Insulin Receptor Dysfunction Impairs Cellular Clearance of Neurotoxic Oligomeric Aβ*

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Accumulation of amyloid β (Aβ) oligomers in the brain is toxic to synapses and may play an important role in memory loss in Alzheimer disease. However, how these toxins are built up in the brain is not understood. In this study we investigate whether impairments of insulin and insulin-like growth factor-1 (IGF-1) receptors play a role in aggregation of Aβ. Using primary neuronal culture and immortal cell line models, we show that expression of normal insulin or IGF-1 receptors confers cells with abilities to reduce exogenously applied Aβ oligomers (also known as ADDLs) to monomers. In contrast, transfection of malfunctioning human insulin receptor mutants, identified originally from patient with insulin resistance syndrome, or inhibition of insulin and IGF-1 receptors via pharmacological reagents increases ADDL levels by exacerbating their aggregation. In healthy cells, activation of insulin and IGF-1 receptor reduces the extracellular ADDLs applied to cells via seemingly the insulin-degrading enzyme activity. Although insulin triggers ADDL internalization, IGF-1 appears to keep ADDLs on the cell surface. Nevertheless, both insulin and IGF-1 reduce ADDL binding, protect synapses from ADDL synaptotoxic effects, and prevent the ADDL-induced surface insulin receptor loss. Our results suggest that dysfunctions of brain insulin and IGF-1 receptors contribute to Aβ aggregation and subsequent synaptic loss.

Abnormal protein misfolding and aggregation are common features in neurodegenerative diseases such as Alzheimer (AD), Parkinson, Huntington, and prion diseases (1–3). In the AD brain, intracellular accumulation of hyperphosphorylated Tau aggregates and extracellular amyloid deposits comprise the two major pathological hallmarks of the disease (1, 4). Aβ aggregation has been shown to initiate from Aβ1–42, a peptide normally cleaved from the amyloid precursor protein (APP) via activities of α- and γ-secretases (5, 6). A large body of evidence in the past decade has indicated that accumulated soluble oligomers of Aβ1–42, likely the earliest or intermediate forms of Aβ deposition, are potently toxic to neurons. The toxic effects of Aβ oligomers include synaptic structural deterioration (7, 8) and functional deficits such as inhibition of synaptic transmission (9) and synaptic plasticity (10–13), as well as memory loss (11, 14, 15). Accumulation of high levels of these oligomers may also trigger inflammatory processes and oxidative stress in the brain probably due to activation of astrocytes and microglia (16, 17). Thus, to understand how a physiologically produced peptide becomes a misfolded toxin has been one of the key issues in uncovering the molecular pathogenesis of the disease.

Aβ accumulation and aggregation could derive from overproduction or impaired clearance. Mutations of APP or presenilins 1 and 2, for example, are shown to cause overproduction of Aβ1–42 and amyloid deposits in the brain of early onset AD (18, 19). Because early onset AD accounts for less than 5% of entire AD population, APP and presenilin mutations cannot represent a universal mechanism for accumulation/aggregation of Aβ in the majority of AD cases. With respect to clearance, Aβ is normally removed by both global and local mechanisms, with the former requiring vascular transport across the blood–brain barrier (20, 21) and the latter via local enzymatic digestions by several metalloproteases, including neprilysin, insulin-degrading enzyme (IDE), and endothelin converting enzymes 1 and 2 (22–24).

The fact that insulin is a common substrate for most of the identified Aβ-degrading enzymes has drawn attention of investigators to roles of insulin signaling in Aβ clearance. Increases in insulin levels frequently seen in insulin resistance may compete for these enzymes and thus contribute to Aβ accumulation. Indeed, insulin signaling has been shown to regulate expression of metalloproteases such as IDE (25, 26), and influence aspects of Aβ metabolism and catabolism (27). In the endothelium of the brain–blood barrier and glial cells, insulin signaling is required to regulate protein–protein interactions in an uptake cascade involving low density lipoprotein receptor-related protein and its ligands ApoE and α2-macroglobulin, a system known to bind and clear Aβ via endocytosis and/or vascular transport (28, 29). Similarly, circulating IGF-1 has been reported to play a role in Aβ clearance probably via facilitating brain–blood barrier transportation (30, 31).

*This work was supported, in whole or in part, by National Institutes of Health Grant R01-AG022547 from NIA. This work was also supported by Intramural funding at Blanchette Rockefeller Neurosciences Institute, Rockville, MD, the American Health Assistance Foundation, the Alzheimer’s Association, Acumen Pharmaceuticals, Inc., South San Francisco, CA, and Intramural funding at NCCAM/NIH, Bethesda, MD.

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‡The abbreviations used are: AD, Alzheimer disease; APP, amyloid precursor protein; IDE, insulin-degrading enzyme; IGF-1, insulin-like growth factor-1; IGF-1R, IGF-1 receptor; ADDL, amyloid β-derived diffusible ligand; bADDL, biotin-labeled ADDL; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; VEH, vehicle; Aβ, amyloid β.
In the brain, insulin signaling plays a role in learning and memory (32–34), potentially linking insulin resistance to AD dementia. Recently we and others have shown that Aβ oligomers interact with neuronal insulin receptors to cause impairments of the receptor expression and function (35–37). These impairments mimic the Aβ oligomer-induced synaptic long term potentiation inhibition and can be overcome by insulin treatment (35, 38). Consistently, impairments of both IR and IGF-1R have been reported in the AD brain (39 – 41).

Based on these results, we ask whether impairment of insulin and IGF-1 signaling contribute to Aβ oligomer build-up in brain cells. To address this question, we set out to test roles of IR and IGF-1R in cellular clearance and transport of Aβ oligomers (ADDLs) applied to primary neuronal cultures and cell lines overexpressing IR and IGF-1R. Our results show that insulin and IGF-1R receptors function to reduce Aβ oligomers to monomers, and prevent Aβ oligomer-induced synaptic toxicity both at the level of synapse composition and structure. By contrast, receptor impairments resulting from “kinase-dead” insulin receptor mutations, a tyrosine kinase inhibitor of the insulin and IGF-1 receptor, or an inhibitory IGF-1 receptor antibody increase ADDL aggregation in the extracellular medium. Our results provide cellular evidence linking insulin and IGF-1 signaling to amyloidogenesis.

**EXPERIMENTAL PROCEDURES**

**Primary Neuronal and Immortal Cell Cultures**—Primary cortical and hippocampal neuronal cultures were prepared from either postnatal day-1 (P1) or embryonic day 18 (E18) rats according to previous descriptions (8, 37). Neurons were maintained for ~3 weeks before experimentation. NIH3T3 cells with or without expression of full-length IR or IGF-1R were cultured in low glucose (1 g/liter glucose) Dulbecco’s modified Eagle’s medium supplement with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured to 80% confluence and serum deprived overnight before using for experimental treatments. All cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA).

**Transient Transfection of Human IR and IR Mutants**—The full-length of human IR and “kinase-dead” IR mutant constructs (42–44) were transiently transfected, respectively, into NIH3T3 cells and primary neurons using Lipofectamine 2000 (Invitrogen). Following transfection (3 h), cells were maintained for 48 h before ADDL (or bADDL) and insulin treatment.

**ADDLs and Pharmacological Treatments**—ADDLs were prepared with synthetic Aβ1–42 (American Peptides, Sunnyvale, CA) according to the procedure described previously (45). For certain experiments, biotin-labeled synthetic Aβ1–42 was used to prepare ADDLs (bADDLs) using the above protocol (7). Cultured neurons and IR- or IGF-1R-expressing cells were treated with 100 nm ADDLs or vehicle at 37 °C for various times in the presence or absence of 100 nm insulin or 100 nm IGF-1. To inhibit IR and IGF-1R activities, cells were pretreated with 100 μM AG1024 for 45 min before ADDL or insulin/IGF-1. A second dose of AG1024 was given together with ADDLs, and/or insulin/IGF-1. A neutralizing IGF-1R antibody (1:50, MAB391, R&D Systems, Minneapolis, MN) was also preincubated with neurons for 15 min to inhibit the IGF-1R activity. After ADDL treatments for different lengths of time, the culture media were collected, and spun at 4,000 rpm for 10 min to remove cell debris. The media were then concentrated using Centricon YM-3 (Millipore, Temecula, CA). To prepare cell lysates, the treated cells were rinsed with 1 × PBS, quick frozen then lysed on ice for 30 min in a lysis buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.8 M EDTA, 0.5 M EGTA, 1% Triton X-100, 0.5% Nonidet P-40, and 1% protease cocktails). The lysates were then centrifuged at 10,000 × g for 15 min, and the supernatant was collected. Protein concentrations were determined and equalized before samples were applied to dot and Western blotting.

**Immunoblotting**—ADDL levels were measured with dot and Western blots. For dot blotting, the concentrated media and cell lysates from different treatments were spot, in triplicates, onto a nitrocellulose membrane. For Western blotting, the concentrated media were resolved on 4–20% SDS-PAGE and transferred to a nitrocellulose membrane. The ADDL immunoreactivity was determined with NU-1 (1:2000) or NU-2 (1:4000) antibodies (46) followed by a chemiluminescent detection process.

**Immunoprecipitation**—Primary neurons or NIH3T3 cells transfected with wild-type or mutant human insulin receptors were treated with 100 nm bADDLs for 30 min in the presence or absence of 100 nm insulin. Upon termination of the treatments, the media were collected and concentrated with Centricon YM-3. bADDLs from the concentrated media were pulled down by 6E10 that was immobilized to agarose resin (AminoLink Plus Coupling Resin, Pierce). The precipitated bADDLs were separated on 4–20% SDS-PAGE and transferred to a nitrocellulose membrane. The bADDL signals were then detected with streptavidin conjugated with horseradish peroxidase followed by chemiluminescent process.

**Surface Biotinylation**—Surface insulin receptor biotinylation was performed using the surface labeling kit (Pierce) according to the manufacturer’s manual. The isolated surface and the total insulin receptors were resolved on 4–20% SDS-PAGE and transferred to nitrocellulose membranes. The amount of receptors was detected with polyclonal antibodies against the α subunit of the insulin receptor. Changes in the amount of surface receptor were measured after normalization with the total amount of receptors in each sample prior to bead pull down.

**ADDL ELISA**—ADDL ELISA was performed in a 96-well Optiplate (PerkinElmer) coated with NU-1 (5 μg/ml) in a carbonate-bicarbonate buffer. After blocking with 5% bovine serum albumin (Sigma-Aldrich), the ADDL-treated media and cell lysate samples were added to each well (100 μl/well) in quadruplicates, and incubated at 4 °C overnight. The unbound samples were removed and plate washed six times with PBS containing Tween 20 (PBS-T) at room temperature. A 6E10 antibody (100 ng/ml) conjugated with alkaline phosphate was added to each well (100 μl/well) and incubated at room temperature for 2 h. After washes with PBS-T, the plate was incubated with the CDP-Star substrate (Applied Biosystems, Foster City, CA) at room temperature for 30 min. The ELISA signal was read on an Envision microplate platform (PerkinElmer Life Sciences).

**Immunocytochemistry**—Immunocytochemical staining were performed as described previously (8, 37). Briefly, the treated
neurons and NIH3T3 cells were fixed with 3.7% formaldehyde at room temperature for 10 min. Following washes with PBS, pH 7.5, blocking/permeabilizing with 10% normal goat serum containing 0.1% Triton X-100, cells were incubated with either NU1 (1:500) or 6E10 (1:200) to detect ADDL binding. A neuronal marker (an anti-GAP43 antibody or fluorescent-labeled cholera toxin B), was simultaneously present in the incubation. The IR-expressing cells were double stained with NU-1 (1:500), an anti-IR antibody (1:200). In the experiments where the surface IR expression was measured, immunolabeling was performed without permeabilizing reagent. In some experiments, biotin-labeled ADDLs (bADDL) were used to treat neurons. To identify astrocytes, an anti-glia fibrillary acidic protein antibody was used. The incubations were carried out overnight at 4 °C. Cells were washed three times with PBS and incubated for 1 h at room temperature with appropriate Alexa fluorophore-conjugated secondary antibodies (1:500). After wash, cells on coverslips were mounted with Prolong Gold mounting medium (Invitrogen) and were observed with Leica TCS SP2 confocal or Nikon TE 1200 fluorescence microscopes.

**Imaging Insulin and IGF-1 Protection of Dendritic Spine Loss**—The ADDL-induced dendritic spine loss was measured as described previously (8). In brief, 21 days *in vitro* hippocampal neurons were treated with vehicle or ADDLs (500 nM) for 24 h in the presence or absence of 1 μM insulin or different concentration (0.1, 0.5, and 1.0 μM) IGF-1. Changes in spine abundance were determined by the immunoreactivity of drebrin, a specific dendritic spine marker, using drebrin antibody (1:500, Stressgen, Victoria, British Columbia, Canada) overnight at 4 °C followed by AlexaFluor488-conjugated secondary antibody incubation. Data obtained from three independent experiments, using three dendritic regions from five individual images containing ADDL-targeted neurons, were quantified with the Metamorph v6.3 software (Universal Imaging Corp., Downingtown, PA). The density of dendritic spines was quantified by the number of drebrin-positive puncta per length of dendrite.

**Data Analyses**—For each experiment, at least three independent replicated experiments were performed. ADDL immunoreactive results from dot and Western blots were acquired with densitometric scan and quantified with ImageJ. The values from triplicates of each treatment from at least three repeated experiments were compared statistically using GraphPad Prism software (San Diego, CA). ADDL binding on neuronal dendritic processes were also quantified with ImageJ and analyzed with 1-way analysis of variance. For spine densities, the quantified values from Metamorph were analyzed statically by GraphPad Prism using One-way analysis of variance and comparison post-hoc tests.

**RESULTS**

**Insulin Signaling Reduces Extracellular ADDLs**—To maintain ADDLs in their native form, we used dot blotting to measure ADDL levels in the medium and cortical cell lysates. Samples from ADDL-treated and control neurons were spotted on nitrocellulose membrane and blotted by a specific ADDL antibody, NU-2. NU-2 showed negative immunoreactivity with samples from vehicle- and insulin-treated cortical cells (Fig. 1A, 1 and 2), but detected a strong immunoreactivity from samples (both medium and lysates) treated with 100 nM ADDLs (Fig. 1A-3). A brief (5 min) insulin stimulation caused a reduction of ADDLs in the medium alone with an increase in ADDLs in the lysates (Fig. 1A-4). In contrast, AG1024, an IR and IGF-1R tyrosine kinase inhibitor (47), caused a striking increase in ADDL immunoreactivity in the medium, but a marked decrease in ADDL reactivity in the cell lysate (Fig. 1, A-5 and A-6). Similar results were also observed with cultured hippocampal neurons (data not shown). A time course study (Fig. 1B) showed that there was an initial reduction in extracellular ADDL immunoreactivity shortly (30 min) after addition to cells (Fig. 1B-1). It was then gradually increased as incubation time was prolonged, probably due to continuous aggregation of unbound ADDL species in the medium. In the cell lysate (Fig. 1B-2) the highest ADDL immunoreactivity was seen immediately after its addition to cells, indicating a rapid binding. The immunoreactivity was reduced by 40% at 24 h after ADDL incubation. Addition of insulin reduced the extracellular ADDL buildup, which was accompanied by an increase in ADDL immunoreactivity in the cell lysate at 30 and 60 min after ADDL incubation. At 24 h after incubation, however, insulin caused a marked reduction in both extra- and intracellular ADDLs.

To verify that the observed ADDL reduction was due to functions of IR, we tested ADDL clearance in NIH3T3 cells with or without IR overexpression. In cells with absence of IR (3T3-IR (−)), the majority of ADDL immunoreactivity was present in the medium (Fig. 1C-1) suggesting a low level ADDL binding. Insulin and AG1024 treatments had no effect on ADDL levels. In contrast, cells with stable overexpression of the human IR (3T3-IR (+)) showed transfer of ADDL from medium to the cell lysates following insulin stimulation (Fig. 1C-2), which was inhibited by AG1024. Together, these results suggest that activation of IR promoted transport of ADDLs from medium to cell lysates, which appeared to be associated with ADDL clearance.

**Insulin Resistance-associated IR Mutation Causes Increases in Extracellular ADDL Levels**—To verify effects of IR activity on ADDL reduction, we transiently infected 3T3 IR (−) cells or neuronal cultures with human wild-type or two “kinase dead” human IR mutations. The first IR mutation was a naturally occurring tyrosine kinase-deficient insulin receptor mutation (42, 43), in which methionine at position 1153 was replaced by isoleucine (Met1153 → Ile). The second was also a human insulin receptor gene with mutation on the ATP-binding site (44) by replacing lysine at position 1030 with alanine (Lys1030 → Ala). Patients with these mutations have impaired insulin receptor functions manifested as obesity, diabetes, and other insulin resistance syndrome (42–44). As shown in Fig. 2A, when transfected into NIH3T3 cells, all IR constructs expressed the IR protein at similar levels (upper panel), except for the eGFP construct, a negative control. However, only the wild-type IR displayed tyrosine phosphorylation in the β subunit in response to insulin (lower panel, indicated by an asterisk).

When treated with ADDLs (Fig. 2B), cells transfected with the wild-type IR showed a moderate level of ADDLs in the medium. Insulin caused a smaller decrease in extracellular bADDL levels, compared with results observed with stable IR-expressing cells in Fig. 1D. This discrepancy could be due to the
low transfection efficiency in lipid-based method, such that the lower IR expression resulted in weaker ADDL binding and insulin-induced effect. With IR mutant transfection, however, the extracellular ADDL levels rose by 1.5- to greater than 2-fold (Fig. 2B). Similar results were also found in neurons transfected with IR mutants, in which the extracellular ADDL levels were increased by 2-fold (Fig. 2C-1). Western blotting showed increased oligomeric species in the medium, including dimer, trimer, tetramer, and a higher band above 52 kDa (Fig. 2C-3). In the cell lysates (Fig. 2C-2), insulin stimulated an increased ADDL signal. The insulin-stimulated effect was not seen in neurons transfected with IR mutants Met1153Ile and Lys1030Ala, although the latter showed increased bADDL levels in the cell lysates regardless of insulin treatment. These results confirm the results in Fig. 1, and clearly show that malfunctioning insulin receptors cause increases in ADDL species in the extra- and/or intracellular compartments.

**IGF-1R Functions to Reduce ADDLs in the Extracellular Compartment**—In the brain, the IR tetramer exists frequently as hybrids of IR and IGF-1R, the latter also responding to insulin. We thus tested the involvement of IGF-1R in ADDL cellular clearance. Unlike insulin, IGF-1 did not increase the ADDL immunoreactivity in the lysate fraction of cortical neurons (Fig. 3A-4). It caused, however, a profound reduction in ADDL reactivity in the medium (Fig. 3, A and D-4), which was also blocked by AG1024 (Fig. 3A-6). When present alone, the AG1024-increased extracellular ADDLs was accompanied by a reduction in intracellular ADDLs, probably due to inhibition of basal insulin receptor action (Fig. 2A-5). Similarly, in human IGF-1R-overexpressing NIH3T3 (NIH3T3 IGF-1R (+)) cells, IGF-1 completely eliminated the extracellular ADDL immunoreactivity (Fig. 3B-4). These results suggest IGF-1R is more effective in ADDL clearance compared with IR. Unlike IR, activation of which mediates ADDL attachment to cells, IGF-1R activation appears to clear ADDLs using an extracellular mechanism.

**Impairments of IR and IGF-1R Promote Extracellular ADDL Aggregation**—To verify that the increased detectable ADDLs on dot blots under IR and IGF-1 inhibition represents oligomers rather than monomers, we measured SDS-resistant ADDL species using Western blots. As shown in Fig. 4A, the majority of ADDLs in the neuronal extracellular media were reduced to monomers (lane 2), that further decreased in the

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**FIGURE 1. Insulin promotes redistribution of ADDLs.** A, cultured rat primary cortical neurons (14 days in vitro) were treated with 100 nM ADDLs for 30 min in the presence or absence of 100 nM insulin, which was added to neurons either simultaneously with ADDLs (30 min) or 5 min prior to termination of the reaction. To inhibit IR tyrosine kinase activity neurons were treated with AG1024 prior to ADDL and insulin addition. At the end of the reaction, ADDL levels from the extracellular medium from each treatment were measured on dot blots with specific ADDL antibody NU2. B, time course of ADDL degradation in rat cortical neurons (14 days in vitro) with and without insulin. B-1, ADDL levels from the medium; B-2, ADDL levels from the cell lysate; C, NIH3T3 cells with (3T3-IR (+)) and without (3T3-IR (-)) overexpression of IR treated with ADDLs as described in A. * * * p < 0.001.
Neuronal Insulin Resistance and Aβ Oligomer Accumulation

The presence of insulin and IGF-1 (lanes 3–6). Several high molecular bands resistant to SDS were also shown in these samples. It is not clear whether they are large ADDL species, or cross-link products of small oligomers with extracellular proteins. In the presence of AG1024, a striking amount of ADDL aggregates highly resistant to reducing reagents was present in the medium (lanes 7–9) along with a reduction in monomers. Insulin and IGF-1 lost effects in the presence of AG1024. In addition, treatment of cortical cultures with a neutralizing IGF-1R antibody, known to inhibit the activity of IGF-1R (48), also caused increases in ADDL aggregates in the medium, although to a lesser degree (lane 10). When incubated in cell-free conditioned neurobasal-A with or without 100 μM AG1024, ADDLs were measured with dot blots using NU1. A, the insulin-stimulated IR tyrosine phosphorylation from NIH3T3 cells transfected with huIR-wt and mutated human IR. Only the huIR-wt showed positive insulin-induced tyrosine phosphorylation on the β-subunit. B, ADDL digestions in the medium were inhibited in the IR mutants-transfected NIH 3T3 cells. C-1, bADDL digestions in the medium were inhibited in the IR mutants-transfected hippocampal neurons. C-2, insulin-induced translocation of ADDLs were not seen in the IR mutant-transfected hippocampal cells. **, p < 0.001, n = 3. C-3, transfected neurons were treated with bADDLs. The concentrated medium from different conditions was pulled down by streptavidin covalently immobilized to agarose resin and detected on Western blots with 6E10. BADDL species were increased in the medium of primary neurons transfected with IR mutants.

FIGURE 2. IR mutations prevent ADDL degradation. The human full-length wild-type IR (huIR-wt), or two IR mutations (IR-1153Ile and IR1030Ala) were transient transfected to primary hippocampal neurons or NIH3T3 cells. Cells were then treated with biotin-labeled ADDLs (bADDLs) in the presence or absence of insulin. The media were collected and concentrated. The cell lysates were prepared with a lysates buffer. Both the extracellular and the cell-attached bADDLs were measured with dot blots using NU1. A, the insulin-stimulated IR tyrosine phosphorylation from NIH3T3 cells transfected with huIR-wt and mutated human IR. Only the huIR-wt showed positive insulin-induced tyrosine phosphorylation on the β-subunit. B, ADDL digestions in the medium were inhibited in the IR mutants-transfected NIH 3T3 cells. C-1, bADDL digestions in the medium were inhibited in the IR mutants-transfected hippocampal neurons. C-2, insulin-induced translocation of ADDLs were not seen in the IR mutant-transfected hippocampal cells. **, p < 0.001, n = 3. C-3, transfected neurons were treated with bADDLs. The concentrated medium from different conditions was pulled down by streptavidin covalently immobilized to agarose resin and detected on Western blots with 6E10. BADDL species were increased in the medium of primary neurons transfected with IR mutants.

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When incubated with NIH3T3 IR (−) cells (Fig. 4C-1), ADDLs from the medium were resolved as monomers and some high molecular weight aggregated species (lane 2). No difference was observed with insulin treatment (lane 3). In the presence of AG1024, an additional band above 52kDa was detected (lane 4). Interestingly a similar band was also observed in the medium of the kinase-dead IR mutants-transfected neurons (Fig. 2C-2). When ADDLs were applied to NIH3T3-IR(−) and -IGF-1R(−) cells (Fig. 4C-3), however, not only were no high ADDL aggregates detectable, the amount of Aβ monomer was also markedly reduced (Fig. 4, C-2 and -3, lane 2). Stimulation of cells with insulin further reduced the amount of Aβ (Fig. 4, C-2 and -3, lane 3) clearly consistent with the dot blotting results. AG1024 again caused a massive increase in ADDL aggregates that are much stronger than those from normal 3T3 cells (Fig. 4, C-2 and -3, lane 4), demonstrating that the aggregation is specifically associated with impairment of IR or
Collectively, these results suggest that activation of IR and IGF-1R can reduce the extracellular ADDLs to monomers. The proper functions of IR and IGF-1R are important to prevent aggregation of Aβ/H9252 in the extracellular compartment.

IR Mediates Internalization of ADDLs in NIH3T3-IR(H11001) Cells—Because insulin induced a translocation of ADDL immunoreactivity to intracellular compartments, we tested whether this was due to internalization of ADDLs. Clear ADDL binding was detected in NIH3T3-IR(H11001) cells (Fig. 5B-2), suggesting an interaction between ADDL and IR while no ADDL binding was shown in parent NIH3T3 cells which had no IR and IGF-1R expression (Fig. 5A, 1A). On the other hand, a brief insulin stimulation (5 min) induced a high ADDL binding and translocation of ADDL immunoreactivity into the intracellular compartments (Fig. 5B-3). The internalized ADDLs were highly colocalized with IR suggesting complex of ADDLs with activated IR. Because the images were acquired via confocal sequential scanning, the colocalization of the immunosignals could not result from filter “bleed-through.” The internalized ADDLs were highly compartmentalized suggesting that the oligomers were transported via a specific pathway. Under prolonged insulin treatment (30 min), the internalized ADDLs and IR traveled further to the nuclear envelop and the nucleus (Fig. 5B-4). We next compared the insulin-induced ADDL internalization in the IR- and IGF-1R-expressing cells. The immunoreactivity of ADDLs, IR, and IGF-1R were observed under an epi-fluorescent microscope. Unlike NIH3T3-IR(H11001) cells (Fig. 5C, upper panel, 2), the binding of ADDLs was relatively sparse.
in NIH3T3-IGF1R(+) cell (Fig. 5C, lower panel, 2). While insulin stimulated ADDL internalization to the nucleus of the IR-expressing cells (upper 3), ADDLs were not internalized by insulin into the IGF-1R-expressing cells, but stuck on the membrane surface (lower 3). In the presence of AG1024, ADDL uptake to the IR-expressing cells was blocked, with extracellular aggregation of ADDLs observed in both types of cells (Fig. 5C-4). These results indicate that, whereas activation of IR promotes internalization of ADDLs, activation of IGF-1R appears to keep ADDLs on the membrane surface. The attachment of ADDLs on the membrane surface may probably explain the appearance of ADDLs in the cell lysates in neuronal cultures following IGF-1 stimulation (see Fig. 4B).

**Insulin Reduces ADDL Binding to Neurons Accompanied by ADDL Internalization**—In cultured neurons, ADDL binding was detected by NU-1 on dendrites (Fig. 6A, panel 5 and 6). Following treatment of insulin for 10 min and 1 h, ADDL binding was significantly (p < 0.001) reduced (Fig. 6A, panels 7 and 8). The decreased binding occurred largely in dendrites proximate to the soma, and was accompanied by an increase in ADDL immunostaining inside the cell body (Fig. 6A, panels 8 and 9). In comparison, 6E10 detected internalized ADDLs in both neuronal and non-neuronal cells under insulin treatment (Fig. 6B-1, panels 8 and 9). Fig. 6B-2 shows quantification of the NU-1-detected ADDL dendritic binding under insulin treatments. In addition, IGF-1 also reduced ADDL binding to dendrites (data not shown).

To verify the expression of IR in the ADDL-containing cells, we double stained the bADDL-treated, and permeabilized neurons with an IR antibody followed anti-rabbit IgG conjugated with Alexa-488 and streptavidin conjugated with Alexa-555. Under this permeabilized condition, both surface and internal IR would be measured. As shown in Fig. 6C, IR was expressed in normal neurons, including dendrites and the soma. When treated with bADDLs, IR only showed a low degree of colocalization (yellow colored, arrows in Fig. 6D-1, Merge) with ADDL binding, probably due to its rapid loss from the surface upon ADDL treatment (37). Indeed, in some neurons, the majority of the IR signals was present inside the ADDL-bound dendrites (Fig. 6D-1, merge), whereas other neurons showed more severe dendritic IR loss (Fig. 6D-2, merge, arrowhead). In addition, abundant IR immunoreactive was seen in astrocytes-like cells adjacent to the neuron (Fig. 6D-2, arrow) that appeared to take up bADDLs. To verify whether uptake of ADDLs occurs in astrocytes under insulin and IGF-1 treatments, we double stained the bADDL-treated hippocampal neurons in the presence or absence of insulin or IGF-1 with an anti-glial fibrillary acidic protein antibody, an astrocyte marker, and the anti-ADDL antibody NU-1 (Fig. 6E). Although a low degree of ADDL binding was observed on astrocytes (Fig. 6E, upper left panel 5 and 6). Following treatment of insulin for 10 min and 1 h, ADDL binding was significantly (p < 0.001) reduced (Fig. 6A, panels 7 and 8). The decreased binding occurred largely in dendrites proximate to the soma, and was accompanied by an increase in ADDL immunostaining inside the cell body (Fig. 6A, panels 8 and 9). In comparison, 6E10 detected internalized ADDLs in both neuronal and non-neuronal cells under insulin treatment (Fig. 6B-1, panels 8 and 9). Fig. 6B-2 shows quantification of the NU-1-detected ADDL dendritic binding under insulin treatments. In addition, IGF-1 also reduced ADDL binding to dendrites (data not shown).

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Insulin and IGF-1 Prevents ADDL-induced Losses of Dendritic Spines and the Surface IR—To test whether the insulin-induced ADDL internalization is harmful to synapses, we test effects of insulin on ADDL-induced synaptic spine loss. As previously reported, ADDLs have a deleterious effect on dendritic spine expression (8). Using immunocytochemistry of drebrin, a cytoskeletal marker highly concentrated in dendritic spine heads, we applied a long term ADDL treatment (500 nM, 24 h) to hippocampal neurons in the presence or absence of 1 μM insulin and different concentrations of IGF-1. Relative drebrin immunofluorescence was quantified and expressed as number of drebrin positive puncta per length of dendrite, which was measured on images, including full neurons that presented ADDLs bound to their dendrites. As shown in Fig. 7A, vehicle (VEH)-treated neurons harbored abundant dendritic spine densities. However, a drastic loss (~85%) in drebrin-positive puncta was observed after ADDL incubation (Fig. 7A-1, AD, p < 0.001). IGF-1 showed a concentration-dependent protection of drebrin: at 1 μM it prevented the ADDL-induced drebrin loss (Fig. 7A-I, AD-IGF1). No significant effects were observed at 0.1 μM (Fig. 7A-I, AD-IGF0.1), and a partial prevention was afforded by 0.5 μM IGF-1 (data not shown). Similarly, 1 μM insulin (Fig. 7A-I, AD-INSI) also fully protected spines from ADDL attack. In conditions of both 1 μM IGF-1 and 1 μM insulin, drebrin levels in spines were comparable to that of vehicle-treated neurons. Note that both IGF-1 (Fig. 7A-J, VEH-IGF0.1 and VEH-IGF1) and insulin on their own (Fig. 7A-J, VEH-INSI) did not seem to have a major impact on spine drebrin expression. A statistical comparison of spine drebrin levels under different treatments were summarized in Fig. 7A-2.

Furthermore, we tested the protective effect of IGF-1 and insulin on ADDL-induced surface IR loss. The surface IR was imaged on ADDL-treated hippocampal neurons without permeabilization. As shown in Fig. 7B, both IGF-1 and insulin at 1 μM completely protected the dendritic surface IRa from ADDLs. These effects were confirmed by surface biotinylation (Fig. 7C). Taking together, although differences exist in ADDL internalization in response to insulin or IGF-1, these results clearly demonstrate both insulin and IGF-1 prevents ADDLs from causing synaptic spine and the surface insulin receptor losses.

Insulin- and IGF-1-induced ADDL Degradation Is Inhibited by IDE Inhibitor Phenanthroline—To understand the mechanism by which IGF-1 cause ADDL clearance in the extracellular medium, we explored the involvement of IDE and neprilysin, two most extensively studied metallopeptidases known to play a role in cellular digestion of Aβ (49–51). Cortical neurons were pretreated with an IDE inhibitor 1, 10-phenanthroline (52) and a neprilysin inhibitor phosphoramidon prior to ADDL, insulin, and IGF-1 treatments. Levels of ADDLs from the medium and lysates were measured using ELISA. As shown in Fig. 8A, phosphoramidon markedly increased the extracellular ADDL levels, but it did not block effects of insulin and IGF-1, implicating that insulin and IGF-1 activated an alternative mechanism to reduce ADDLs. In contrast, although phenanthroline alone did not cause significant increases in extracellular ADDLs, it completely blocked the effect of IGF-1, and to a lesser degree, of insulin. These results suggest that neprilysin
Neuronal Insulin Resistance and Aβ Oligomer Accumulation

**DISCUSSION**

Accumulation of neurotoxic soluble Aβ oligomers in the CNS appears to be responsible for synaptic damage in early stages of AD (10, 13, 53). An important goal is to identify factors that may prevent or promote accumulation of these neurotoxins. A great deal of interest has been drawn to the role of insulin signaling in the etiology and pathogenesis of AD (54, 55). Not only is the type 2 diabetes mellitus a risk factor for AD (56, 57), but these two diseases also share several common molecular pathological features such as amyloidogenesis (1, 58–60) and insulin resistance (18750).

In the extracellular compartment, insulin and IGF-1 play a predominant role in Aβ oligomer degradation. However, when it is inhibited, insulin and IGF-1 compensate via different enzymes such as IDE. In the cell lysates, on the other hand, both phenanthroline and phosphoramidon caused increased Aβ oligomer levels suggesting inhibition of Aβ oligomer degradation (Fig. 8B). By inhibiting degradation of Aβ monomers, phenanthroline and phosphoramidon may shift the oligomer-monomer equilibrium toward higher oligomer levels. Interestingly, insulin did not cause further increase in intracellular Aβ oligomers. Cells might increase Aβ oligomer internalization to compensate for the inhibition of extracellular Aβ oligomers. In this case, any insulin-induced Aβ oligomer internalization might be masked. However, further studies are required to clearly understand the cellular clearance of Aβ oligomers.

**FIGURE 7.** Insulin prevents Aβ oligomer-induced spine loss. A, drebrin labeling of mature hippocampal cells treated with vehicle (VEH, equivalent volume to 500 nM Aβ oligomers (ADDLs)) or soluble Aβ oligomers (ADDLs, 500 nM) in combination or not with 0.1 μM IGF-1 (IGF-1 0.1), 1 μM IGF-1 (IGF-1 1), and 1 μM insulin (INS 1) for 24 h. Images of Aβ oligomer-bound neurons were captured on a confocal microscope and quantified with Metamorph. A-1, representative images of drebrin immunolabeling obtained in the different treatment groups. A-2, bar graph illustrating the dendritic spine density obtained from drebrin immunolabeling quantified with Metamorph image sets as shown in A-1. Data represent the number of drebrin positive puncta after threshold applied and integrated analysis morphology of selected dendritic regions and are expressed as spine density per length of dendrites with Vehicle serving as control; data are expressed as % over control. Dendrites of neurons treated with ADDLs for 24 h (ADDL) exhibit decreased drebrin immunofluorescence when compared with neurons treated with vehicle for 24 h (Veh). Neurons treated with ADDLs in the presence of 1 μM IGF-1 (AD-IGF-1 1) and 1 μM insulin (AD-INS 1) exhibit drebrin staining comparable to the VEH-treated neurons, demonstrating that IGF-1 and insulin have a protective effect on Aβ oligomer-induced drebrin loss. IGF-1 (VEH-IGF-1) and insulin alone (VEH-INS) did not show much effect on dendritic spine density. Differences between VEH versus ADDL as well as differences between ADDL versus ADDL plus IGF-1 or, ADDL plus INS are highly significant (**, p < 0.001). B, ADDL-induced loss of surface IR was prevented by insulin and IGF-1: Rat hippocampal neurons were treated with 500 nM ADDLs for 24 h in the presence or absence of 1 μM IGF-1 or insulin. Cells were fixed but without permeabilization. The surface IR was detected with an anti-IR antibody. IGF-1 and insulin demonstrated complete protection of the surface IR against ADDLs. C, surface biotinylation of IR confirming the protective effects of IGF-1 and IR: Rat hippocampal neurons were treated with 500 nM ADDLs for 1 h. The surface IR was labeled with biotin before isolation with streptavidin immobilized to agarose beads. The isolated surface IR and the total IR from the cell lysates were resolved on SDS-PAGE and detected with an anti-IR antibody on Western blots. ADDL caused a significant reduction in the surface IR. Insulin and IGF-1 treatment prevent this loss. **, p < 0.01.

**FIGURE 8.** Effects of IDE and neprilysin on extra- and intracellular Aβ oligomer degradation. Cortical neurons were preincubated with phosphoramidon (0.1 μM) and phenanthroline (2 μM) at 37 °C for 30 min. ADDLs (100 nM) was added to neurons in the presence or absence of 100 nM of insulin or 100 nM IGF-1. After 1-h treatment, the medium and lysates were collected and measured with 20C2–6E10 ELISA. A, extracellular ADDL levels from the medium; B, ADDL levels from the cell lysates. ***, p < 0.001; **, p < 0.01; *, p < 0.05.
insulin resistance (54, 60, 61). Here we show that cultured nerve cells possess a capacity of degrading exogenously added ADDLs in a time-dependent manner. The degradation in the extracellular milieu can be accelerated by activation of IRs and IGF-1Rs. Conversely, dysfunctional IR mutants that manifest insulin resistance syndrome in clinic, or pharmacological inhibition of the receptor activities prevent ADDL removal and promote its further aggregation. These results provide compelling evidence linking insulin resistance to Aβ abnormality, strongly supporting the hypothesis that dysfunctional CNS insulin or IGF-1 signaling may significantly contribute to the onset and progression of AD pathology (40, 41, 61, 62, 85, 86).

Expressions of IR and IGF-1R Confer on Cells the Capability to Reduce ADDLs to Monomers—ADDLs, added exogenously to culture medium, are poorly eliminated by cells without IR or IGF-1R. However, cells overexpressing either of these receptors gained the capacity to decrease ADDL levels. A basal reduction in ADDLs by transfected cells was readily detectable, but the elimination occurred more rapidly upon IR or IGF-activation. Our results demonstrate a cellular mechanism by which ADDLs and Aβ peptides can be eliminated locally. Neuronal cultures with endogenous IR and IGF-1R expression also showed an ability to reduce ADDLs, although this was more moderate than the receptor-overexpressing cells, suggesting abundance of IRs and IGF-1Rs influence ADDL clearance capability.

Mechanisms by Which Insulin and IGF-1 Mediate ADDL Degradation—Our results showed that extracellular ADDLs levels were markedly increased by phosphoramidon, suggesting that neprilysin plays a major role in degrading the extracellular ADDLs. This is consistent with the membrane surface land presynaptic terminal localization of neprilysin (49, 63). However, insulin- and IGF-1-induced ADDL reduction was blocked only by phenanthyline, suggesting that the effects of insulin and IGF-1 on ADDL reduction in the medium are mediated by IDE. IDE has been shown to reside in the detergent-resistant membranes (64) and is transported to the extracellular domain via a distinct pathway (65). It is therefore possible that insulin and IGF-1 stimulate IDE secretion, which acts as a complementary mechanism for ADDL clearance in the extracellular compartment. Although IDE and neprilysin have been thought to be effective toward mainly Aβ monomers (66–68), through degradation of monomers they may shift the equilibrium between monomers and soluble oligomers toward left. Although either insulin or IGF-1 reduced ADDL levels, different characteristics were observed. IR activation produced increased ADDLs in cell lysate fractions and intracellularly, suggesting induced internalization. These patterns were not seen with IGF-1R signaling, which retains ADDLs on the membrane surface. Thus the IGF-1 induced ADDL clearance may occur mainly on the cell surface or in the extracellular matrix. Although IR and IGF-1R respond to each other’s ligand and share common structures, they activate distinct substrates and pathways (69), which might determine their differences in interaction with ADDLs. These differences are consistent with previous observations that oligomers of Aβ bind to IR-associated complexes but not to IGF-1Rs (37). Insulin caused ADDL immunoreactivity to be translocated to specific intracellular loci, including the nucleus in both NIH3T3 IR(+ ) and mixed cortical cells. The internalization was accompanied by a reduction in ADDL binding to dendrites most notably when cells were sensitized to insulin by prior withdrawal of exogenous insulin from the medium. Overall, insulin-induced ADDL internalization was observed more routinely in glial-rich cultures prepared from postnatal rat brains. Although binding of ADDLs or Aβ to IR has been detected (35–37), only a low degree of colocalization between IR and ADDL were seen in neuronal dendrites and astrocytes. This could be explained by rapid loss of the dendritic surface IR upon ADDL binding (37). Intracellular accumulation of exogenously applied Aβ has been previously observed in other types of cells such as human skin fibroblasts (70), although the mediating mechanism may be different. Although uptake is required for Aβ clearance in non-neuronal cells such as microglia and endothelium involving functions of LPR and its ligand ApoE and α2-macroglobulin (29, 71, 72), the nature and significance of insulin-induced neuronal and astrocytes ADDL uptake remain to be understood.

Protection of Dendritic Spines from ADDLs by Insulin and IGF-1—ADDLs are known to cause significant deterioration of synaptic spines (8), a pathology expected to underlie cognitive failure in AD (10). Our results thus raise an important question: is insulin-induced ADDL internalization harmful to synapses? We showed that, although insulin stimulated ADDL internalization, at the same time, it reduced ADDL binding to neurons, prevented the surface IR loss, and blocked ADDL-induced decreased loss in dendritic spines. This finding is in harmony with previous reports showing insulin rescues the Aβ oligomer-induced long term potentiation impairment (35, 38), and suggest that in the presence of insulin, internalized ADDLs failed to cause spine loss. Although it differs in ADDL internalization, IGF-1 showed a similar efficacy to insulin in protecting the surface IR and the spine loss triggered by ADDLs. It is possible that these neuroprotective effects of insulin may involve activation of IGF-1Rs, (or vice versa). IGF-1 has been reported to protect neurons from Aβ (73, 74). In behavioral studies, insulin and insulin sensitizers are known to improve cognitive performance both in AD patients and animals with AD pathology (75, 76). The protection of spines by insulin against ADDLs may provide a mechanism at the synapatic level underlying the beneficial effect of insulin on long term potentiation in animal AD models and its memory-enhancing effects in AD patients.

Impairment of IR and IGF-1R Exacerbates Aβ Oligomerization: A Possible Link with Type 2 Diabetes—The current findings also reveal new pathogenic consequences of diminished brain cell insulin signaling. Converse to the ability of normal IR and IGF-1Rs to mediate a robust clearance of ADDLs, cells with impaired IR and IGF-1R activities show markedly reduced clearance capacity. Reduced ability to clear these potent CNS neurotoxin provides a mechanism that may account, at least in part, for indications that dysfunctional insulin and IGF-1 signaling in the brain contributes to AD pathophysiology (1, 2, 5, 33, 35–37, 60).

An intriguing additional finding that is germane to the relationship between insulin resistance and Aβ abnormality is the altered oligomerization of Aβ occurring in the presence of cells with impaired IRs. Because there is no endogenous production
of human Aβ from the cell types used, the increased ADDL immunoreactivity observed in all IR/IGF-1R inhibitory conditions (expression of kinase dead IR mutation; inhibition of IR/IGF-1R by AG1024; and inhibition of IGF-1R by an inhibitory antibody) is due either to an overall increase in Aβ oligomerization or generation of highly immunoreactive oligomeric species. In pathological studies, both small oligomers (dimer-tetramer) and large oligomers (12-mers) have been found in AD brain and AD mouse models (11, 12, 15, 77–80). The possibility exists that these different oligomers may instigate different neurological deficits associated with AD. Seen here, inhibition of IRs promotes accumulation of oligomers, including the 12-mer range, which are pathogenic synaptic ligands (8). Interestingly, in cells lacking IR and IGF-1R, although ADDLs were not efficiently digested, no significant enhancement of aggregation or reorganization took place. This raises the interesting possibility that interactions of soluble forms of Aβ with dysfunctional IR and IGF-1R might play a “gain-of-function” role in Aβ oligomerization, actively promoting protein misfolding and/or mis-assembly. Precedent that interactions between fibrillogenic proteins and cell surfaces can alter the nature of oligomerization was recently reported for human amylin oligomers, formation of which is linked to diabetes (58, 81).

A Theoretical Pathogenic Loop Involving Insulin Resistance and Aβ Aggregation in AD Pathogenesis—Recently, we and others have observed that Aβ oligomers down-regulate IR function and surface expression, thereby causing brain cell insulin resistance (35–38). Thus, although normal IR activity helps to defend against the accumulation of toxic Aβ oligomers, the insulin receptors themselves are vulnerable to oligomer-initiated dysfunction. This relationship has the potential to generate an insidious pathogenic loop. Deficits in IR function, which could arise from multiple factors, would contribute to increased ADDL formation/accumulation in the brain, and the increasing ADDL levels would further suppress IR activity and worsen insulin insensitivity. It seems likely that the pathologically reinforcing loop between these two abnormalities exacerbate one another to drive the progression of the AD process, although it will require considerable effort to clarify the relationship in vivo. Given the potential of interactions between accumulation of Aβ oligomers and impairment of insulin/IGF-1 signaling for driving disease progression, there may be a synergistic benefit to treating patients with drugs that enhance CNS insulin signaling in tandem with those that neutralize ADDLs. Steps toward each are now in progress individually (82–84). The current results suggest further that understanding the precise relationship between IR/IGF-1R signaling and APP processing/Aβ clearance may lead to identification of additional cellular targets for prevention and treatment of AD.

Acknowledgments—We thank Sara Fernandez, Pauline Velasco, and Kirsten Viola of the Department of Neurobiology and Physiology, Northwestern University, for assistance with neuronal culture and ADDL preparation.

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