3.1 | Fibrillogenesis of purified synthetic Aβ peptides

Previous studies have documented the ability of different amyloid peptides to promote platelet activation and aggregation, but significant discrepancies in the measured responses, potency of the peptides, and mechanism of action still persist. A major technical limitation and a possible explanation for some incongruences is that the chemical properties of the peptides used was poorly characterized. Aβ peptides form amyloid fibrils in aqueous solution, but, typically, they were added to platelets directly from stock solutions in DMSO or after a brief dilution in buffer, and both procedures do not allow to ascertain whether platelets are actually stimulated by Aβ in the form of monomers, oligomers, or fibrils.

To overcome this problem, we first characterized the ability of synthetic Aβ40 and Aβ42 peptides to polymerize into amyloid fibrils upon dilution from the DMSO stock solutions into PBS buffer. To this purpose, we adopted two different and complementary approaches, based on ThT and Congo Red dye staining, respectively. Aβ40, Aβ42, and their respective scrambled peptides were diluted at 0.25 mmol/L in PBS and incubated at 37°C for 72 hours. Aliquots of the samples were plated on a glass coverslip and stained with Congo Red dye, which confers a typical green birefringence to amyloid fibrils. Analysis on a polarized microscope revealed that both Aβ40 and Aβ42 were able to form amyloid fibrils in aqueous solution. By contrast, the scrambled peptides were negative to Congo Red staining, confirming their inability to form fibrils (Figure 1A).

To investigate the kinetics of fibril formation, Aβ40 and Aβ42 peptides were diluted at 0.25 mmol/L in PBS at 37°C in the presence of ThT, a benzothiazole dye, whose fluorescence increases upon binding to amyloid fibrils. Fluorescence was monitored for 72 hours. Figure 1B shows that after 12 hours both Aβ40 and Aβ42 peptides, but not their respective negative control Aβ40 and Aβ42 scrambled peptides, are able to form amyloid fibrils. Fibril formation of Aβ42 is faster than Aβ40 and reach a plateau after 12 hours, whereas Aβ40 is slightly retarded, and shows a significant increase in amyloid formation starting 24 hours after dilution and reaching a plateau at 48 hours (Figure 1B). These results indicate that although both Aβ40 and Aβ42, but not the scrambled peptides, form amyloid fibrils, the fibrillogenesis of Aβ40 is significantly delayed compared with Aβ42. The ThT assay provides useful information on the kinetics of fibrillation, thus allowing to determine how this process evolves and when it is completed. Therefore, to stimulate platelets with peptides of comparable fibrillar morphology, Aβ peptides were routinely incubated at 37°C in PBS for 48 hours, a time sufficient to obtain complete fibrillation of both Aβ peptides, before being used in all the subsequent experiments.

3.2 | Fibrillar Aβ peptides induce platelet aggregation through GPIbα and FcγRIIA

The Aβ active fragment encompassing amino acids 25 through 35 of Aβ peptides (Aβ25-35) is a strong stimulator of platelet aggregation.[10] Conversely, the entire Aβ40 or Aβ42, when added to platelets immediately after dilution, cause limited platelet aggregation, although they are able to potentiate the response to other agonists.[18] We now compared the ability of Aβ40 and Aβ42, both in a fibrillar form, to induce aggregation of washed human platelets.

Figure 2A shows that, after fibrillation and in the presence of 1 mmol/L $CaCl_2$, both Aβ40 and Aβ42 were equally able to stimulate platelet aggregation in a comparable dose-dependent manner. Platelet aggregation was evident already at 5 μmol/L of each peptide and reached the maximal level at 20 μmol/L (Figure 2A). No significant differences in the intensity of platelet aggregation induced by comparable doses of Aβ40 and Aβ42 were observed, and, for both peptides, the maximal aggregation was about 60% compared with the response triggered by the strong platelet agonist thrombin (Figure 2A and B). Importantly, scrambled Aβ40 or Aβ42 peptides completely failed to induce platelet aggregation, even at the highest dose of 50 μmol/L (Figure 2A). Scrambles peptides represent suitable negative controls because they reproduce in a random sequence the same amino acid composition of Aβ40 or Aβ42. However, because they are not amyloidogenic, they do not form fibrils, as shown in Figure 1. To verify whether platelet aggregation induced by Aβ peptides was merely from their fibrillar conformation, we tested a different, unrelated amyloidogenic protein, D76N β2 microglobulin, whose ability to form fibrils is comparable to that of Aβ peptides and has been previously characterized.[25] Figure 2C shows that, differently from fibrillar Aβ40 or Aβ42, stimulation of platelets with 20 μmol/L fibrillar D76N β2 microglobulin failed to induce any platelet aggregation. We also found that aggregation induced by fibrillar Aβ40 and Aβ42 was dependent on fibrinogen binding to integrin αIIbβ3, as it was completely suppressed by preincubation with RGDS (Figure 2D). Moreover, the presence of extracellular $CaCl_2$ was necessary for fibrillar Aβ peptides to induce platelet aggregation. In the presence of extracellular EGTA, aggregation stimulated by 20 μmol/L Aβ40 or Aβ42 was dramatically reduced (Figure 2E), as previously seen for Aβ25-35.[10]

Misfolded amyloid proteins have been proposed to activate platelets by binding to GPIb-IX-V and CD36 receptors.[17] We thus analyzed the possible role of GPIbα on platelet aggregation induced by fibrillar Aβ40 and Aβ42. To this purpose, we used mocarhagin, a metalloproteinase that specifically cleaves GPIbα from the platelet surface.[26] Immunoblotting with anti-GPIbα monoclonal antibody confirmed that treatment of washed platelets with 15 μg/mL mocarhagin completely cleaved GPIbα (data not shown). We found that mocarhagin completely prevented aggregation induced by fibrillar Aβ40 or Aβ42 peptides (Figure 2F) because the minimal residual aggregation was comparable to that seen in RGDS-treated platelets. Importantly, platelet aggregation by the Aβ25-35 fragment occurred normally in mocarhagin-treated platelets (Figure 2G). These results point to a major role of GPIbα as the receptor that mediates platelet activation in response to amyloid peptides. We and others had previously shown that GPIb-IX-V associates to the ITAM-bearing FcγRIIA, which plays an important role as a signaling elements for platelet activation by von Willebrand factor, the

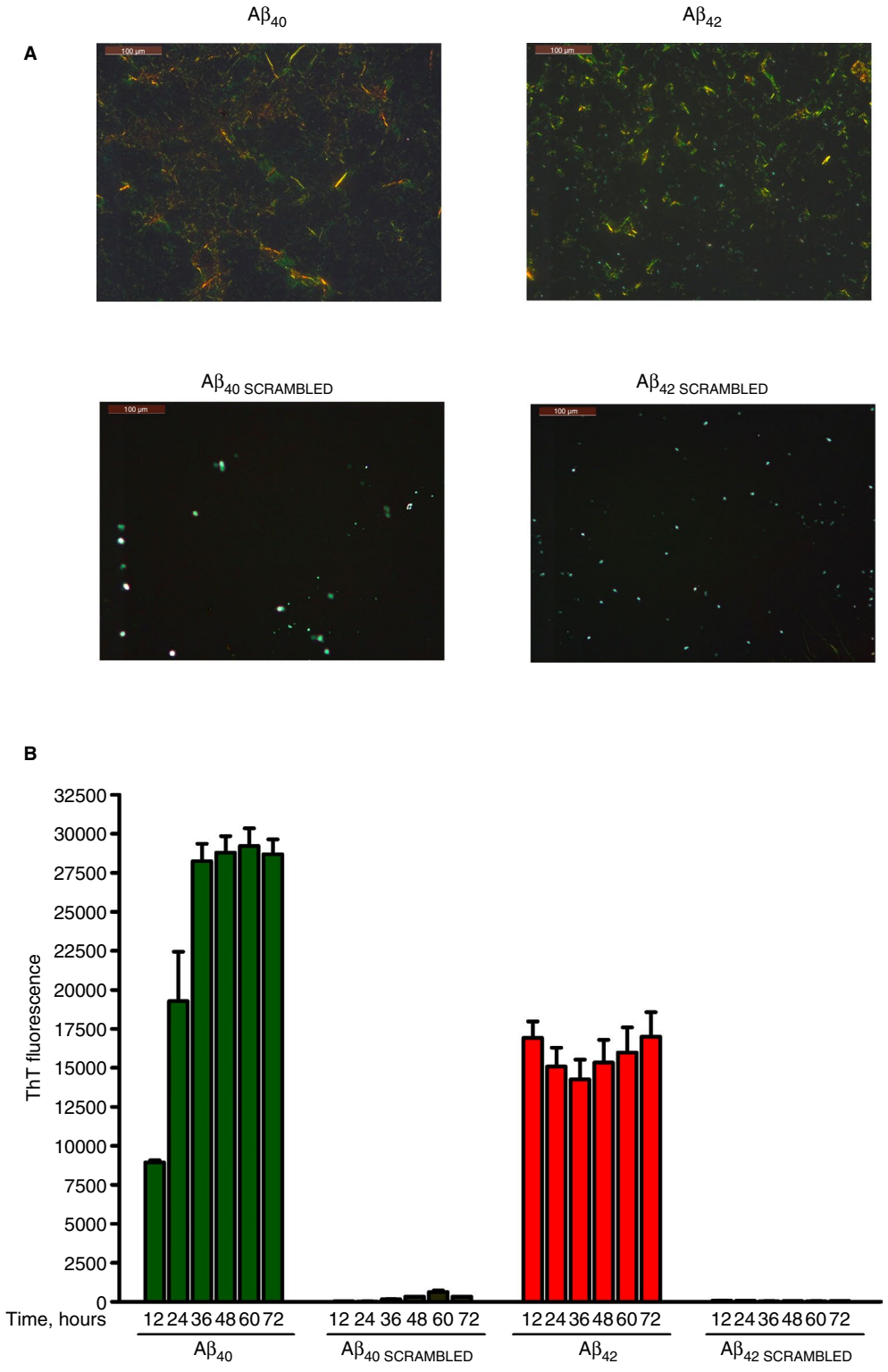
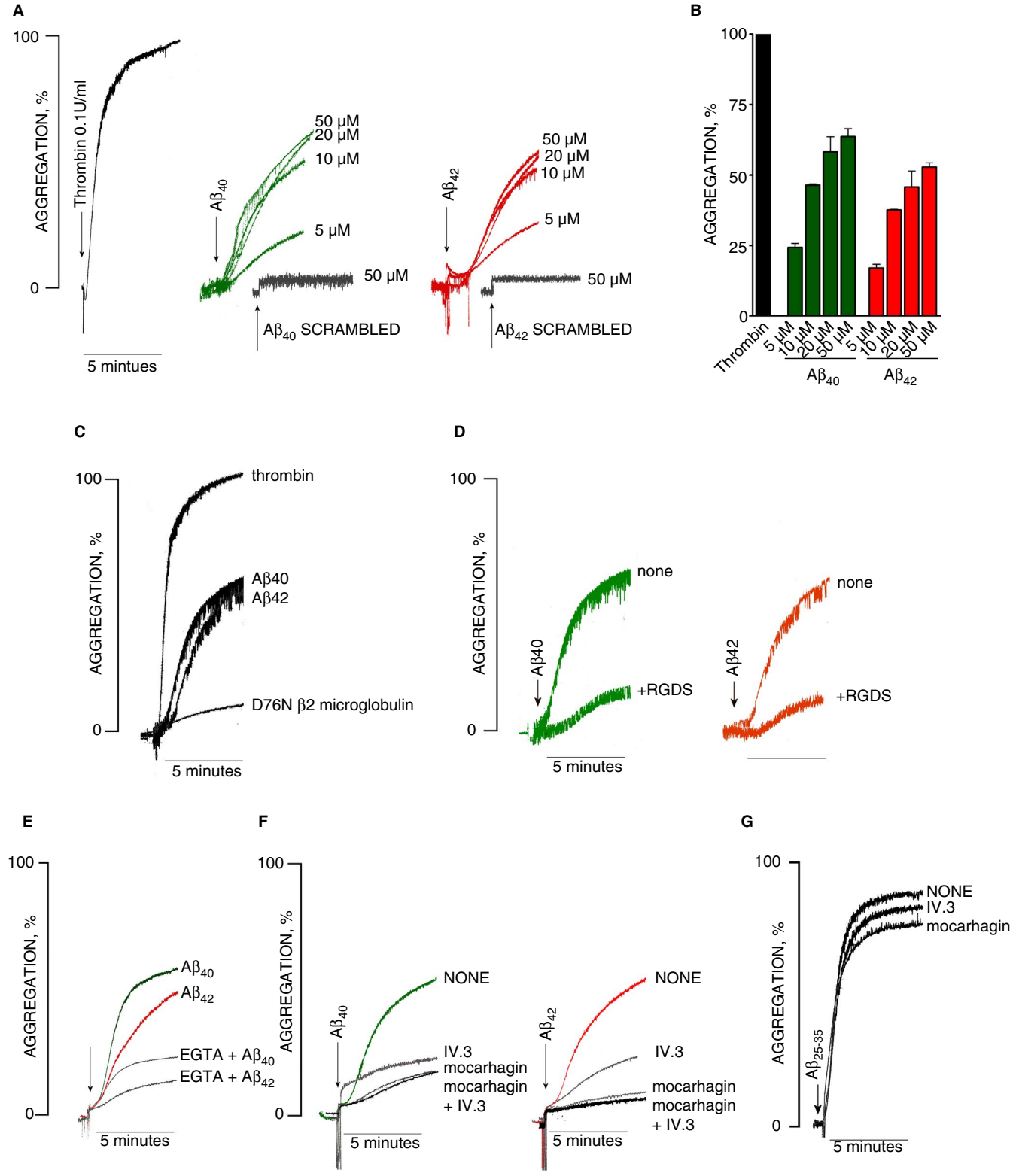


FIGURE 1 Fibrillogenesis of amyloid peptides. Amyloid peptides Aβ40, Aβ42, and controls scrambled peptides were dissolved in 5 mmol/L DMSO, and then diluted at the final concentration of 0.25 mmol/L with PBS, pH 7.4. A, Fibrils formation upon 72 hours of incubation was visualized by Congo red staining and analyzed in a polarized microscope. Representative images are reported. B, Kinetics of fibril formation was evaluated by the Thioflavin T assay. Fluorescence signal was recorded over a period of 72 hours

physiological GPIb-IX-V complex ligand.[27,28] To investigate the possible role of FcγRIIA in amyloid peptideinduced platelet aggregation, platelets were pretreated with the FcγRIIA blocking monoclonal antibody IV.3 and then stimulated with fibrillar Aβ40 and Aβ42 peptides. Figure 2F shows that IV.3 mAb severely reduced, but did not suppress, platelet aggregation induced by both



peptides. As expected, the IV.3 mAb did not display any additive effect when GPIbα was cleaved by mocarhagin (Figure 2F). Moreover. losartan, a recently characterized GPVI inhibitor that at low doses does not cross-react with FcγRIIA,[29] has minimal or negligible effects on platelet aggregation induced by fibrillar Aβ peptides (Figure S1). These results indicate that amyloid peptides activate platelets through the GPIb-IX-V/FcγRIIA axis.

3.3 | Activation of ITAM-dependent signaling pathways by fibrillar Aβ peptides

The inhibitory effect of the anti-FcγRIIA antibody IV.3 on platelet aggregation suggests that fibrillar Aβ40 and Aβ42 activate platelets through an ITAM- and tyrosine kinase-based signaling pathway. Immunoblotting with anti-phosphotyrosine antibody

FIGURE 2 Fibrillar amyloid peptides induce platelet aggregation. A, Washed platelets (3×10^8 platelets/mL) were stimulated with the indicated concentrations thrombin, Aβ40, and Aβ42 in the presence of 1 mmol/L $CaCl_2$. Scrambled peptides were used as controls. Representative traces are reported in A, whereas quantification of maximal aggregation is shown in (B), as mean ± standard error of the mean (SEM) of three different experiments. C, Washed platelets were stimulated with 0.1 U/mL thrombin of with 20 μmol/L fibrillar Aβ40, Aβ42, or D76N β2 microglobulin, as indicated and aggregation was monitored for 5 minutes. Representative traces are reported. D, Platelet aggregation induced by 20 μmol/L fibrillar Aβ40 and Aβ42 was measured in the absence (none) or in the presence of 0.5 mmol/L RGDS peptide. E, Effect of extracellular EGTA (2 mmol/L final concentration) on platelet aggregation induced by 20 μmol/L fibrillar Aβ40 and Aβ42. F, Washed platelets were preincubated with 15 μg/mL mocarhagin for 30 minutes or 20 μg/mL anti-FCγRIIA blocking antibody IV.3 for 5 minutes, alone or in combination, and then stimulated with 20 μmol/L fibrillar Aβ40 and Aβ42, as indicated. Aggregation was monitored for 5 minutes. Representative traces are shown. G, Washed platelets were pretreated with buffer (none) or with 15 μg/mL mocarhagin for 30 minutes or incubated with 20 μg/mL anti-FcγRIIA blocking antibody IV.3 for 5 minutes and then stimulated with 20 μmol/L Aβ25-35. Aggregation was monitored for 5 minutes

confirmed that fibrillar Aβ40 and Aβ42, but not the scrambled control peptides, dose-dependently induced a marked tyrosine phosphorylation of multiple substrates in platelets (Figure 3A). Moreover, inhibition of Src family kinases (SFKs) by PP2 almost completely suppressed aggregation induced by both peptides (Figure 3B). To verify whether SFKs initiate an ITAM-based signaling pathway through FcγRIIA, we analyzed the ability of fibrillar Aβ40 and Aβ42 to activate this receptor. Washed platelets were stimulated with 20 μmol/L fibrillar Aβ40 and Aβ42, or with the scrambled peptides, and FcγRIIA was immunoprecipitated with the IV.3 antibody. Immunoblotting analysis revealed that Aβ40 and Aβ42 promoted the phosphorylation of an immunoprecipitated protein with a molecular mass corresponding to authentic FcγRIIA, as revealed by immunostaining with a specific antibody. FcγRIIA phosphorylation by control scrambled peptides was negligible (Figure 3C). As typically reported for ITAM-based signaling, FcγRIIA-mediated platelet stimulation is also characterized by the phosphorylation and activation of PLCγ2. Immunoprecipitation experiments showed that treatment of platelets with 20 μmol/L fibrillar Aβ40 and Aβ42 induced the phosphorylation of PLCγ2 (Figure 3D). This event was more pronounced for Aβ40 but still clearly evident for Aβ42.

The activation of phosphorylated PLCγ2 was assessed by analyzing the activity of the downstream effector PKC. Platelet stimulation with increasing concentrations of fibrillar Aβ peptides caused the phosphorylation of multiple PKC substrates, as evaluated in immunoblotting with a specific antibody. These included also the p47 kDa protein pleckstrin (Figure 3E).

In a separate set of experiments, we also verified that fibrillar Aβ40 and Aβ42 peptides were able to activate the phosphoinositide 3-kinase (PI3K) pathway. Figure 3F shows the phosphorylation of the PI3K downstream effectors Akt and GSK3α/β induced by fibrillar Aβ40 and Aβ42, but not by the respective control scrambled peptides.

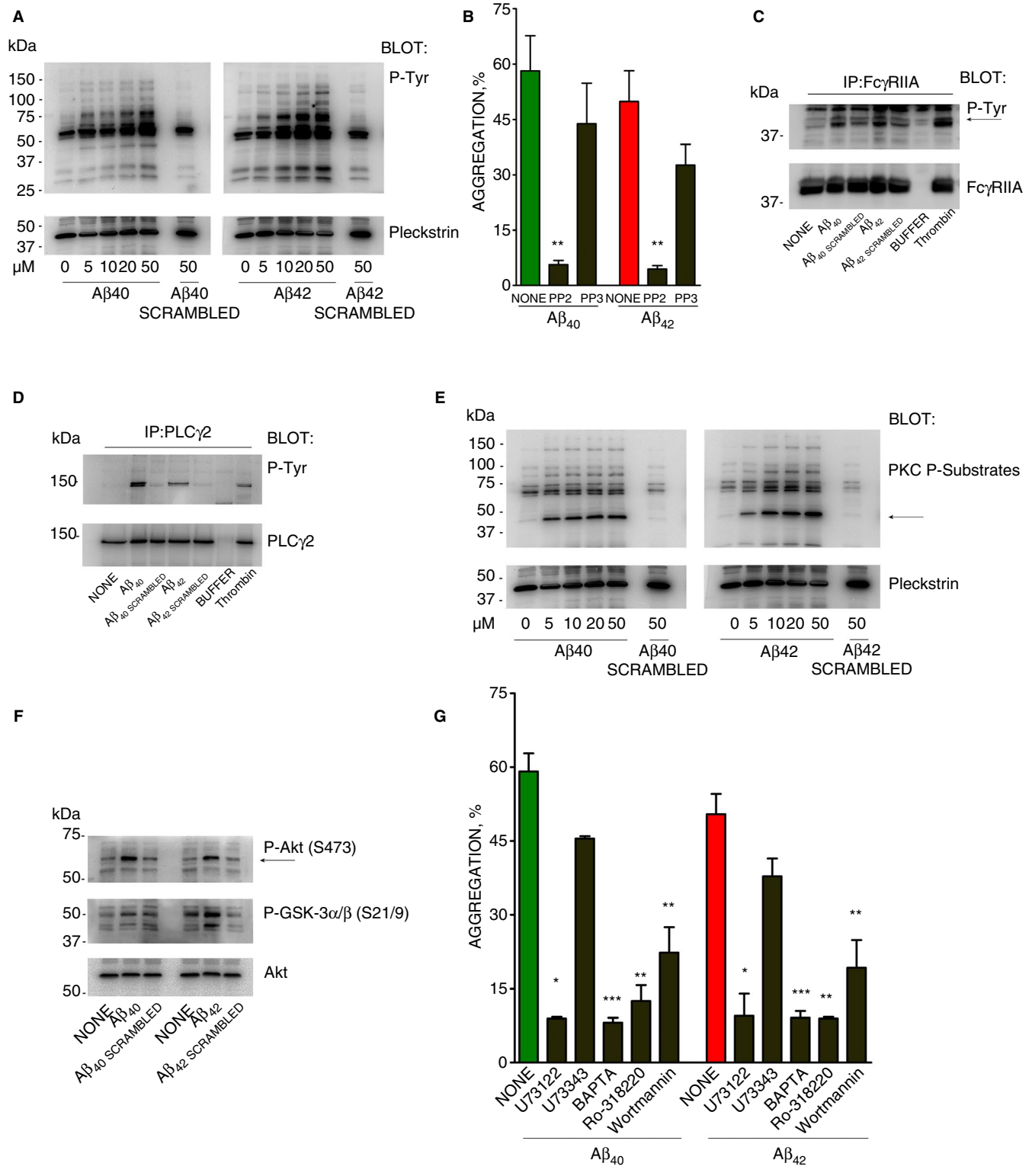
To verify the contribution of these signaling elements to the platelet response, we used specific pharmacological inhibitors and we demonstrated that both PLC and PI3K are required for platelet aggregation induced by fibrillar Aβ40 and Aβ42. Figure 3G shows that the PLC inhibitor U73122, but not the inactive analogue U73343, almost completely prevented platelet aggregation induced by both peptides and that a similar effect was also produced by the neutralization of the PLC-generated intracellular messengers through Ca^{2+} chelation by BAPTA-AM or PKC inhibition by Ro-318220. Moreover, blockade of PI3K activity by wortmannin partially reduced platelet aggregation induced by fibrillar Aβ40 an Aβ42 (Figure 3G). Altogether, these results demonstrate that fibrillar Aβ40 and Aβ42 trigger platelet aggregation through ITAM-based platelet activation initiated by recruitment of FcγRIIA.

3.4 | Fibrillar amyloid peptides promote ROS generation

Previous studies had reported that Aβ42, but not Aβ40, promotes the formation of ROS in platelets, through the activation of NADPH oxidase NOX1 and NOX2.[14,15] However, in these studies, the chemical form of the peptides was not characterized. Therefore, we reconsidered the ability of amyloid peptides to induce ROS production by using fibrillar Aβ40 and Aβ42, generated under standardized conditions. Platelets were preincubated with a redox-sensitive fluorescent dye (H_2DCF-DA) and stimulated with 20 or 50 μmol/L fibrillar Aβ40 and Aβ42 (or corresponding scrambled peptides) for 15 minutes. Intracellular ROS production was then evaluated in flow cytometry, as described in the Methods. Figure 4A shows that both Aβ40 and Aβ42 in a fibrillar form, but not the scrambled peptides, promoted a significant ROS production in platelets. The potency of Aβ42 was clearly stronger than that of Aβ40, which was able to induce a more modest, albeit significant, ROS production. This was also confirmed in time-course experiments: although ROS accumulation induced by fibrillar Aβ42 was already evident after 5 minutes of stimulation, the effect of fibrillar Aβ40 was delayed and clearly measurable only after 15 minutes (Figure 4B).

Preincubation of platelets with the ROS scavenger NAC strongly decreased ROS production in platelets stimulated with fibrillar Aβ40 or Aβ42, confirming that these peptides induce oxidative stress in platelets. Moreover, in line with previous observations,[14] we found that ROS production was also significantly reduced by the pan-NOX inhibitor VAS 2870, indicating that NOXs are implicated in ROS generation by fibrillar amyloid peptides (Figure 4C).

Because ROS may act as second messengers,[30] we investigated their contribution to platelet aggregation induced by



amyloid peptides. Figure 4D shows that the ROS scavenger NAC was able to significantly, albeit not completely, inhibit platelet aggregation induced by fibrillar Aβ40 and Aβ42. Moreover, both peptides were totally unable to induce aggregation of platelets treated with the pan-NOX inhibitor VAS2870. Although ROS production by Aβ40 before 5 minutes of stimulation is hardly detectable with the method used in this study (Figure 4B), it seems plausible that traces of ROS are rapidly produced and support platelet aggregation.

We next investigated whether activation of NOXs and generation of ROS were new elements of the SFKs-initiated, ITAM-mediated signaling pathway that promotes platelet aggregation in response to fibrillar amyloid peptides. Figure 4E indicates that in platelets stimulated with either fibrillar Aβ40 or Aβ42, ROS accumulation was

FIGURE 3 Activation of intracellular signalling pathways by fibrillar amyloid peptides. A, Washed platelets in the presence of 1 mmol/L $CaCl_2$ were stimulated with increasing doses of fibrillar Aβ40 and Aβ42 or with 50 μmol/L of their respective scrambled peptides for 3 minutes. Protein tyrosine phosphorylation was analyzed by immunoblotting with a specific antibody (blot: P-Tyr). Anti-pleckstrin was used as equal loading control (blot: pleckstrin). B, Role of SFKs on platelet aggregation induced by fibrillar Aβ40 and Aβ42 peptides. Platelet aggregation induced by 20 μmol/L fibrillar Aβ40 and Aβ42 was analyzed upon incubation with vehicle (none), 20 μmol/L PP2, or 20 μmol/L PP3 for 10 minutes, as indicated. The histograms show the mean ± SEM of the percentage of aggregation measured in three different experiments. C, Washed platelets were stimulated with 20 μmol/L fibrillar Aβ40 and Aβ42 or with their respective scrambled peptides for 3 minutes. FcγRIIA was immunoprecipitated with the IV.3 antibody and analyzed by immunoblotting with anti-phosphotyrosine antibody (P-Tyr). Membranes were subsequently probed with anti-FcγRIIA antibody, as indicated on the right. The arrow in the upper panel indicates the band corresponding to phosphorylated FcγRIIA. D, PLCγ2 was immunoprecipitated from platelets stimulated with 20 μmol/L fibrillar Aβ40 and Aβ42 with their respective scrambled peptides or with 0.1 U/mL thrombin. Tyrosine phosphorylation of the immunoprecipitated protein was analyzed by immunoblotting with specific antibodies, as indicated on the right. E, Platelets were stimulated with increasing doses of fibrillar Aβ40 and Aβ42 or with 50 μmol/L of their respective scrambled peptides for 3 minutes and analyzed in immunoblotting with anti-PKC phospho-substrates (blot: PKC P-substrates). Reprobing with anti-pleckstrin was used as control for equal loading (blot: pleckstrin). The arrow in the upper panel indicates the band corresponding to phosphorylated pleckstrin. F, Immunoblotting analysis of Akt and GSK3 α/β phosphorylation in platelet stimulated with 20 μmol/L fibrillar Aβ40 and Aβ42 and their respective scrambled peptides were analyzed in immunoblotting with anti P-Akt (S473) and P-GSK3α/β (S21/9). Control for equal loading was performed with anti-Akt antibody. G, Effect of 10 μmol/L U73122, 10 μmol/L U73343, 10 μmol/L Ro-318220, 30 μmol/L BAPTA, 100 nmol/L wortmannin on platelet aggregation induced by 20 μmol/L fibrillar amyloid peptides as indicated. Maximal aggregation was quantified after 5 minutes. Data are the mean ± SEM of three different experiments

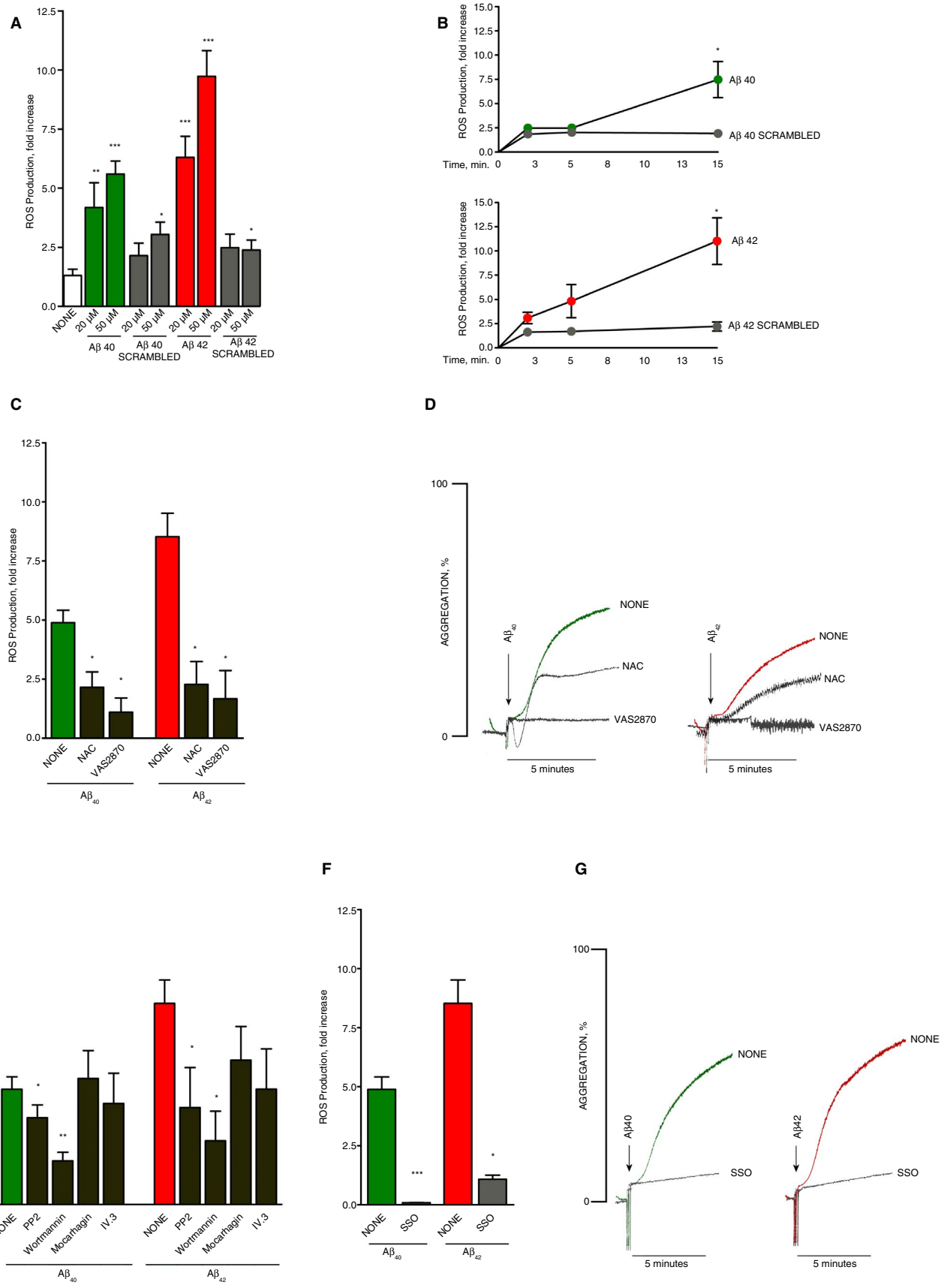
partially inhibited by PP2 and by wortmannin, indicating a contribution of SFKs and PI3K. However, cleavage of GPIbα from the platelet surface by mocarhagin or blockage of FcγRIIA by the IV.3 monoclonal antibody did not affect ROS generation induced by fibrillar Aβ40 or Aβ42. These results suggest the involvement of a receptor different from GPIbα in the generation of ROS. A previous work has demonstrated that oxidized LDL stimulate SFK-mediated and NOX-dependent ROS generation in platelets by binding to the scavenger receptor CD36[31]; CD36 has also been proposed as a receptor for misfolded proteins.[17] Therefore, we used the pharmacological inhibitor SSO, a long chain fatty acid that irreversibly binds to CD36,[32] and that has been previously characterized in studies on blood platelets.[31,33] Importantly, the specificity of SSO was validated by a comparative analysis of platelets from CD36 knockout mice in the study by Magwenzi et al.[31] We found that the CD36 inhibitor SSO strongly reduced ROS accumulation in platelets stimulated with fibrillar Aβ40 or Aβ42 (Figure 4F). Moreover, consistently with the effect of ROS scavenger or pan-NOX inhibitor, the treatment of platelets with SSO almost completely prevented platelet aggregation induced by fibrillar amyloid peptides (Figure 4G).

4 | DISCUSSION

The ability of amyloid peptides to act as platelet agonists has gained increasing interested in recent years, because of the implication not only in thrombosis and inflammation but also in the progression of AD.[34,35] In fact, Aβ peptides have been found in atherosclerotic plaques, and their accumulation in vessel walls during the onset of AD gives rise to a pathological condition known as CAA. Currently, however, most of the information available on the molecular mechanism for platelet activation promoted by Aβ peptides derives from studies that have used the artificial Aβ fragment Aβ25-35, which recapitulates many of the biological effect the natural peptides.[36] By contrast, the few available studies using the full peptides Aβ40 and Aβ42 are incomplete and have generated controversial results on their effective ability and potency to trigger platelet responses.[11,14,15,19] The current incompleteness and incongruences are reasonably because amyloid peptides tend to form fibrils in aqueous solutions, with specific intrinsic kinetics, and the implications of their biochemical conformation on biological activities was not properly considered. Amyloid Aβ fibrils are present in atherosclerotic plaques, and Aβ peptides are also detected in the circulation.[5,34] The structural conformation of Aβ in plasma has not been characterized yet, however, because fibrillogenesis is a spontaneous process in aqueous solution, it seems reasonable that Aβ oligomers and fibrils can be generated also in the circulation.

In the present work, we report the first accurate comparison of the ability of Aβ40 and Aβ42, under controlled conditions of fibrillogenesis, to stimulates platelet activation and aggregation. The major novel findings of our study are the following: (a) fibrillar Aβ40 and Aβ42 are equal efficient in supporting platelet aggregation; (b) both peptides require intact membrane GPIbα and initiate an ITAM-based signaling pathway by recruiting FcγRIIA; moreover, they stimulate NOX-dependent ROS production downstream of CD36; and (c) intracellular ROS integrate ITAM-based signals and full platelet aggregation in response to fibrillar amyloid peptides requires the integration of coordinated signals through both GPIbα and CD36. A cartoon summarizing the model arising from the results of the present study and showing the contribution of the different receptors and signaling proteins is provided in Figure 5.

The precise characterization of the fibrillar conformation of the amyloid peptides used to stimulate platelets allowed us to obtain a reliable comparison of the ability of Aβ40 and Aβ42 to induce platelet aggregation. Such a procedure has never been adopted in previous studies, and a concomitant analysis of the two peptides as platelet agonists has never been provided. In fact, the two previously available studies that have analyzed platelet activation by full amyloid peptides in controlled fibrillar forms have been performed exclusively with Aβ40 in one case or with Aβ42 in the other.[15,37]



Importantly, we have demonstrated here that although Aβ42 rapidly assembles into fibrils when diluted in aqueous solution, Aβ40 requires a prolonged lag time and fibrillation initiates after 24 hours. This evidence explains, for instance, why Aβ40 was excluded in the study by Elaskalani et al, where peptide fibrillation was stopped after 24 hours,[37] and also explains why different platelet responses were

FIGURE 4 Fibrillar amyloid peptides induce ROS production. A, Platelets were preloaded with the fluorescent probe H_2DCF-DA stimulated with increasing doses of fibrillar Aβ40 and Aβ42 or respective scrambled peptides, as indicated. ROS production was analyzed in flow cytometry and reported as fold increase of the positive cells over nonstimulated cells (none). Results are the mean ± SEM of three different experiments. B, Time-course of ROS production induced by 50 μmol/L Aβ40 (upper panel) or Aβ42 (lower panel) in comparison with control scrambled peptides. C, Effect of platelets preincubation with 1 mmol/L NAC or with 10 μmol/L VAS 2870 on ROS production induced by 50 μmol/L fibrillar Aβ40 and Aβ42. D, Effect of platelet preincubation with 1 mmol/L NAC or with 10 μmol/L VAS 2870 on platelet aggregation induced by 20 μmol/L fibrillar Aβ40 and Aβ42. E, Effect of SFKs inhibition by PP2 (20 μmol/L), PI3K inhibition by wortmannin (100 nmol/L), GPIbα cleavage by mocarhagin (15 μg/mL) or FcγRIIA blockade by the IV.3 mAb (20 μg/mL) on ROS production induced by 50 μmol/L fibrillar Aβ40 and Aβ42. F, Effect of the nonantibody CD36 inhibitor SSO (50 μmol/L) on ROS production induced by 50 μmol/L fibrillar Aβ40 and Aβ42. G, Analysis of platelet aggregation by 20 μmol/L fibrillar Aβ40 and Aβ42 in the absence (none) or in the presence of 50 μmol/L non antibody CD36 inhibitor SSO

observed when Aβ peptides were directly added to platelet suspension from a stock solution in DMSO.[14]

We provided various evidence supporting the notion that if both peptides are in comparable fibrillar forms, their potency in stimulating platelet aggregation and ITAM-based signaling through FcγRIIA are comparable. FcγRIIA is a versatile signaling receptor in platelets because it can be activated to initiate ITAM-based signaling pathways in different contexts of platelet activation, including

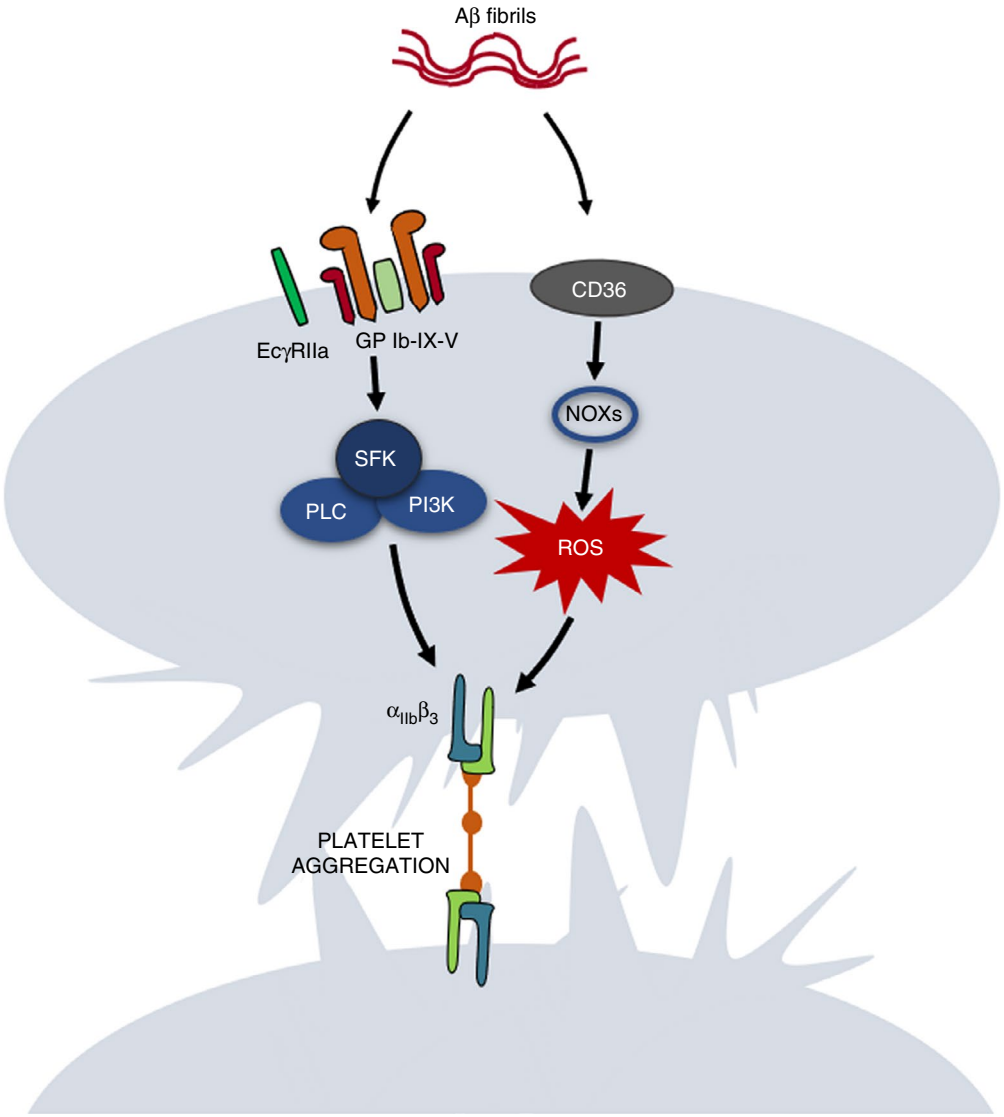


FIGURE 5 Schematic representation of the mechanism for platelet activation by fibrillar Aβ peptides. Stimulation of human platelets by fibrillar Aβ40 and Aβ42 activates at least two different membrane receptors: GPIb-IX-IV/FcγRIIA, which promotes SFK-dependent activation of PLCγ2 and PI3K, and CD36, which leads to NOX-dependent generation of ROS. Both pathways are concomitantly necessary to trigger integrin αIIbβ3-mediated platelet aggregation

integrin αIIbβ3 ligation,[38] von Willebrand factor binding to GPIb-IX-V,[28] and thrombin receptors stimulation.[39] Although we cannot exclude alternative mechanisms for FcγRIIA activation by fibrillar Aβ40 and Aβ42, the complete inhibition of aggregation upon GPIbα cleavage by mocarhagin strongly support the conclusion that FcγRIIA is engaged in association with GPIb-IX-V, by a mechanism comparable to that previously reported in von Willebrand factor-stimulated platelets.[28]

We have detected a significant difference in the amount of ROS generated by fibrillary Aβ40 or Aβ42. This difference is unlikely to be due to a different degree of fibrillation of the two peptides because after 48 hours of incubation in PBS, fibrillation was complete for both of them. It seems likely that Aβ42 possesses an intrinsic stronger ability to stimulate ROS formation than Aβ40. In support of this possibility, it is interesting to note that previous studies have been able to measure ROS production exclusively upon platelet challenge with Aβ42.[14,15] Interestingly, however, we found that ROS generation by fibrillar Aβ peptides in platelets is not mediated by recruitment of GPIbα and activation of the FcγRIIA but requires CD36 engagement. In this context, it is interesting to outline that CD36 represents a recognized receptor for misfolded proteins, including amyloid peptides, and has been shown to stimulate SFK-dependent generation or ROS in response to oxidized LDL.[17,31,33] The different efficiency of fibrillar Aβ40 and Aβ42 in stimulating ROS generation could reflect a different binding to CD36, a possibility that deserves further investigation.

A previous study has demonstrated that ROS generation by Aβ42 is mediated by NOX1 and NOX2.[15] Accordingly, we found that the pan-NOX inhibitor VAS2870 suppressed ROS generation by both fibrillar Aβ40 and Aβ42 peptides, and concomitantly also totally prevented platelet aggregation. It should be noted, however, that platelet aggregation is only partially reduced by the ROS scavenger NAC, indicating that either VAS2870 has off-target effects or that NOX activity supports aggregation by a mechanism in part independent of ROS generation. Nevertheless, our results clearly indicate that ROS are important messengers supporting platelet aggregation induced by both fibrillar Aβ40 and Aβ42. We have also shown that platelet aggregation is significantly reduced upon blockade of FcγRIIA and that a similar effect is also caused upon inhibition of intracellular effectors of the ITAM-dependent pathway, including SFKs and PI3K. Therefore, it is reasonable to conclude that platelet aggregation induced by fibrillary Aβ peptides integrates independent signals downstream recruitment of multiple membrane receptors. In this line, it should be considered that previous work using less characterized forms of Aβ peptides has suggested the existence of a wider range of platelet receptors for Aβ40 or Aβ42 including GPVI, PAR1, or integrin αIIbβ3.[18,19,37]

As previously mentioned, a large part of our knowledge on platelet activation induced by amyloid peptides derives from studies using the fragment Aβ25-35.[10,18] The results of the present study indicate that the mechanism for fibrillary Aβ40- and Aβ42-promoted platelet aggregation and activation differ from those previously reported for this shorter Aβ fragment. Although not present in biological fluids, this peptide has been commonly used in functional studies because it encompassed the neurotoxic sequence of genuine Aβ peptides, is easier to solubilize and, importantly, is much cheaper. Aβ25-35 has been shown to induce potent platelet aggregation through the initial influx of extracellular Ca^{2+} leading to platelet secretion and subsequent stimulation of platelets by released ADP.[10] The membrane receptor mediating Aβ25-35-induced platelet activation has never been identified and its existence is uncertain considering its ability to form pores in the plasma membrane.[20] In this work, we definitively demonstrate that although genuine Aβ40 and Aβ42 in fibrillar forms activate platelets similarly to Aβ25-35, the mechanism implicated is significantly different. In contrast to Aβ40 and Aβ42, Aβ25-35-mediated platelet aggregation is independent of GPIbα and FcγRIIA engagement. These observations support the notion that Aβ25-35 may activate platelets through a membrane receptor-independent mechanism and suggest caution with the use of this tool.

In conclusion, the present study demonstrates that both Aβ40 and Aβ42, when used in a comparable fibrillary state, are potent platelet agonists able to activate multiple signal transduction pathways through membrane receptor recruitments. Because amyloid peptides accumulate as fibrils in physiopathological contexts, such as in atherosclerotic plaques or in CAA, fibrillar Aβ40 and Aβ42 must be considered true and potent pro-thrombotic agents.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Caterina Visconte designed and performed the experiments, analyzed the data, and wrote the manuscript; Jessica Canino and Gianni Francesco Guidetti performed experiments, analyzed the data, and edited the manuscript; Mauro Vismara performed the experiments; Sara Raimondi provided technical support and vital reagents; Giordano Pula edited the manuscript; and Mauro Torti and Ilaria Canobbio designed the research, analyzed the data, wrote the manuscript, and guided the overall direction of the study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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