Alzheimer's β-Amyloid Peptides Compete for Insulin Binding to the Insulin Receptor

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The amyloid-β (Aβ) peptide is neurotoxic and associated with the pathology of Alzheimer's disease (AD). We investigated the effect of Aβ peptides on insulin binding to the insulin receptor because it is known that (1) Aβ and insulin are both amyloidogenic peptides sharing a common sequence recognition motif, (2) Aβ and insulin are substrates for the same insulin degrading enzyme, and (3) impaired glucose metabolism is a characteristic event in the pathology of AD. We discovered that Aβ_{1-40} and A$\beta_{1-42,}$ the main physiological forms, reduced insulin binding and receptor autophosphorylation. The reduction in binding was caused by a decrease in the affinity of insulin binding to the insulin receptor. This reduction was independent of the receptor concentration. The reverse, control peptide Aβ_{40-1} did not reduce insulin binding or insulin receptor autophosphorylation. These results demonstrate that Aβ is a direct competitive inhibitor of insulin binding and action. We speculate that the increased levels of Aβ in Alzheimer's disease may be linked to the associated insulin resistance that has been observed previously in this disease.

Key words: amyloid-β peptide; insulin binding; insulin receptors; Scatchard analysis; Alzheimer's disease; diabetes

The deposition and accumulation of the amyloid-β (Aβ) protein [Aβ as plaques in brains is a hallmark of the pathology of Alzheimer's disease (AD) (Masters et al., 1985)]. Aβ_{1-40} is the main protein constituent in the amyloid associated with the cerebral vasculature, whereas Aβ_{1-42} is mainly found in the senile plaque cores in the AD brain. The neurotoxicity of Aβ may be related to its propensity to aggregate into β-pleated sheet structures in amyloid fibrils at high concentrations of the peptide (Pallitto et al., 1999; Lorenzo et al., 2000), although an emerging view is that the soluble peptide, including dimers and small aggregates, is also toxic (Klein et al., 2001). Interestingly, there is a novel homology among amyloid-forming peptide sequences that include insulin (Turnell and Finch, 1992). Examination of the sequences of Aβ insulin and islet amyloid polypeptide reveals that they share a consensus sequence that is in common with the substrates for insulin-degrading enzyme (Kurochkin, 1998). The insulin degrading-enzyme has been associated with AD because of its ability to degrade Aβ (Qiu et al., 1998). Considering these links between insulin and Aβ, it is interesting to note that a characteristic event of AD is reduced glucose metabolism in the brain (Minoshima et al., 1999) that may be associated with a defect of insulin action (Frolich et al., 1998). In this study, we explore the possibility that Aβ may directly compete for insulin binding to insulin receptors, and in doing so may in itself contribute directly to the impaired glucose utilization of the AD brain.

MATERIALS AND METHODS

Human Aβ_{1-40}, Aβ_{40-1}, and Aβ_{1-42} were synthesized, purified, and then characterized by HPLC, amino acid analysis, and mass spectroscopy by the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). ^{125}I-sodium iodide, γ^{32}P-ATP, Sephadex G50, Amersham-ECL film, and anti-mouse immunoglobulin horseradish peroxidase-linked F(ab$'$)$_2$ fragment were purchased from Amersham Biosciences. Wheat germ agglutinin (WGA) Sepharose 6B, ATP, and human insulin were purchased from Sigma (St. Louis, MO). The anti-β subunit of insulin receptor antibody, IRβ29 B4, and Dynabeads M-450 cross-linked by anti-IRβ29-B4-IgG (anti-mouse) were purchased from Dynal Pty Ltd. The luminal enhancer was purchased from Pierce (Rockford, IL). The monoclonal antibody, W02 (class IgG2b), was kindly donated by Prof. Colin Masters (University of Melbourne, Melbourne, Australia) and Prof. Konrad Beyreuther (Heidelberg University, Heidelberg, Germany).

Preparation and size exclusion chromatography of Aβ solutions. Aβ_{1-40} (2.3 mg) was dissolved in 1 ml of 50 mM HEPES, pH 7.4, and kept on ice. A 20 μl aliquot of this Aβ_{1-40} solution was diluted with 180 μl of 50 mM HEPES, pH 7.4, in the presence or absence of 0.1% bovine serum albumin (BSA). The Aβ solutions were incubated for 16 hr at 4°C and then centrifuged for 10 min at 13,000 $\times$ g at 4°C. The supernatants

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containing soluble Aβ then were resolved on a Sephadex G-50 column (1×30 cm). The elutes were collected at a constant flow rate of 30 ml/hr with 50 mM HEPES, pH 7.4, in either the presence or absence of 0.1% BSA. The column was previously calibrated with dextran blue ($V_o = 30$ kDa), cytochrome c (12.4 kDa), aprotinin (6 kDa), and potassium dichromate ($V_s = 1500$ kDa). The calibration curve was used to determine the molecular mass of each Aβ peak. Immunoblotting and quantitation of Aβ in the fractions were performed using the method of Fonte et al. (2001).

Preparation of human placental plasma membranes. Human placental plasma membranes (HPPM) were used as a rich source of human insulin receptors that are essentially indistinguishable from insulin receptors derived from other tissues, including brain (Roth et al., 1986). Placentas were collected from King Edward Memorial Hospital (Subiaco, WA, Australia) within 1 hr of delivery. Placental plasma membranes were prepared using the method of Davies et al. (1981).

Analysis of the Aβ chromatographic data. The relative distribution of Aβ between the chromatographic peaks was estimated by assuming that the data were composed of three peaks. Both fully Gaussian and fully Lorenzian iterative fits to the data were evaluated using the Leverson-Marquart algorithm. The Gaussian distribution provided the best fit because the Lorenzian function had too much head/tail intensity and lead to unrealistically high "peak maxima." There was no evidence of significant leading/tailing, and the normally distributed model was applicable. The distribution of Aβ between the three peaks was calculated from the areas determined by integration of the Gaussian fitted peaks.

Preparation of partially purified insulin receptor. Chinese hamster ovary cells overexpressing the human insulin receptor (CHO_{IR}) were cultured in Hams–F12 medium, pH 7.4, containing 2 mM glutamine, 14 mM $NaHCO_3$, 19 mM penicillin, 10 μM geneticin, and 30 μM streptomycin with 10% fetal calf serum in 5% CO_2 at 37°C. The confluent cells were washed in ice-cold PBS and solubilized in 20 mM HEPES, pH 7.4, containing 1.5 mM Triton X-100, 5 mM EDTA, 50 mM NaCl, 50 mM NaF, 7.7 μM aprotinin, 0.1 μM leupeptin, 1 mM phenylmethylsulfonylfluoride (PMSF), and 30 mM β-glycerophosphate. The solubilized protein was then added to a column containing 1 ml of WGA Sepharose and rotated at 4°C for 2 hr. The insulin receptors were eluted in 5 mM HEPES, pH 7.8, containing 150 mM NaCl, 0.1 mM PMSF, 1.5 mM Triton X-100, and 300 mM N-acetyl-D-glucosamine at 4°C. The eluted insulin receptors were then stored at −80°C.

3-(^{125}I) iodotyrosyl A14 insulin binding to HPPMs in the presence or absence of competitors. Human insulin was iodinated, and then the 3-(^{125}I)iodotyrosyl-A14-insulin was purified from the iodination mixture following the procedure of De Leo and Helmerhorst (1992). Human placental plasma membranes (50 μl) were incubated with 50 μl of 3-(^{125}I) iodotyrosyl A14-insulin (5 fmol), 50 μl of human insulin (0–1 nmol), and 50 μl of Aβ (0, 4, or 10 nmol). All solutions were buffered in 50 mM HEPES, pH 7.8, containing 5 U of bacitracin and 0.15 μM BSA. After the incubation, the insulin receptors bound with ^{125}I-insulin were precipitated by the addition of 300 μl of an ice-cold solution of 28 mM polyethylene glycol 6000 and 27 mM bovine-γ-globulin. This solution was mixed thoroughly and incubated for 15 min on ice. The solution was then centrifuged at 4°C for 10 min at $7000 \times g$. The pellet was measured for radioactivity in a Hewlett Packard gamma counter.

Immunoprecipitation and autophosphorylation of insulin receptor. Autophosphorylation of insulin receptors was based on the method of Van Obberghen and Kowalski (1982). Insulin receptors (12.5 μl) purified by WGA affinity chromatography were incubated for 15 min at 25°C with 2.5 μl of insulin (0 or 40 nM) in the presence or absence of a final concentration of 50 μM Ab. Then 2.5 μl of γ^{32}P-ATP (18.5×10^4 Bq) in phosphorylation solution containing 0.1 mM ATP and 10 mM sodium orthovanadate was added. The reaction was terminated after 25 min at 25°C by the addition of a mixture of 1.5 μl of 0.5 M EDTA and 3.4 μl of 1.5 M NaCl. Insulin receptors were immunoprecipitated by incubation with 10 μl of anti-β subunit of insulin receptor antibody (IRβ-29-B4) (1 μg in PBS) for 16 hr at 4°C and then incubation for 90 min at 4°C with 25 μl of Dynabeads M-450 (1:1 in PBS) cross-linked by anti-IRβ-29-B4 (anti-mouse IgG). The immunoprecipitates were electrophoresed on 8% SDS gels. After the gels were dried, they were exposed to x-ray film overnight at −84°C and developed with Kodak developer solution. The bands were analyzed using NIH image-68 software.

Analysis of binding data. Binding data were analyzed using the Biosoft EBDA and LIGAND software. A nonlinear least-square curve fitting technique was used to obtain the parameters of binding affinity and insulin receptor concentration. All points were equally weighted, and
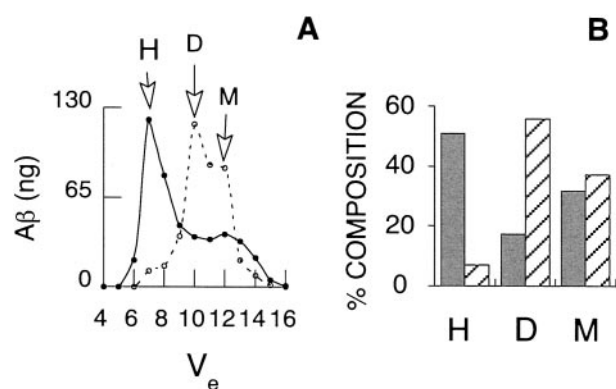

Figure 1. Size-exclusion chromatography of soluble Aβ$_{1-40}$. Soluble Aβ$_{1-40}$ (50 μM) was prepared in 50 mM HEPES, pH 7.4, in the presence (*A, continuous line*) or absence (*A, broken line*) of 0.1% bovine serum albumin as described in Materials and Methods. The chromatograms were fitted as described in Materials and Methods and assumed three normally distributed Gaussian peaks corresponding to monomers (*M*), dimers (*D*), and higher (*H*) level complexes of Aβ in the presence (*B, solid columns*) or absence (*B, cross-hatched columns*) of albumin.

nonspecific binding was handled as a computer-fitted parameter. The data were analyzed by fitting a one-site model to the data and determining the goodness of fit using a run test (Munson and Rodbard, 1980).

RESULTS

The Aβ peptides used in this study were relatively insoluble, with only approximately half of Aβ$_{1-40}$ remaining in solution over the duration of our experiments. The remaining soluble Aβ$_{1-40}$ solution was resolved into three separate peaks on a Sephadex-G50 column (Fig. 1). On the basis of a comparison against a range of protein standards, the molecular masses of the three resolved peaks were estimated to be ~4, 10, and 30 kDa corresponding to monomers, dimers, and higher level associates of the peptide, respectively. The distribution of Aβ$_{1-40}$ between these three peaks was calculated as described in Materials and Methods. In the absence of bovine serum albumin, the Aβ$_{1-40}$ solution comprised ~17.4% monomers, 26.3% dimers, 3.3% higher level associates, and 53% precipitated out of solution over the duration of the experiment. In the presence of bovine serum albumin, the Aβ$_{1-40}$ solution comprised 17% monomers, 9% dimers, and 27% higher level, soluble associates. The remaining 47% Aβ$_{1-40}$ precipitated out of solution. The Aβ$_{1-42}$ peptide behaved in a similar manner to the Aβ$_{1-40}$ peptide in solution.

3-(^{125}I) Iodotyrosyl A14 insulin binding to human placental plasma membranes was specific and competitively displaced by unlabeled human insulin. The presence of either Aβ$_{1-40}$ or Aβ$_{1-42}$ reduced the specific binding of insulin in a concentration-dependent manner (Fig. 2*A,C*). Scatchard representations of this data are illustrated in Figure 2, *B* and *D*. A model assuming a single class of homogeneous binding sites provided an adequate fit to the data from each experiment (run test > 0.05).

The effects of varied concentrations of Aβ$_{1-40}$ and Aβ$_{1-42}$ on the Scatchard estimated equilibrium dissociation constants defining insulin binding to its receptor are illustrated in Figure 3*A*. Both Aβ peptides reduced the affinity of insulin for its receptor in a concentration-dependent manner (Fig. 3*A*). There was a linear relationship between the equilibrium constant (K_d) and concentration of competing Aβ peptide, which is typical of a competitive inhibition of a ligand-binding interaction. The inhibitory constant (K_i) ($K_{dapp} = K_d + [i] K_d/K_i$) for Aβ$_{1-40}$ and Aβ$_{1-42}$ was indistinguishable and calculated on the basis of total soluble Aβ or
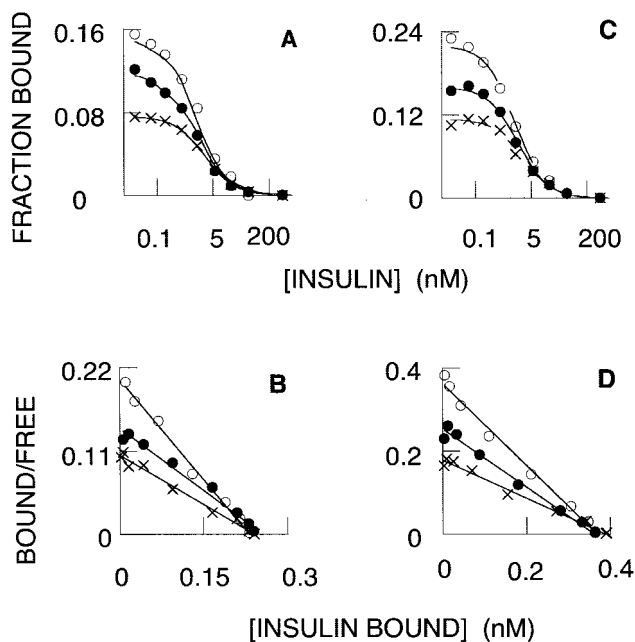
Figure 2. The effect of Aβ on insulin binding. Insulin binding to human placental plasma membranes was determined in the absence of Aβ (○) or presence of 20 μM (●) and 50 μM (×) of Aβ. The competitive displacement of $A\beta_{1-40}$ or $A\beta_{1-42}$ on insulin binding is illustrated in *A* and *C*, respectively. The plots derived by Scatchard analysis of the $A\beta_{1-40}$ or $A\beta_{1-42}$ data are illustrated in *B* and *D*, respectively.

monomeric Aβ was estimated to be ~25 or 8 mM, respectively. The effects of $A\beta_{1-40}$ and $A\beta_{1-42}$ on insulin binding were independent of receptor concentration (Fig. 3*B*). The reverse peptide sequence $A\beta_{40-1}$ failed to affect specific insulin binding at any concentration tested (Fig. 3*C*).

In vitro, 40 nMM insulin promoted approximately a fourfold increase in phosphorylation of a 90 kDa band corresponding to the autophosphorylated tyrosine kinase subunit of the insulin receptor (Fig. 4*A*) ($p < 0.01$). $A\beta_{1-40}$ (50 mM) inhibited the ability of insulin to promote this autophosphorylation of the receptor (Fig. 4*A,B*) ($p < 0.01$). In contrast, 50 mM of the reverse peptide $A\beta_{40-1}$ was ineffective in inhibiting the autophosphorylation of the insulin receptor (Fig. 4*B*) ($p = 0.84$).

DISCUSSION

The propensity of Aβ to form amyloid fibrils is shared in common with insulin and other proteins that share a consensus sequence (Pallitto et al.,1999). Insulin and Aβ are also common substrates for the insulin-degrading enzyme (Kurochkin and Goto, 1994), which is active in various tissues including brain (Authier et al., 1996) and may play a role in eliminating toxic amyloidogenic peptides (Perez et al., 2000; Vekrellis et al., 2000).

Considering the similarities in molecular structure and behavior of insulin and Aβ and that insulin resistance may be an early sign of AD (Frolich et al., 1998), we considered the possibility that Aβ directly competes with insulin for binding to the insulin receptor and thereby contributes directly to the impaired glucose utilization evident in the AD brain. In concert with this prediction, we found that both major forms of Aβ directly competed for the binding of insulin to human insulin receptors (Figs. 2, 3*A*). This competition was specific because the reverse peptide sequence $A\beta_{40-1}$ did not affect insulin binding at any concentration tested.

It is highly probable that the consensus sequence shared between insulin and the Aβ peptides (Pallitto et al., 1999) explains the molecular basis of the competition observed in this study. Indeed, the binding of Aβ to the insulin binding site of the insulin receptor probably involves the residues 16–25 of Aβ because this sequence shares a common pattern recognition motif with residues 21–30 of the B-chain of insulin (Kurochkin, 1998), which are implicated in the binding of insulin to its receptor (Yip, 1992).

Aβ also binds phosphofructokinase (Bigl and Eschrich, 1995); however, the significance of this observation is uncertain because the specific activity of phosphofructokinase actually increases in Alzheimer's patients (Bigl et al., 2000). Interestingly, Aβ also shares a sequence common to tachykinins, and it has been suggested that it also may act as a neuromodulatory peptide by binding to tachykinin receptors (Kimura and Schubert, 1993). The activity of a range of other proteins that lead to metabolic changes and the downregulation of glucose metabolism also has been reported, but the mechanisms involved are unclear and may be caused by indirect effects of Aβ (Hoyer, 1992; Schulze et al., 1993). Thus, Aβ may directly affect the action of several different proteins, including the insulin receptor, and indirectly affect the action of many other proteins, leading toward many of the outcomes of Alzheimer's disease.

There is emerging evidence that small oligomers are in themselves a toxic agent (Klein et al., 2001). Considering that Aβ probably binds to the insulin receptor because it presents a sequence motif in common with insulin (which is active itself as the monomer), it is likely that monomers and small oligomers of Aβ are the active species in this study. On the basis of the monomeric concentration of Aβ determined by chromatography in this study (Fig. 1), the apparent inhibitory dissociation constant (K_i) of Aβ binding to the insulin receptor was estimated to be ~8 μM (the true K_i, however, will be lower than this value because the proportion of monomeric Aβ is overestimated as the aggregates dissociate toward the monomeric species during the chromatographic separation). Thus, a low micromolar concentration of monomeric Aβ will interfere with insulin binding. It is interesting that albumin had little effect on the available monomeric Aβ in solution but seemed to complex with Aβ dimers and co-elute in the void volume of the column (Fig. 1).

In contrast to control brains in which nanomolar concentrations of Aβ are detected, micromolar concentrations of Aβ are detected in the AD brain (Cherny et al., 1999; Fonte et al., 2001). Micromolar concentrations of Aβ also are needed to promote its aggregation and neurotoxic effects in cortical neuronal cultures (Estus et al., 1997). Therefore, we believe that it is highly plausible that increased Aβ levels may be linked with decreased binding of insulin resulting in impaired insulin action in AD.

Several lines of evidence are highly consistent with this concept. First, several studies support a link between AD and features of insulin resistance, including higher fasting glucose and insulin levels (Fujisawa et al., 1991; Kuusisto et al., 1997; Craft et al., 1998; Carantoni et al., 2000). Moreover, a number of population-based studies strongly support a link between type 2 diabetes and AD (Leibson et al., 1997; Ott et al., 1999).

Second, numerous studies implicate impaired glucose metabolism as an early event in the progression of AD (Meier-Ruge et al., 1996; Reimain et al., 1996; Minoshima et al., 1999) that may be predictive of cognitive impairment (Arnaiz et al., 2001). Hoyer and Nitsch (1989) report on a 44% reduction in cerebral glucose metabolism in early onset AD where β amyloid deposition would be marked. Furthermore, the major genetic risk factor for AD,

Figure 3. The effect of Aβ on the insulin binding parameters. The Scatchard plots of insulin binding to human placental plasma membranes shown in Figure 2 were resolved into estimates of the affinity of insulin for the insulin receptor (*A*) and the receptor concentration (*B*), as a function of varied concentrations of $A\beta_{1-40}$ (■) and $A\beta_{1-42}$ (×). The effect of the reverse $A\beta_{40-1}$ peptide on the specific binding of radiolabeled insulin was determined from the difference in binding in the absence and presence of 2 μM insulin (*C*).
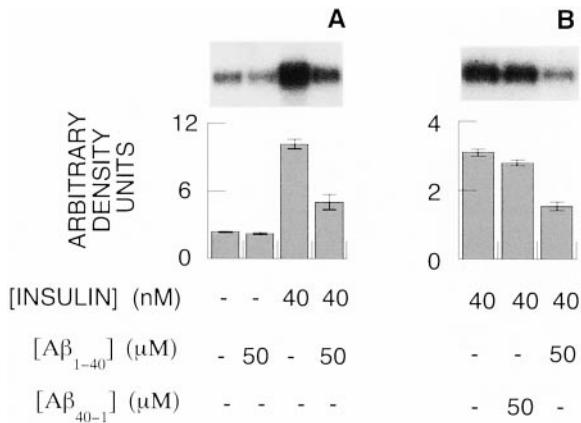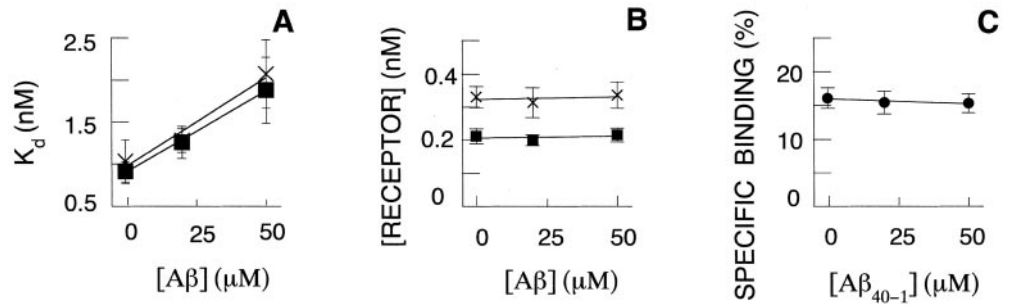

Figure 4. The effect of Aβ on insulin promoted autophosphorylation of insulin receptors. Insulin receptors purified by chromatography on wheat germ agglutinin Sepharose were incubated in a buffer containing 40 nM insulin and 50 μM $A\beta_{1-40}$ or $A\beta_{40-1}$. The autophosphorylated insulin receptors were immunoprecipitated and resolved on 8% reducing SDS-polyacrylamide gels. The *top panels* of *A* and *B* each show a representative autoradiograph of the labeled 90 kDa band. The mean relative densitometric values are plotted in the histograms with the SEs determined from three experiments.

the ε-4 allele of apolipoprotein E, is associated with lowered cerebral glucose metabolism in patients with a clinical diagnosis of AD (Small et al., 2000). Impaired glucose utilization leads to characteristic AD pathology in cultured skin fibroblasts from AD patients (Blass et al., 1991), probably by impairing the processing of the amyloid precursor protein (Gasparini et al., 1999; Solano et al., 2000). Interestingly, Aβ also decreases glucose uptake in endothelial cells (Blanc et al., 1997) and in synaptosomes purified from rat brain (Keller et al., 1997). An elevated level of $A\beta_{1-42}$ in cerebrospinal fluid is associated with the severity of impaired glucose metabolism in the AD brain (Okamura et al., 1999).

Third, it would be expected that increasing concentrations of insulin should relieve the competitive inhibitory effect of Aβ and the associated insulin resistance. Indeed, raising plasma insulin by intravenous infusion induces memory improvement in AD subjects (Craft et al., 1999), presumably by promoting glucose metabolism, which is impaired in AD brain (Meier-Ruge and Bertoni-Freddari, 1996).

Fourth, plasma and cerebrospinal fluid hyperinsulinemia is associated with AD (Fujisawa et al., 1991; Razay and Wilcock, 1994). This may be the compensation for the impaired insulin action associated with Aβ competition against insulin binding.

Finally, insulin receptor sites are upregulated, and the insulin receptor tyrosine kinase activity is inhibited in the sporadic AD brain (Frolich et al., 1998). The binding of Aβ to the insulin

receptor to antagonize insulin-promoted autophosphorylation *in vitro* (Fig. 4) is consistent with the reduced tyrosine kinase activity of the insulin receptor in the AD brain.

In conclusion, this study demonstrates that *in vitro*, Aβ is a competitive inhibitor of insulin binding to its receptor. As a consequence, Aβ antagonizes the effect of insulin in promoting the autophosphorylation of the insulin receptor, an event that is inextricably linked to the downstream effects of insulin. Thus, events that lead to an increased production of Aβ may lead to an impairment of glucose utilization as an early event in the pathogenesis of AD. However, we cannot exclude the possibility that the altered glucose utilization observed in AD brain may be an effect of widespread neural dysfunction and loss caused by other aspects of the underlying pathological process. This issue can best be addressed in an animal model of AD.

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