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Objective—Protein misfolding diseases result from the deposition of insoluble protein aggregates that often contain fibrils called amyloid. Amyloids are found in Alzheimer disease, atherosclerosis, diabetes mellitus, and systemic amyloidosis, which are diseases where platelet activation might be implicated.

Methods and Results—We induced amyloid properties in 6 unrelated proteins and found that all induced platelet aggregation in contrast to fresh controls. Amyloid-induced platelet aggregation was independent of thromboxane A_2 formation and ADP secretion but enhanced by feedback stimulation through these pathways. Treatments that raised cAMP (iloprost), sequestered Ca^{2+} (BAPTA-AM) or prevented amyloid-platelet interaction (sRAGE, tissue-type plasminogen activator [tPA]) induced almost complete inhibition. Modulation of the function of CD36 ($CD36^{-/-}$ mice), $p38^{MAPK}$ (SB203580), COX-1 (indomethacin), and glycoprotein Ibα (Nk-protease, 6D1 antibody) induced ≈50% inhibition. Interference with fibrinogen binding (RGDS) revealed a major contribution of $\alpha_{IIb}\beta_3$-independent aggregation (agglutination).

Conclusions—Protein misfolding resulting in the appearance of amyloid induces platelet aggregation. Amyloid activates platelets through 2 pathways: one is through CD36, $p38^{MAPK}$, thromboxane A_2–mediated induction of aggregation; the other is through glycoprotein Ibα–mediated aggregation and agglutination. The platelet stimulating properties of amyloid might explain the enhanced platelet activation observed in many diseases accompanied by the appearance of misfolded proteins with amyloid. (***Arterioscler Thromb Vasc Biol.*** **2007;27:1657-1665.)**

Key Words: amyloid ■ platelet activation ■ sRAGE ■ tissue plasminogen activator ■ CD36 ■ glycoprotein Ibα

Proteins typically adopt a well-defined 3-dimensional structure. There is now an increasing amount of evidence that abnormalities in this process have far reaching consequences for human health. Certain mutations and posttranslational modifications such as glycation and oxidation interfere with proper folding, resulting in protein misfolding, aggregation, and ultimately polymerization into insoluble fibrils called amyloid.[1,2] The term amyloidosis defines a group of systemic and localized diseases associated with the deposition of amyloid in different tissues. Alzheimer disease is caused by abnormal folding of amyloid-β and formation of amyloid-rich plaques that obstruct neurons and microvessels of the brain.[3] One has argued that these plaques cause the hyperreactivity of platelets observed in these patients as illustrated by P-selectin positive platelets and increased levels of urinary thromboxane A_2 metabolite.[4,5] An environmental risk factor for Alzheimer disease is Herpes Simplex virus. It contains glycoprotein B, a protein fragment that assembles into fibrils that are ultrastructurally indistinguishable from amyloid-β.[6]

Protein misfolding is not restricted to Alzheimer disease but is a common feature in the pathology of atherosclerosis, diabetes mellitus, and systemic amyloidosis.[7–9] Atherosclerotic plaques contain oxidized low density lipoprotein (LDL) which has amyloid properties[10] and activates platelets.[11] Interestingly, Herpes Simplex virus also contributes to initiation and progression of coronary atherosclerosis.[12] In diabetic mellitus type 2 the high blood glucose glycates hemoglobin and albumin introducing amyloid properties[13] that might contribute to the hyperactivity of platelets in these patients.[14] Several types of systemic amyloidosis are also known to be associated with thrombosis.[15,16]

Amyloids are filamentous protein structures rich in β-sheets that share a structural motif, the cross-β structure. Amyloids have been defined in a number of different ways: operationally in terms of their capacity to bind dyes like Congo Red and thioflavin derivatives, morphologically as 6- to 10-nm filaments, and structurally as "cross-β structure" fibrils in X-ray diffraction. The term cross-β refers to the stacking of β-sheets perpendicular to the fibril axis. Proteins with amyloid may meet these definitions independent of amino acid sequence. We recently showed that tissue-type plasminogen activator (tPA) selectively binds to amyloid.[17]

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Structural characterization and biochemical analysis of fibril assembly revealed that amyloid forms through a transition of soluble oligomers into intermediate elements that form the fibrils. Consequently, most amyloids are heterogenous in nature. We here use the term amyloid properties to refer to nonnative protein aggregates, containing extended β-sheet arrangement, including oligomeric intermediates, amorphous aggregates, and end-stage fibrils. There is growing consensus that misfolding and formation of amyloid is an intrinsic property of any protein. Amyloid formation can be caused by mutations that affect protein folding, an increase in temperature, changes in pH, oxidation, glycation, and contact with a negatively charged surface.[10,13,18,19] These conditions enhance the relative β-sheet structure content of proteins that subsequently aggregate and polymerize into fibrils. This may occur within the cells and at extracellular locations and greatly interferes with proper cell function.[20]

In the present study we addressed the question whether protein aggregates with amyloid properties have the capacity to activate platelets. To avoid bias by other peptide domains, a number of unrelated proteins was modified and the appearance of amyloid properties was evaluated in platelet activation assays. The results reveal that proteins with amyloid properties have potent and specific platelet activating properties that might underlie the development of atherothrombosis observed in diseases known to be associated with formation of amyloid.

Methods

For the preparation of fresh and misfolded proteins, the characterization of amyloid properties and the biochemical and functional determinants in human and mice platelets, see the Methods section http://atvb.ahajournals.org.

Results

Appearance of Amyloid Properties in a Number of Unrelated Proteins

Treatment of freshly prepared amyloid-β with a trifluoroacetic acid/hexafluor propanol mixture to introduce monomers followed by evaporation and prolonged incubation in PBS introduced amyloid as illustrated by the binding of Thioflavin T and Congo Red,[21,22] the capacity to activate tPA,[17] and a fibrillar structure in electron micrographs (supplement Figure I, available online at http://atvb.ahajournals.org). These observations indicate that the modified amyloid-β met the criteria for amyloid-containing proteins. Freshly prepared amyloid-β did not show these characteristics illustrating that is was devoid of amyloid. Also Hb-AGE, BSA-AGE, modified glycoprotein B and fibrin peptides 12 and -13 met these requirements whereas Hb, BSA, and fibrin peptide 10 were negative in these assays (data not shown). Thus, there was a clear separation between protein samples with and without amyloid properties in these unrelated proteins.

Amyloid Proteins Induce Platelet Aggregation

Amyloid-β induced platelet aggregation (Figure 1). At 12.5 μg/mL amyloid-β there was a slight shape change followed by little aggregation. Higher concentrations induced a dose-dependent increase in aggregation reaching a maximum at 50 to 100 μg/mL amyloid-β. A similar response was induced by glycohemoglobin (Hb-AGE) although aggregation was slightly weaker than with modified amyloid-β. Also glycated BSA (BSA-AGE) induced aggregation but there was a delay of about 500 seconds at low and 250 seconds at high concentration. Aggregation induced by Herpes Simplex glycoprotein-B was rapid, but responses were weaker than seen with the other proteins. The fibrin peptides 12 and -13 also induced aggregation, but higher concentrations were required to obtain an effect. None of the fresh control proteins/peptides had platelet-activating properties, and also fibrin peptide 10 which lacks amyloid failed to induce aggregation (not shown). Using aggregation by a suboptimal concentration of TRAP as an interassay reference, studies were repeated in platelets from 5 different donors for calculation of dose-response relationships (Figure 1B), maximal aggregation (Figure 1C), and EC_{50} data (Figure 1D). Amyloid-β and Hb-AGE induced the highest aggregation. BSA-AGE, modified glycoprotein-B, and fibrin peptide 12 were slightly less effective, and fibrin peptide 13 induced the lowest response. Hb-AGE and BSA-AGE had the lowest EC_{50} (about 10 μg/mL), amyloid-β, glycoprotein-B and fibrin peptide 12 showed an intermediate activity (about 30 μg/mL), and fibrin peptide 13 had the highest EC_{50} (about 100 μg/mL). Thus, different proteins with only amyloid properties in common induce platelet aggregation and the extent of aggregation varies with individual amyloid proteins.

Effect of Inhibitors on Amyloid-Induced Platelet Aggregation

Because amyloid proteins possess a fibrillar structure and therefore might trap platelets resulting in agglutination rather than aggregation, platelets were treated with the stable prostacyclin analog iloprost to raise cAMP.[23] This treatment completely abolished responses induced by modified amyloid-β, Hb-AGE, and BSA-AGE as well as the aggregation by TRAP (Figure 2A). Thus the change in light transmission of platelet suspensions stimulated with amyloid proteins depends on undisturbed platelet activating sequences. Aggregation is known to be enhanced by formation of thromboxane A_2, followed by stimulation of TP-receptors and by release of ADP from δ granules followed by stimulation of $P2Y_1$ and $P2Y_{12}$ receptors. Blockade of thromboxane A_2 synthesis with the COX-1 blocker indomethacin inhibited aggregation induced by amyloid-β by 30%. Interference with the $P2Y_{12}$ receptor with AR-C69931MX reduced the response by 40%. When both inhibitors were present there was a further reduction of the aggregation. Similar results were found with Hb-AGE and BSA-AGE (Figure 2B and 2C). Thus, depending on the type of amyloid protein, 20% to 50% of the aggregation was the result of direct platelet activation by these proteins, which was enhanced by feedback stimulation by thromboxane A_2 and ADP.

To investigate whether other activating pathways contributed to amyloid-induced aggregation, platelets were treated with different metabolic inhibitors. There was about 50%

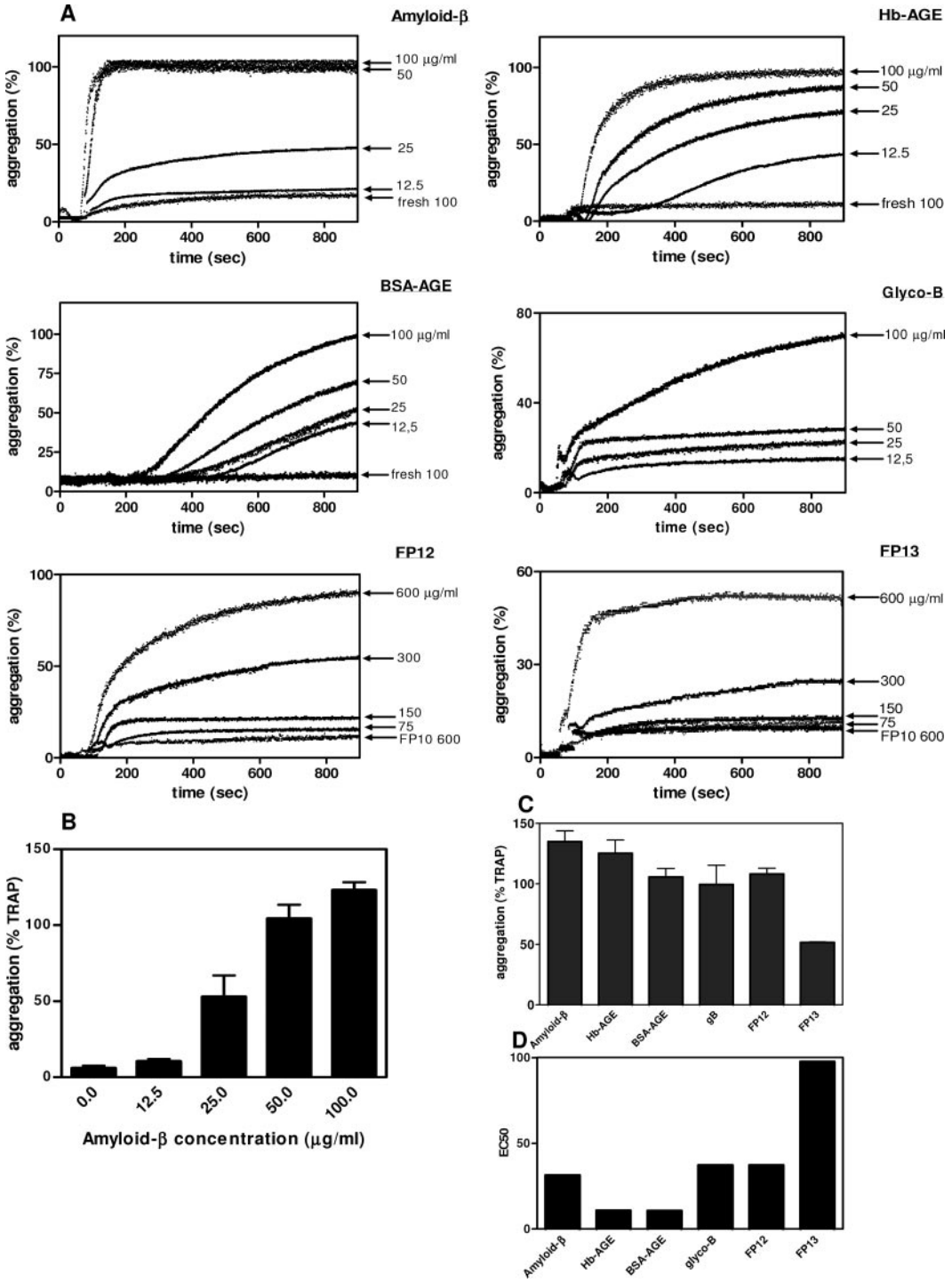

Figure 1. Amyloid-like proteins induce platelet aggregation. A, Platelets were stimulated with Amyloid-β, Hb-AGE, BSA-AGE, and glycoprotein B (Glyco-B) at indicated final concentrations. Freshly dissolved Amyloid-β, Hb, BSA, and amyloid-free fibrin-derived peptide 10 (FP10) served as controls. Note that aggregation scales are different. B, Dose-response relation of Amyloid-β induced aggregation expressed as percentage of aggregation induced by 8 μmol/L TRAP. C, Maximal platelet aggregation induced by 100 μg/mL Amyloid-β, Hb-AGE, BSA-AGE, Glyco-B, and 600 μg/mL fibrin peptide 12 (FP12) and fibrin peptide 13 (FP13). D, EC_{50} values calculated from dose–response curves of each amyloid protein or peptide. (Means±SD, n=5).

inhibition by an inhibitor of mitogen activated kinase (PD98059), an upstream regulator of ERK1/2, 80% to 90% inhibition by an inhibitor of Src family kinases (PP1) and a Ca^{2+} chelator (BAPTA-AM), whereas an inhibitor of the $P2Y_1$ ADP receptor (A3P5PS) induced only 15% to 30% inhibition (supplemental Table I).

Amyloid-Induced Platelet Aggregation Is Inhibited by sRAGE and tPA

Amyloids, including amyloid-β and glycated proteins, are ligands for the receptor for advanced glycated end products (RAGE).[24–27] The presence of soluble RAGE strongly interfered with platelet aggregation induced by amyloid-β, Hb-

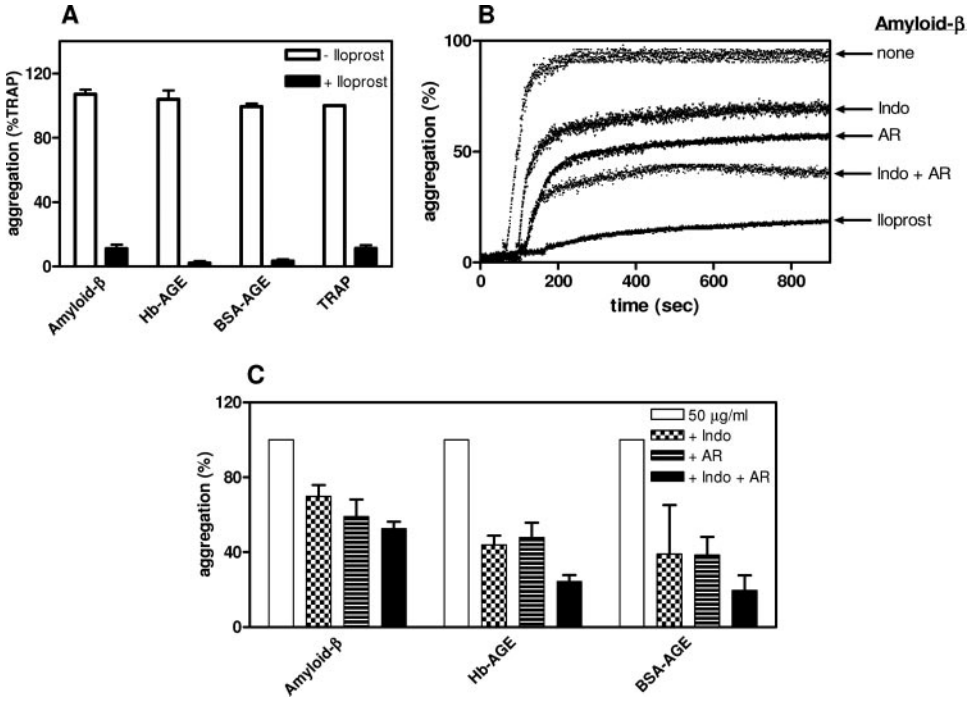

Figure 2. Effect of metabolic inhibitors on amyloid-induced platelet aggregation. A, The PGI_2 analogue Iloprost (20 ng/mL, 2 minutes preincubation, 37°C) inhibits aggregation induced by 50 μg/mL Amyloid-β, 100 μg/mL Hb-AGE, 100 μg/mL BSA-AGE, or the positive control 4 μmol/L TRAP. Data are percentages of TRAP-induced aggregation. B and C, Indomethacin (Indo, 30 μmol/L), AR-C69931MX (AR, 50 nmol/L), or the combination inhibits amyloid-β–induced aggregation. Data are expressed as arbitrary units (B) and percentage of aggregations without additions (C; Means±SD, n=3).

AGE, and BSA-AGE, demonstrating that binding of sRAGE to amyloid-β neutralized its platelet activating properties. In contrast, sRAGE did not interfere with the aggregation induced by TRAP and collagen (Figure 3A, 3C, and 3E). Also tPA binds with high affinity to proteins with amyloid.[17] Again, the platelet activating properties in amyloid-β, Hb-AGE, and BSA-AGE were abolished by tPA but TRAP- and collagen-induced aggregation were not affected (Figure 3B, 3D, and 3F). Taken together, these findings illustrate that amyloid proteins activate platelets through the epitope for sRAGE and tPA binding, which is apparently absent in collagen and TRAP.

Soluble Amyloid Proteins Retain Platelet-Activating Properties

Amyloid proteins show varying degrees of multimerization ranging from oligomers to matured fibrils. To determine the effect of multimerization on the platelet activating properties, preparations of amyloid-β and related proteins were centrifuged to separate soluble and insoluble fractions. The soluble fractions of amyloid-β, Hb-AGE, and BSA-AGE contained fibrils and retained aggregation-inducing properties (supplemental Figure II).

Amyloid Proteins Activate Platelets in Part Through $p38^{MAPK}$, COX-1, and CD36

Because amyloid proteins are known to bind to scavenger receptors,[28] which in many cell types are regulators of MAP-kinases,[29] we investigated whether amyloid-containing proteins and peptides initiated platelet aggregation through $p38^{MAPK}$. Amyloid-induced platelet aggregation was strongly inhibited by the $p38^{MAPK}$ inhibitor SB203580 (Figure 4A). The presence of indomethacin left this inhibition unchanged suggesting that part of the amyloid induced aggregation was the result of thromboxane A_2 formation through $p38^{MAPK}$ and COX-1. Indeed, there was a potent dose-dependent activation of $p38^{MAPK}$ by modified amyloid-β, Hb-AGE, and BSA-AGE, but not by the fresh controls (Figure 4B).

A major scavenger receptor on platelets is CD36 (glycoprotein IV), which is a class-B receptor. To address the role of CD36 in platelet activation by amyloid, platelets from wild-type and CD36-deficient mice were incubated with amyloid-β, Hb-AGE, and BSA-AGE and fresh controls, and the activation of $p38^{MAPK}$ was measured. Again, amyloid proteins induced a 3- to 4-fold activation of $p38^{MAPK}$ in wild-type platelets as seen in their human counterparts (Figure 4C). In CD36-deficient platelets $p38^{MAPK}$ activation was strongly impaired and reached the range found after addition of buffer or fresh proteins. Aggregation experiments showed that amyloid-β–induced aggregation was ≈50% lower in CD34-deficient platelets compared with wild-type controls (Figure 4D). Thus, a major part of amyloid-induced platelet aggregation is the result of signaling through CD36, $p38^{MAPK}$, and COX-1, which are upstream steps in the formation of thromboxane A_2.

Amyloid Proteins Activate Platelets in Part Through Glycoprotein Ibα

Because inhibition of CD36 signaling blocked only part of the amyloid-induced aggregation, the nature of CD36-independent aggregation was investigated in more detail. An inhibitor of fibrinogen binding to αIIbβ3 caused 80% inhi-

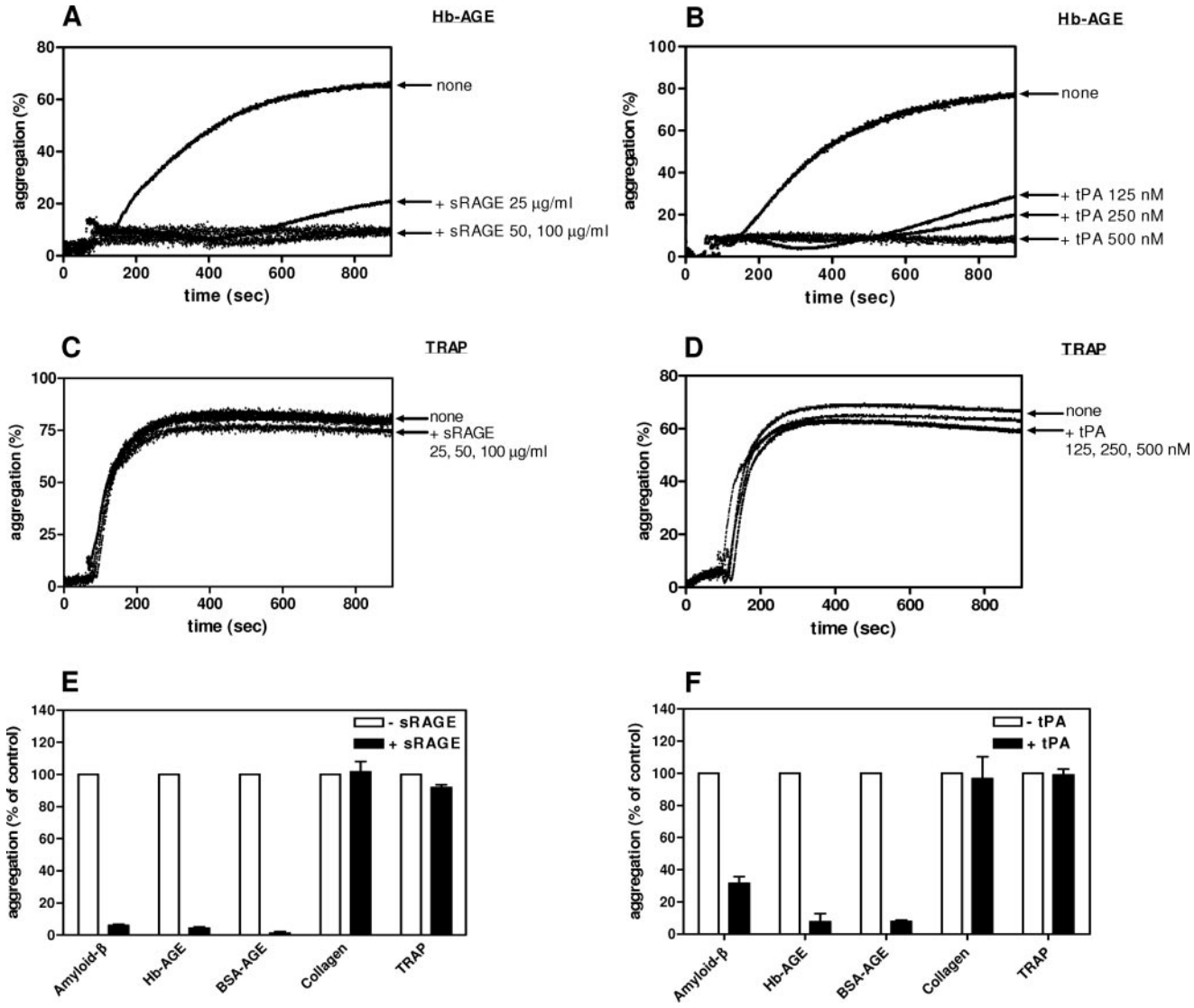

Figure 3. Amyloid-induced platelet aggregation is inhibited by soluble RAGE and tPA. A through D, Platelet aggregation induced by 25 μg/mL Hb-AGE is dose-dependently inhibited by soluble (s)RAGE (A) and tPA (B), but TRAP (4 μmol/L)-induced aggregation is not disturbed (C and D). E and F, sRAGE (100 μg/mL, E) and tPA (500 nmol/L, F) inhibit aggregation induced by Amyloid-β (50 μg/mL), Hb-AGE (25 μg/mL), and BSA-AGE (25 μg/mL), but aggregation by TRAP (4 μmol/L) and collagen (4 μg/mL) is not changed.

bition of TRAP-induced aggregation but left about half of amyloid-induced aggregation unchanged (Figure 5A). This αIIbβ3-independent aggregation is generally referred to as agglutination and typically observed when fixed platelets are treated with a von Willebrand factor (vWF)-ristocetin mixture. In contrast to activated vWF, amyloid proteins failed to induce agglutination of fixed platelets. In intact platelets activated vWF triggers agglutination/aggregation by signal transduction through the vWF receptor glycoprotein (GP) Ibα, which is part of the $GP(Ib)_2V(IX)_2$ complex. Removal of the extracellular part of GPIbα by treatment with Nk-protease reduced aggregation to 40%, the remainder probably reflecting CD36-mediated aggregation (Figure 5B). A similar effect was seen in the presence of anti-GPIbα antibody 6D1 (Figure 5C). RDGS reduced aggregation by Nk-protease–treated platelets to the range found with iloprost-treated platelets, suggesting that signaling through CD36 induced αIIbβ3-mediated aggregation (Figure 5B). When platelets were treated with indomethacin to halt CD36-mediated signaling to thromboxane A_2, RDGS inhibited only half of the remaining aggregation. This indicates that signaling through GPIbα induced aggregation as well as agglutination.

Discussion

The present work demonstrates that a number of unrelated proteins, but with common amyloid properties, induce platelet aggregation. This capacity is absent in the fresh proteins and neutralized by sRAGE and tPA which are specific high-affinity blockers of amyloid. Platelet activation by amyloid-containing proteins is mediated through 2 mutually independent pathways. One pathway signals through CD36, activation of $p38^{MAPK}$, and COX-1, which are intermediates in thromboxane A_2 formation, and starts a normal αIIbβ3-mediated aggregation response. The second pathway signals through the vWF receptor GPIbα and triggers aggregation as well as agglutination.

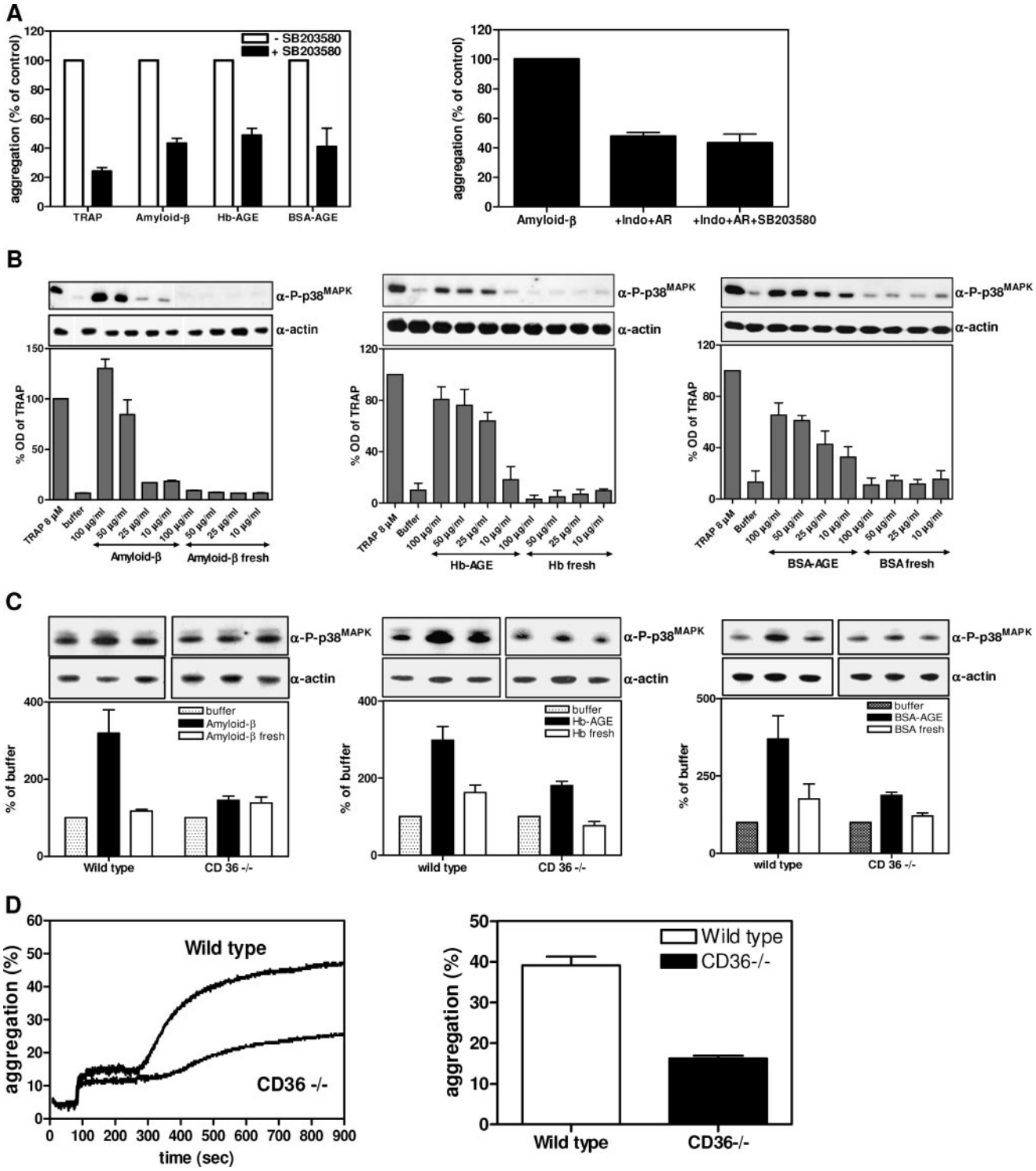

Figure 4. Amyloid proteins activate platelets in part through p38^{MAPK}, COX-1, and CD36. A, left, Platelet aggregation without and with the p38^{MAPK} inhibitor (SB203580, 10 μmol/L) induced by TRAP (8 μmol/L) and by Amyloid-β, Hb-AGE, and BSA-AGE (50 μg/mL each). Right, SB203580 does not induce more inhibition in indomethacin-treated platelets. B, Platelets were stimulated with Amyloid-β, Hb-AGE, and BSA-AGE (1 minute, 37°C). Samples were analyzed by SDS-PAGE and immunoblotted with an antibody against phosphorylated P38^{MAPK}. C, Similar experiments in wild-type and CD36-deficient mice. Densities were expressed as a percentage of samples after addition of vehicle (buffer) and are means±SD, n=4. D, Platelet aggregation induced by Amyloid-β (100 μg/mL) in wild-type and CD34-deficient mice platelets (n=6).

Proteins with amyloid properties initiate a minor shape change response followed by a rather weak aggregation, which is enhanced by thromboxane A_2 formation and ADP secretion. Because CD36-induced aggregation and GPIbα-induced aggregation/agglutination start immediately after platelet contact with amyloid, both pathways signal hand in hand. It is possible that the agglutination induced by GPIbα interferes with the shape change induced by amyloid and

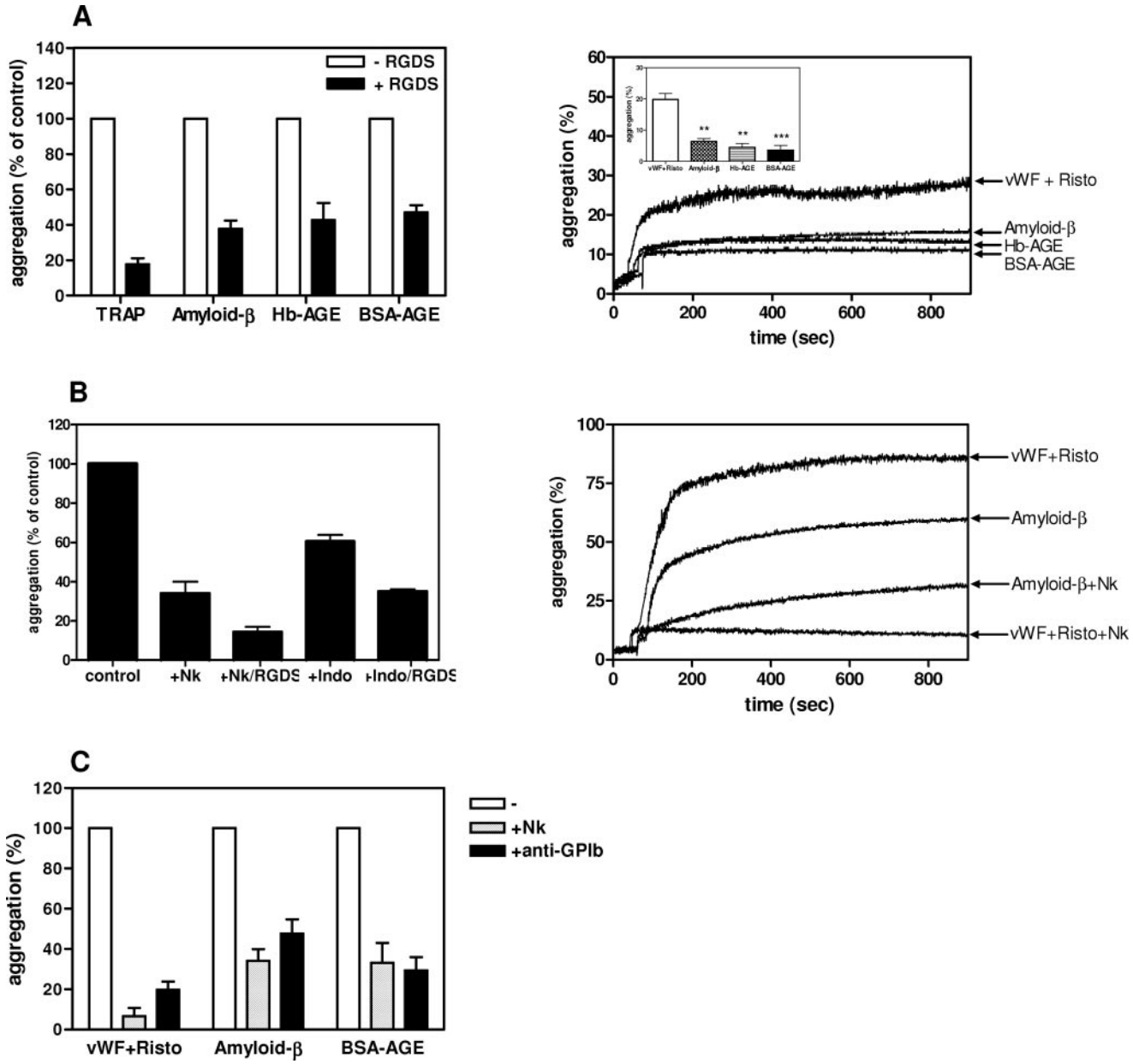

Figure 5. Amyloid proteins activate platelets in part through glycoprotein Ibα. A, left, Effect of αIIbβ3 blockade by RGDS on aggregation induced by TRAP and amyloid proteins. Right, vWF–ristocetin mixture induces agglutination of fixed platelets but amyloid proteins do not. B, left, Cleavage of GPIbα by the snake venom Nk-protease reduces amyloid-β (100 μg/mL)–induced aggregation; RDGS induces a further inhibition. Right, Amyloid-β (100 μg/mL) induces aggregation that is inhibited in part by Nk-protease; vWF–ristocetin induced aggregation is fully inhibited by Nk-protease. C, The anti-GPIbα antibody 6D1induces the same inhibition as Nk-protease.

reduces the fall in light transmission normally seen with platelet activating agents. Aggregation is inhibited by prostacyclin analog, probably reflecting inhibition of $p38^{MAPK}$ signaling because this enzyme is extremely sensitive to elevated cAMP levels.[30] Of interest is the observation that inhibition by iloprost is complete, indicating that also the GPIbα-mediated aggregation/agglutination is blocked by high cAMP. Studies with RDGS reveal that ≈50% of the GPIbα-induced response reflects αIIbβ3-mediated aggregation, which is blocked by elevated cAMP. The finding that also agglutination is absent suggests that it is the result of an earlier, cAMP-sensitive platelet aggregation initiated by GPIbα. Also Ca^{2+} sequestration by BAPTA-AM and inhibition of Src-family kinases by PP1 led to almost complete inhibition, confirming the importance of signaling steps in the induction of aggregation and agglutination by amyloid proteins.

Whereas these inhibitors of intracellular activation pathways induced complete inhibition of amyloid-induced aggregation/agglutination, the interference by extracellular inhibitors was incomplete. Indomethacin and an inhibitor mimicking the action of the clopidogrel metabolite Act-Met induced a 50% to 60% reduction, suggesting that platelet activation by amyloid through GPIbα is largely insensitive to interference with $P2Y_{12}$ signaling. This observation might have important clinical implications because it indicates that

in vivo activation through this pathway is partially unresponsive to aspirin and clopidogrel, which are important drugs against arterial thrombosis. In addition, the agglutination occurs independent of αIIbβ3 and is therefore unresponsive to abcixymab and other antagonists of ligand binding to αIIbβ3.

Although each of the amyloid proteins activates platelets, their capacity to do so varies considerably. Modified amyloid-β and Hb-AGE induce the strongest aggregations whereas fibrin peptide 13 is relatively weak. In contrast, EC_{50} data show that Hb-AGE and BSA-AGE are the most effective proteins inducing aggregation at relatively low concentrations. Combinations of different amyloid proteins added in suboptimal concentrations showed that one protein could enhance the activation by the other. Saturating concentrations failed to show additive effects. Each of the modified proteins was positive in the Thioflavin T binding and Congo Red assays, but it is difficult to assess the role of amyloid in quantitative terms. Amyloid preparations are highly heterogenous in nature, varying from small soluble oligomeric species and amorphous aggregates to large insoluble fibrils. Their structural similarities are limited to binding to Congo Red, Thioflavin T, and tPA, but the precise nature of the binding epitopes for these compounds and for the platelet receptors that respond to amyloid remains to be elucidated.

A better insight in the activating properties of amyloid proteins is also crucial for our understanding of conformational diseases. In Alzheimer disease, amyloid plaques correlate poorly in number, appearance, and distribution with the clinical progression of brain injury, and the small oligomeric species generated from a variety of proteins are better inducers of neuronal damage than the mature amyloid fibrils.[31] Removal of insoluble fractions by high speed centrifugation preserved the property to activate platelets in most of the amyloid containing proteins. An exception was fibrin peptide 12 which lost most of its biological properties after centrifugation. Thus, both the soluble and insoluble form of amyloid proteins contain platelet activating epitopes.

CD36 is a multiligand receptor that binds modified proteins such as amyloid-β and glycated proteins, each known to contain amyloid properties. The recent demonstration of amyloid in oxidized low-density lipoprotein (oxLDL) is in line with these observations[32] because oxLDL is a potent platelet activator.[11] CD36 has a short cytosolic tail and no recognized signaling motif. It is therefore possible that CD36 is simply functioning as an adhesion receptor and thereby bringing the misfolded proteins to other low affinity signaling receptors. Candidates for such a role are multiligand receptors such as low density lipoprotein receptor-related protein (LRP) and RAGE[33] which bind proteins with amyloid, but their contribution to platelet activation is still uncertain. The results also raise the possibility that the absence of CD36 in 5% of the Asian population offers protection against the platelet-activation component of amyloid-based diseases, a result that has important implications.[34]

Recent publications described the presence of amyloid-β in platelet-derived microparticles in healthy subjects[35] and in patients with atherosclerotic disease[36] and type 2 Diabetes Mellitus.[37] These particles are a source of tissue factor, which is the prime initiator of coagulation. This property, together with the capacity of amyloid to activate platelets shown in this study, makes these particles potent triggers for a combined activation of the coagulation cascade and formation of a platelet thrombus.

Protein misfolding and the generation of amyloid occurs in Alzheimer disease, atherosclerosis, diabetes mellitus, and systemic amyloidosis.[38] Our observation that amyloid activates platelets suggests that protein misfolding should be considered as a risk factor for thrombotic disease.

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Disclosures

Declaration: B.B. and M.F.B.G.G. are employees and shareholder of Crossbeta Biosciences B.V., a biotech company developing diagnostics and therapeutics for protein misfolding diseases. Other authors declare not to have conflicting interests.

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