ACS Chemical Neuroscience

Letter

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Reciprocal Molecular Interactions between the Aβ Peptide Linked to Alzheimer's Disease and Insulin Linked to Diabetes Mellitus Type II

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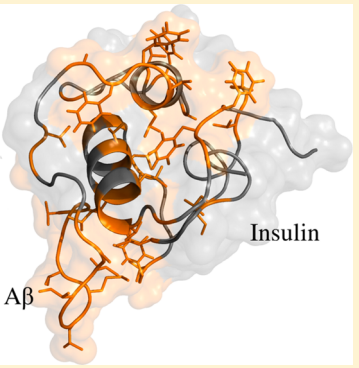
Supporting Information

ABSTRACT: Clinical studies indicate diabetes mellitus type II (DM) doubles the risk that a patient will also develop Alzheimer's disease (AD). DM is caused by insulin resistance and a relative lack of active insulin. AD is characterized by the deposition of amyloid β (Aβ) peptide fibrils. Prior to fibrillating, Aβ forms intermediate, prefibrillar oligomers, which are more cytotoxic than the mature Aβ fibrils. Insulin can also form amyloid fibrils. In vivo studies have revealed that insulin promotes the production of Aβ, and that soluble Aβ competes with insulin for the insulin receptor. Here, we report that monomeric insulin interacted with soluble Aβ and that both molecules reciprocally slowed down the aggregation kinetics of the other. Prefibrillar oligomers of Aβ that eventually formed in the presence of insulin were less cytotoxic than Aβ oligomers formed in the absence of insulin. Mature Aβ fibrils induced fibrillation of soluble insulin, but insulin aggregates did not promote Aβ fibrillation. Our study indicates that direct molecular interactions between insulin and Aβ may contribute to the strong link between DM and AD.



KEYWORDS: *Amyloid β peptides, Alzheimer's disease, insulin, diabetes mellitus type II, cross amyloid interaction, fibrillation*

Diabetes Mellitus type II (DM) and Alzheimer's disease (AD) are severe and eventually fatal disorders affecting mostly elderly people.[1] AD is associated with deposition of aggregates of amyloid β (Aβ) peptides and tau proteins, while DM results from metabolic dysfunctions of insulin secretion or insulin action.[2] Clinical and epidemiological studies have demonstrated a strong link between the two disorders: DM patients have, for example, a doubled risk of developing AD.[3−5] Moreover, cerebrovascular dysfunction induced by DM or other factors aggravates the loss of cognitive functions associated with AD.[6] For DM, patient treatment with insulin may increase the risk of AD, although a positive effect of insulin on cognitive performance has also been found,[5,7,8] including a report on synapse protection from Aβ toxicity by insulin signaling.[9]

At a molecular level, in vivo and in vitro studies have shown that insulin and Aβ affect each other's production, function, and degradation.[2,7] Insulin can increase the level of extracellular Aβ by stimulating the activity of γ-secretase in Aβ secretion,[10,11] and may inhibit Aβ degradation by blocking the activity of the insulin-degrading enzyme, which in neuronal and microglial cell cultures also degrades Aβ.[12] Insulin furthermore causes the Aβ-clearing lipoprotein receptor-related protein 1 (LRP-1) to translocate to the membrane in hepatocytes, thereby facilitating hepatic clearance of plasma Aβ.[13] Insulin signal transduction plays a role in the pathogenesis of AD, as it regulates the activity of hyperphosphorylated tau in the formation of AD neurofibrillary tangles via its signaling factor, glycogen synthase kinase (GSK).[2,7] Aβ, on the other hand, competes with insulin for binding to the insulin receptor, and inhibits the effect of insulin on the secretion of the Aβ precursor protein.[14,15] By reducing the signal transduction activity of the insulin receptor, Aβ may induce insulin resistance.[16] Although Aβ and insulin mutually modulate each other's functions in the brain, the underlying molecular mechanisms of insulin/Aβ cross-regulation remain to be fully clarified.

Here, we studied molecular interactions between Aβ and insulin in vitro. Both Aβ and insulin aggregate into amyloid fibrils under suitable conditions,[17] and we identified fibrils either by solid state atomic force microscopy (AFM) images or by thioflavin T (ThT) fluorescence assays in solution.[18] We chose to study AB/insulin interactions in the three Aβ aggregation forms known to be relevant in vivo: monomeric, oligomeric, and fibrillar. (I) Monomeric Aβ/insulin interactions were investigated via NMR spectroscopy and HTRF insulin assays. (II) The morphology and toxicity of oligomeric Aβ/insulin aggregates and coaggregates were examined by AFM imaging and cell viability assays. (III) The fibrillation of Aβ in presence and absence of insulin was studied by kinetic ThT fluorescence assays, AFM imaging, and CD spectroscopy.

RESULTS

I. Interactions between Monomeric Aβ and Monomeric Insulin. The molecular interactions between Aβ40 and insulin were monitored by NMR spectroscopy. Solution state

Received: December 8, 2015
Accepted: January 19, 2016
Published: January 19, 2016

DOI: 10.1021/acschemneuro.5b00325
ACS Chem. Neurosci. 2016, 7, 269−274

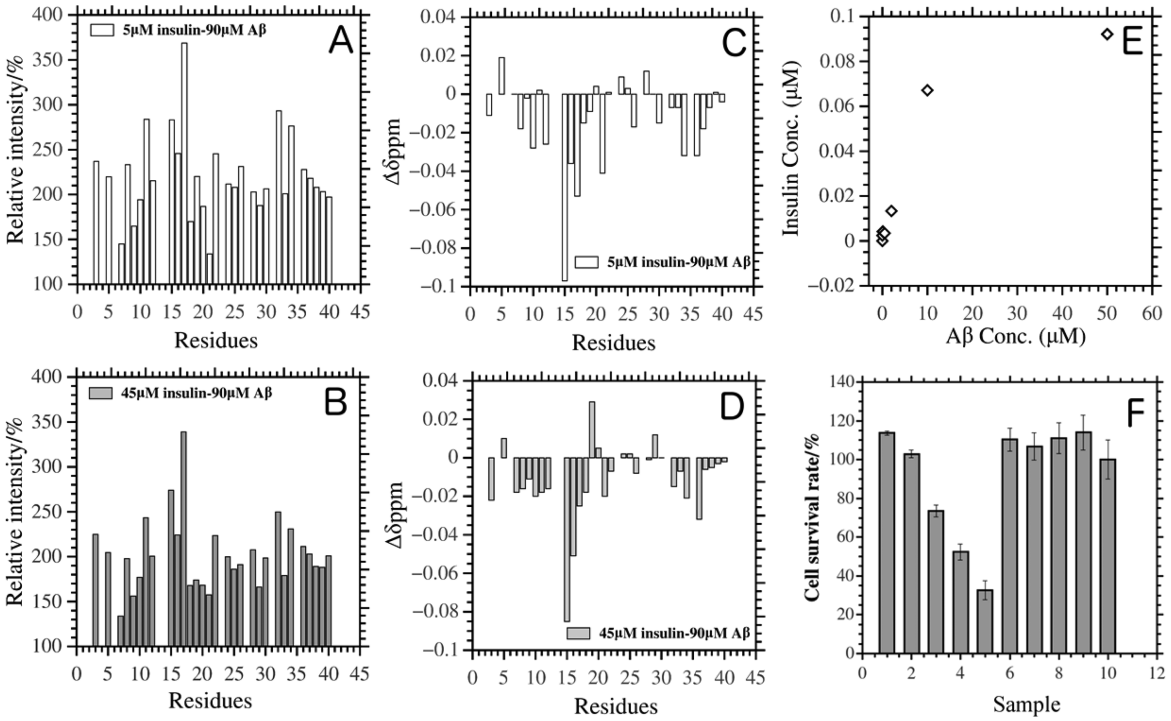


Figure 1. (A–D) NMR spectroscopy data. Relative crosspeak intensities (A,B) and chemical shift change (C,D) differences in $^{1}H-^{15}N$ HSQC spectra of 100 μM ^{15}N-labeled Aβ(1–40) in 20 mM sodium phosphate buffer at pH 7.3 and +5 °C, measured before and after addition of 5 μM (A,C) and 45 μM (B,D) insulin. (E) HTRF insulin assay of active insulin. 0.1 μM insulin was incubated for 30 min at room temperature with 0, 0.5, 2, 10, or 50 μM monomeric Aβ. The sample was centrifuged for 5 min at 20 000*g*. The supernatant of soluble insulin was measured using the HTRF kits. The loss of signal in the absence of Aβ was not due to nonspecific absorption (see Table S1). (F) Luminescent cell viability assay of SH-SY5Y cells exposed to varying levels of insulin and Aβ oligomers (Aβ with/without insulin oligomers were obtained by using the same incubation methods as prepared for AFM experiments (Figure S3) by incubation for 100 min at 25 °C without shaking conditions and then were added to the cell wells at the different concentrations). Sample 1, 20 μM insulin/20 μM Aβ; sample 2, 5/20 μM insulin/Aβ; sample 3, 1/20 μM insulin/Aβ; sample 4, 0.2/20 μM insulin/Aβ; sample 5, 20 μM Aβ; sample 6, 20 μM insulin; sample 7, 5 μM insulin; sample 8, 1 μM insulin; sample 9, 0.2 μM insulin; sample 10, control. Cell survival was measured after 48 h.

$^{1}H-^{15}N$ HSQC spectra for 90 μM ^{15}N-labeled Aβ(1–40) together with 0, 5, or 45 μM unlabeled insulin show typical random coil resonances (Figure S1, Supporting Information), in agreement with our previous studies where these $^{1}H-^{15}N$ amide crosspeaks have been assigned.[19] Upon addition of 5 or 45 μM insulin the intensities of these crosspeaks increased up to ~200% (Figure 1A,B). A corresponding intensity increase is visible also in the 1D ^{1}H spectra (Figure S2). As large protein aggregates are "invisible" to direct NMR measurements,[20] these results suggest that insulin can release NMR-detectable monomeric Aβ peptides from more aggregated states. Adding insulin also induced certain chemical shift changes for the NMR crosspeaks, especially for Aβ residues 15–17 and 34–36 (Figure S1a,b and 1C,D). These regions, which are known to be selectively involved in Aβ self-aggregation,[21] also displayed the largest intensity changes, suggesting this is where insulin preferentially interacts.

To investigate whether low molecular weight Aβ affects the aggregation of insulin, we used an immunoassay that provides a measure for the amount of soluble insulin: the HTRF. Two monoclonal antibodies, one labeled with Eu^{3+}-cryptate and the other with XL665, recognize distinct epitopes of insulin. When the two antibodies simultaneously bind an insulin molecule, their close proximity allows Förster resonance energy transfer (FRET) to occur between Eu^{3+}-cryptate and XL665. We found the FRET signal to increase proportionally with the concentration of monomeric insulin. We found also that incubated insulin aggregated into clusters large enough to spin down at 20 000*g* for 5 min (and ascertained that the loss of signal was not due to nonspecific absorption to the incubation vessels, see Table S1). When increasing amounts of monomeric Aβ was added to the sample, the FRET results showed increasing amounts of monomeric insulin present after incubation and spinning (Figure 1E), indicating that Aβ inhibits insulin aggregation. We conclude that monomeric Aβ and monomeric insulin mutually interfere with each other's aggregation, and reciprocally shift each other's aggregation equilibria toward monomeric forms.

II. Insulin Provides Protection against Cell-Toxic Aβ Oligomers. To test the effect of insulin on Aβ oligomerization, 100 μM Aβ with and without 20 μM insulin, as well as 20 μM insulin alone, were incubated in PBS buffer at room temperature for 100 min.[22] AFM images showed that the globular Aβ oligomers that formed both with and without insulin had very similar morphologies, with sizes in the range 2–15 nm (without insulin) and 2–12 nm (with insulin) (Figure S3a,c). Formation of some fibrils was also observed (Figure S3a,c), and the control experiment with 20 μM insulin but without Aβ showed some smaller oligomers as well (Figure S3b).

To assess the toxicity of these aggregates, we incubated human neuroblastoma cells (SH-SY5Y) with the Aβ/insulin oligomers. Light microscopy showed the cells to adopt abnormal shapes in the presence of oligomers formed by Aβ alone (Figure S3d). In contrast, cells exposed to oligomers

DOI: 10.1021/acschemneuro.5b00325
ACS Chem. Neurosci. 2016, 7, 269–274

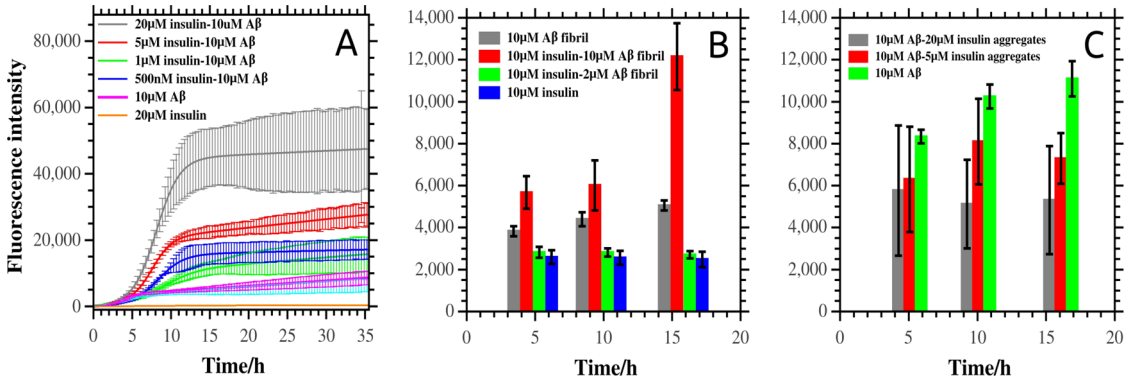


Figure 2. ThT fluorescence results for Aβ(1−40) and insulin coaggregation (37 °C, 50 mM Tris buffer, pH 7.4, without agitation). (A) Fibrillation kinetics of 10 μM monomeric Aβ in the presence of 500 nM, 1 μM, 5 μM, or 20 μM insulin (37 °C, 50 mM Tris buffer, pH 7.4, without agitation). The kinetic curve for 20 μM insulin alone lies on the baseline. (B) ThT fluorescence intensity at different times for 10 μM insulin incubated (37 °C, 50 mM Tris buffer, pH 7.4 without agitation) together with 2 μM or 10 μM already fibrillated Aβ (50 μM Aβ aggregated for 18 h, 200 rpm agitation, 30 °C, 50 mM Tris buffer, pH 7.4). (C) ThT fluorescence intensity at different times for 10 μM Aβ incubated (37 °C, 50 mM Tris buffer, pH 7.4 without agitation) together with 5 μM or 20 μM insulin aggregates (100 μM insulin aggregated for 18 h, 37 °C, in 5 mM EDTA, 50 mM Tris buffer, pH 7.4, without agitation[17]). Error bars show standard deviations based on three measurements. After 15 h, 10 μM Aβ fibril in panel (B) gave a signal of 5000 units (rising by 25% compared to the signal after 5 h), whereas in panel (C), 10 μM Aβ after 15 h gave a signal of 11 500 units (rising by 30% compared to the signal after 5h). The experiments in panels (A), (B), and (C) were performed on different days and the difference in absolute signal was probably caused by slightly different conditions of fibril preparation and the sensitivity of ThT fluorescence. The data in panel (A) was obtained by subtracting buffer reference for fitting, but the data in panels (B) and (C) was presented without subtraction.

Table 1. Lag Times and Transition Times for Amyloid Fibrillation of 10 μM Aβ(1-40) Peptide in the Presence and Absence of Insulin[a]

	20 μM insulin/10 μM Aβ	5 μM insulin/10 μM Aβ	1 μM insulin/10 μM Aβ	0.5 μM insulin/10 μM Aβ	0.1 μM insulin/10 μM Aβ	10 μM Aβ
transition time (h)	5.97	5.10	8.42	6.52	4.44	3.01
lag time (h)	11.08	10.05	13.41	11.58	6.32	5.35

[a]Time constants were obtained from fitting to a modified Boltzmann equation[19] (see the Supporting Information).

formed by Aβ and insulin (Figure S3f) or by insulin alone (Figure S3e) exhibited normal morphologies. Cell viability assays revealed that Aβ-containing oligomers (whether grown in presence or absence of insulin) induced cell death, compared to cells that were left untreated or exposed to insulin alone (Figure 1F). However, oligomers formed by Aβ and insulin together were less toxic to the cells than pure Aβ oligomers formed from the same Aβ concentration, and the higher the insulin concentration was, the more cells survived.

Taken together, these results showed that Aβ oligomers grown in the presence and in the absence of insulin differed in toxicity, despite their similar morphologies. However, the molecular mechanisms by which insulin/Aβ coaggregates display reduced cytotoxicity remain to be explored.

III. Aβ Fibrils Promote Insulin Aggregation. To investigate possible Aβ/insulin coaggregation, kinetic ThT fluorescence measurements were carried out (Figure 2). Induced ThT fluorescence is used here as a probe for the presence of amyloid/fibril structures.[23,18] The observed ThT fluorescence curves were fitted to a modified Boltzmann equation to yield transition and lag times (Table 1).[19] Fibrillation of 10 μM Aβ(1−40) with 0−20 μM monomeric insulin showed that the lag time, transition time, and final ThT fluorescence intensity all increased with increasing insulin concentrations. These observations suggested two conclusions: (i) the increase in lag time indicated that insulin makes Aβ less prone to aggregate into amyloid as probed by ThT fluorescence, which is in line with our results reported in subsection I, and (ii) the increase in final fluorescence intensity: indicated that more amyloid fibrils were formed, so insulin should have been incorporated into the fibrils or caused to fibrillate by presence of the Aβ fibrils (see below). This demonstrated Aβ/insulin interaction during Aβ aggregation, and indicated that in the presence of Aβ, insulin forms ThT-active aggregates (or coaggregates) under conditions where it otherwise does not aggregate. Under these conditions 20 μM insulin alone did not give rise to measurable induced ThT fluorescence (Figure 2A).

When we added monomeric insulin to 2 or 10 μM preformed Aβ fibrils, we observed a clear increase in final fluorescence intensity especially with the higher Aβ fibril concentration (Figure 2B). The most likely explanation is again that insulin formed ThT-active aggregates under the influence of Aβ fibrils, either by fibril seeding or by secondary nucleation, for example, on the fibril surfaces, or that Aβ and insulin formed mixed coaggregates with different properties as to ThT binding and/or induction of ThT fluorescence. When monomeric Aβ was incubated together with preincubated insulin aggregates, however, the resulting ThT fluorescence levels were lower than for pure Aβ samples, suggesting that insulin aggregates prevent amyloid Aβ fibrillation (Figure 2C). As insulin contains stabilizing Zn(II) ions,[17] and as Zn(II) is known to interact with Aβ and modulate the aggregation and toxicity of Aβ aggregates,[24] we investigated whether insulin-bound zinc might be interacting with Aβ and affect its fibrillation. With the addition of 5 mM EDTA, which would chelate any free zinc ions, however, EDTA had no effect on the fibrillation assays (Figure S4). We conclude that Aβ aggregation was not affected by insulin-bound zinc.

DOI: 10.1021/acschemneuro.5b00325
ACS Chem. Neurosci. 2016, 7, 269−274

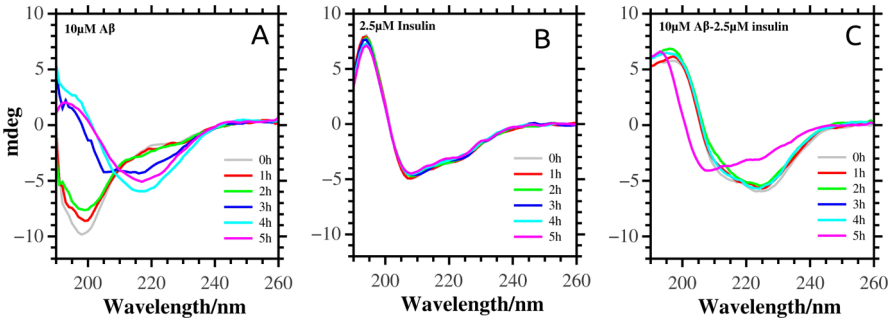


Figure 3. CD spectra showing secondary structure transitions for Aβ(1−40) and insulin incubated over time in 5 mM sodium phosphate buffer at pH 7.3, at 37 °C with magnetic stirring at 908*g*. (A) 10 μM Aβ alone; (B) 2.5 μM insulin alone; and (C) 10 μM Aβ together with 2.5 μM insulin.

CD spectroscopy was used to measure secondary structure transitions of Aβ in the presence and absence of insulin (Figure 3). Without insulin, Aβ retained its mainly random coil CD signature for about 2 h, and then began to shift to a β sheet conformation (Figure 3A). Insulin alone, on the other hand, did not undergo changes under these experimental conditions, but remained α-helical throughout (Figure 3B). When 10 μM Aβ was mixed with 2.5 μM insulin, however, a β-sheet CD signal with a minimum around 225 nm was immediately observed (Figure 3C). This β-sheet CD signal was very different from the random coil and α-helical signals for isolated Aβ and insulin, respectively, and it remained constant for about 3 h. We conclude that mixing Aβ with insulin induces immediate conformational transitions into β-sheet structures, most likely for both peptides. At the end of the observation period the CD spectrum strongly resembles that of insulin alone, albeit with lower intensity. A plausible explanation is that the Aβ peptides and some insulin then has formed aggregates too large to give a CD signal; what remains is a fraction of free and soluble insulin.

The effect of insulin on Aβ aggregate morphology was visualized with AFM imaging. In the presence of insulin, seemingly normal Aβ fibrils were formed (Figure 4), however

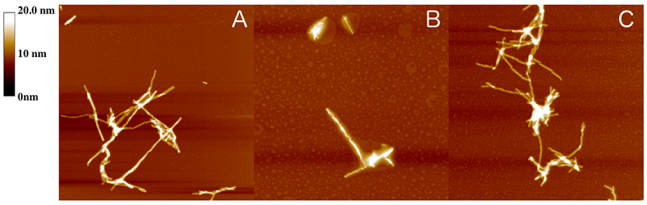


Figure 4. AFM images (1.5 × 1.5 μm^2) of Aβ(1−40) with/without insulin fibrillated in 50 mM Tris buffer, pH 7.4, at 30 °C and shaking at 200 rpm. (A) 50 μM Aβ alone after 5 h; (B) 50 μM Aβ together with 25 μM insulin after 5 h; and (C) 50 μM Aβ together with 100 μM insulin after 18 h.

often in conjunction with more globular structures (Figure S5). The control morphologies of insulin alone has been shown as globular structures in Figure S6. Presumably, Aβ fibril is in conjunction with insulin globular oligomer and forms the conjunction structure. Eventually, the globular oligomer can be coassembled into fibril and more ThT fluorescence intensity was observed in Figure 2A.

DISCUSSION

Our experiments revealed various interactions between monomeric and aggregated forms of Aβ and insulin, indicating links between AD, insulin resistance, and DM at a molecular level. The insulin activity and fibrillation process were affected by Aβ, and insulin and Aβ coaggregated into fibrillar oligomers. Insulin affected the structure, solubility, and toxicity of Aβ and its aggregates. Although aggregated insulin did not strongly affect Aβ aggregation, monomeric insulin immediately induced conformational transitions in monomeric Aβ. Both the NMR and HTRF results showed that insulin/Aβ interactions increased the solubility of monomeric Aβ and insulin, with the monomeric forms being released from oligomers. Prefibrillar Aβ oligomers were also less toxic when formed in the presence of insulin. The ThT assays showed increased lag and transition times for Aβ/insulin mixtures, which is consistent with insulin shifting the Aβ aggregation equilibrium toward monomers. The final ThT intensities are however higher, suggesting that also the added insulin eventually forms amyloid material.

Recently, Saio et al.[25] and Karagöz et al.[26] found that trigger factor chaperone and heat shock protein 90 interact with transiently exposed regions of aggregates of tau and certain other proteins. These interactions increased the solubility and further inhibited the aggregation of unfolded proteins.[25,26] Our NMR data support a similar mechanism in which the interaction of insulin with aggregation-prone Aβ regions increases Aβ solubility, suggesting that insulin has chaperone-like qualities, at least when interacting with Aβ. This observation is not unique: we recently reported similar behavior for other proteins without known chaperone activity.[27,28] Our FRET assay confirms the reciprocal solubilization of insulin by Aβ. However, unlike the chaperone proteins, insulin itself can form amyloid fibrils. We therefore propose that the reciprocal solubilization of insulin and Aβ enables them to form less aggregation-prone complexes. Our ThT fluorescence assays confirmed that the fibrillation kinetics of Aβ in the presence of insulin were significantly slower than that of Aβ alone.

The concentration of insulin is ∼0.06−1 nM in the blood[29] and about 10−100 times higher in cerebrospinal fluid (CSF).[30] There is still no consensus as to the accurate levels of insulin in the AD brain. While one study reported lower insulin levels in CSF of Alzheimer patients,[31] another study found no significant differences in insulin levels between AD and healthy brains.[32] For Aβ, the CSF levels of Aβ40 are slightly higher in AD than in healthy brains (both in the range 1−10 nM), while the Aβ42 levels are lower (100/200 pM for AD/normal brains).[33] Diabetes patients do not display significantly altered Aβ levels, although the CSF tau levels are higher.[34,35] The concentrations used in our experiments are higher due to the requirements of our instruments and assays, but the observed interactions should occur also at lower concentrations and thereby in vivo,

DOI: 10.1021/acschemneuro.5b00325
ACS Chem. Neurosci. 2016, 7, 269−274

although possibly at slower reaction rates. It is clear from our measurements that insulin is captured by Aβ fibrils and incorporated into Aβ-amyloid through cross-β interactions. It is likely that misfolded insulin accumulates in Aβ amyloid in vivo, as the off-rate of insulin from fibrils must be very small. Even at (sub-)micromolar levels, the Aβ peptide had an observable positive effect on the conversion from insulin aggregates to antibody detectable insulin, as indicated by our immunoassay, suggesting that the reciprocal interactions of the Aβ peptide and insulin have relevance also at physiological concentrations.

Combining our observations with literature reports, we propose the following functional interactions between insulin and Aβ. (1) Insulin increases the production of Aβ by activating γ-secretase[10,11] and inhibits Aβ degradation by the insulin degrading enzyme (IDE).[12] (2) Insulin decreases the cytotoxicity of Aβ aggregates and kinetically stabilizes Aβ in a soluble monomeric form. (3) Fibrillar Aβ promotes the fibrillation of insulin. Aβ has previously been suggested to compete with insulin for binding to the insulin receptor.[14,15] Our current results however suggest that reduced receptor binding might not only be caused by competition, but also by formation of Aβ/insulin complexes that do not fit the receptor binding site. Furthermore, the observation that Aβ can promote insulin fibrillation prompts the intriguing possibility that AD might contribute to DM progression.

In conclusion, the present results suggest that Aβ and insulin coaggregation promotes overall amyloid formation, that these coaggregates are less toxic than pure Aβ oligomers, and that insulin shifts the Aβ aggregation equilibrium toward soluble monomers, away from harmful aggregates. Although at least the latter two interactions are expected to mitigate Aβ toxicity, insulin is known to promote AD progression by inducing overproduction of Aβ peptide in vivo.[5,7,8] This is most likely the dominating effect underlying DM and AD co-occurrence. Yet, the contrasting and reciprocal functional molecular interferences demonstrated here between insulin, Aβ, and their amyloid formation pathways may provide mechanistic clues to help in understanding the details of the connection between the two diseases.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschemneuro.5b00325.

Experimental details; additional NMR, ThT, AFM measurements, cell morphology, and FRET results as Figures S1−S6 (PDF)

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Author Contributions

Conceived and designed the experiments: J.L., S.K.T.S.W., A.G., and J.P.A. Performed the experiments and Analyzed the data: J.L. and S.K.T.S.W. Contributed reagents/materials/analysis tools: J.P.A. Wrote the paper: J.L. and J.P.A. Commented on and edited the manuscript: S.K.T.S.W. and A.G.

Funding

The work was funded by grants from the Magnus Bergvall foundation to S.K.T.S.W., and from the Swedish Research Council and the Brain Foundation to A.G.

Notes

The authors declare no competing financial interest.

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DOI: 10.1021/acschemneuro.5b00325
ACS Chem. Neurosci. 2016, 7, 269−274