Insulin Has Multiple Antiamyloidogenic Effects on Human Neuronal Cells

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Alzheimer's disease is increased in diabetic patients. A defective insulin activity on the brain has been hypothesized to contribute to the neuronal cell dysregulation leading to AD, but the mechanism is not clear. We analyzed the effect of insulin on several molecular steps of amyloid precursor protein (APP) processing and β-amyloid (Aβ) intracellular accumulation in a panel of human neuronal cells and in human embryonic kidney 293 cells overexpressing APP-695. The data indicate that insulin, via its own receptor and the phosphatidylinositol-3-kinase/AKT pathway, influences APP phosphorylation at different sites. This rapid-onset, dose-dependent effect lasts many hours and mainly concerns dephosphorylation at the APP-T668 site. This effect of insulin was confirmed also in a human cortical neuronal cell line and in rat primary neurons. Cell fractionation and immunofluorescence studies indicated that insulin-induced APP-T668 dephosphorylation prevents the translocation of the APP intracellular domain fragment into the nucleus. As a consequence, insulin increases the transcription of antiamyloidogenic proteins such as the insulin-degrading enzyme, involved in Aβ degradation, and α-secretase. In contrast, the transcripts of pro-amyloidogenic proteins such as APP, β-secretase, and glycogen synthase kinase (Gsk)-3β are decreased. Moreover, cell exposure to insulin favors the nonamyloidogenic, α-secretase-dependent APP-processing pathway and reduces Aβ40 and Aβ42 intracellular accumulation, promoting their release in the extracellular compartment. The latter effects of insulin are independent of both Gsk-3β phosphorylation and APP-T668 dephosphorylation, as indicated by experiments with Gsk-3β inhibitors and with cells transfected with the nonphosphorylatable mutated APP-T668A analog. In human neuronal cells, therefore, insulin may prevent Aβ formation and accumulation by multiple mechanisms, both Gsk-3β dependent and independent. **(*Endocrinology* 154: 375–387, 2013)**

Alzheimer's disease (AD) is a neurodegenerative disorder that typically occurs late in life and is characterized by memory loss and cognitive impairment. The disease is associated with progressive neuronal degeneration and the accumulation of neurofibrillary tangles and insoluble β-amyloid (Aβ) plaques in the central nervous system. AD pathogenesis is classically attributed to Aβ overproduction due to abnormal APP processing and to τ-protein hyperphosphorylation in the central nervous system.

Amyloid plaques are aggregates of insoluble Aβ peptides derived from the proteolytic cleavage of amyloid precursor protein (APP), a cell transmembrane protein that is important for neuronal synaptogenesis, synapse remodeling, and neurite outgrowth (1). APP metabolism can follow two distinct pathways. In the nonamyloidogenic path-

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Abbreviations: Aβ, β-Amyloid; AD, Alzheimer's disease; AICD, APP intracellular domain; APP, amyloid precursor protein; BACE1, β-secretase; Cdc2, p34cdc2 protein kinase; Cdk5, cyclin-dependent protein kinase 5; HEK, human embryonic kidney; HR, hybrid receptor; IDE, insulin degrading enzyme; IR, insulin receptor; P, phosphorylated; PI3K, phosphatidylinositol 3-kinase; sAPPα and sAPPβ, extracellular soluble amino-terminal fragments of APP; T668, threonine at position 668 of APP.

way APP is cleaved by an α-secretase that releases an extracellular NH_2-terminal soluble fragment with neurotrophic activity (sAPPα) and the intracellular COOH-terminal fragment (CTFα) that does not generate Aβ. In the amyloidogenic pathway APP is cleaved by a β-secretase that releases a soluble extracellular domain (sAPPβ) with unknown function, and an intracellular fragment (CTFβ) that is further cleaved by the γ-secretase to form Aβ and the APP intracellular domain (AICD) (2, 3). AICD can translocate inside the nucleus where it exerts transcriptional activity. The prevalence of the amyloidogenic pathway of APP processing may lead to Aβ accumulation.

In addition to the secretase enzymes, APP metabolism is regulated by an intricate signaling network that can depend on the phosphorylation state of APP (4). T668, one of the eight potential phosphorylation sites in the APP intracellular domain, is constitutively phosphorylated in the brain (5) and thought to be important for the neuronal function of APP (6). T668 can be phosphorylated by a number of kinases, including glycogen synthase kinase-3β (GSK-3β), cyclin-dependent protein kinase 5 (Cdk5), p34cdc2 protein kinase (Cdc2), and c-jun N terminal kinase (JNK) (5, 7-9). AICD phosphorylation at T688 influences the peptide transcriptional activity (10-12) which favors neuronal cell apoptosis (13). This deleterious AICD effect may be prevented when the nuclear translocation of the fragment is inhibited (14), suggesting that its harmful action is linked to intranuclear activity.

Diabetes is associated with a 1.4- to 4.3-fold (15-18) increased risk of AD (17, 19, 20), and many AD patients have impaired glucose tolerance, hyperinsulinemia, and insulin resistance (21). AD patients also have a reduced cerebrospinal fluid to plasma insulin ratio (22). Moreover, memory improvement has been observed in AD patients after intranasal insulin administration (23-25). These observations suggest a possible role of impaired insulin action (*i.e.* insulin resistance and/or insulin deficiency) in the pathogenesis of AD. Insulin has important functions in brain regions involved in learning and memory (26, 27). In addition to its typical metabolic effects, insulin also activates mechanisms related to neuronal activity, including APP processing, Aβ generation, and τ protein phosphorylation, all major components of AD pathogenesis.

To clarify the possible mechanistic role of insulin in AD pathogenesis, we studied *in vitro* the effect of insulin on APP processing and Aβ accumulation. We found that in neuronal cells insulin activates multiple mechanisms that antagonize Aβ formation and accumulation.

Materials and Methods

Cell media and chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO). Fetal calf serum and geneticin (G418) were from Invitrogen Laboratories (Paisley, UK). IGF-I, IGF-II, LY294002, and PD98059 were purchased from Calbiochem (San Diego, CA). Anti-phospho-APP (T668), anti-APP/Aβ, anti-phospho-ERK1/2 (T202/Y204), anti-phospho-AKT (S473), anti-GSK3β and anti-phospho-GSK3β (S9) antibodies were from Cell Signaling Technology (Danvers, MA). Anti-APP (22C11) antibody was from Chemicon (Millipore, Billerica, MA) and anti-sAPPα and anti-sAPPβ antibodies were from IBL (Hamburg, Germany). Anti-phospho-APP (S198, S655, and Y682) antibodies were from Abgent (San Diego, CA). Anti-APP C-terminal and anti-Flag M2 antibodies were from Sigma. Anti-phospho-Cdc2 (T14/Y15), anti-phospho-Cdk5 (Y15), anti-phospho-JNK (T183/Y185), anti-Histone H2B, anti-α-tubulin, and anti-β-tubulin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). pcDNA3.1-APP-695, encoding the human APP short isoform, was kindly provided by Dr. V. M.-Y. Lee (Philadelphia, PA). The cDNA encoding the mutated form of human APP, pAPP-695-T668A, was kindly provided by Dr. T. Suzuki (Sapporo, Japan).

Cells and receptors

H4 human neuroglioma cells, SK-N-SH and SH-SY5Y neuroblastoma cells, SK-N-MC neuroepithelioma cells, and human kidney 293 cells (HEK293) were obtained from the American Type Culture Collection (Manassas, VA). The nontransformed human cortical neuronal cell line (HCN-2) was kindly provided by Dr. G. Cantarella (Catania, Italy). Primary cultures of pure cortical neurons were obtained from rats at embryonic d 15 as previously described (28). Animal care and experimentation were in accordance with institutional guidelines.

The HEK293 cell line is widely used as a mammalian expression system to study regulatory proteins. HEK293 cells were transfected with an expression vector containing the human APP-695 cDNA that encodes for either the APP short isoform or the mutated APP-695-T668A cDNA. The transfected cells showed an approximately 10- to 15-fold higher expression of APP than wild-type HEK293 cells (Fig. 1A).

In studied cells, insulin receptor (IR), IGF-I receptor (IGF-IR), and hybrid IR/IGF-IR receptor (HR) were measured by ELISA as previously described (29). For immunodepletion studies, cells were preincubated for 45 min at 37 C with 50 nM of the appropriate blocking antibody (MA-10 for IR; αIR-3 for IGF-I-R). IR isoform transcript was measured using oligonucleotide primers spanning nucleotides 2246-2265 (5′-CCA-AAG-ACA-GAC-TCT-CAG-AT-3′) and 2391-2410 (accession NM_000208.2) (5′-AAC-ATC-GCC-AAG-GGA-CCT-GC-3′) of the human IR. After PCR amplification and electrophoresis of the PCR products, the 165- and 129-bp DNA fragments representing the Ex11+ and Ex11− of IR isoforms were analyzed by scanning densitometry and compared with the standards.

Real-time PCR

Total RNA (5 μg) was reverse transcribed with ThermoScript RT (Invitrogen) and Oligo dT primers. Synthesized cDNA (25 ng) was then combined in a PCR using the appropriate primers (Table 1). Quantitative real-time PCR was performed on an ABI

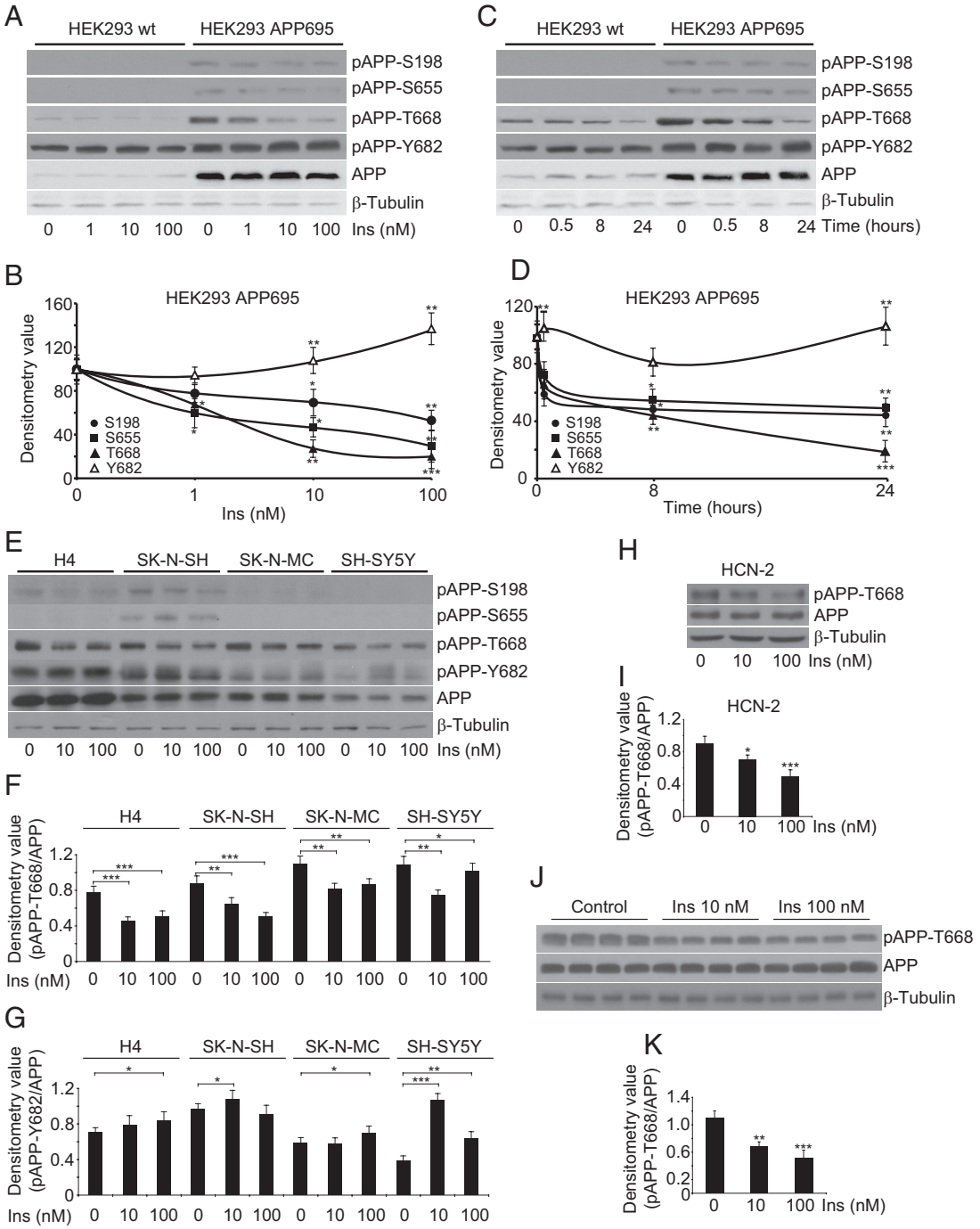

FIG. 1. APP phosphorylation at serine 198 and 655, threonine 668, and tyrosine 682 in HEK293, in human neuronal cell lines of and in rat primary neurons. Serum-starved cells were incubated in the presence or absence of insulin for 24 h. Cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Membranes were reblotted with an anti-APP antibody and an anti-*β*-tubulin antibody to control for protein loading. A representative result of three independent experiments is shown. A, The dose effects of insulin exposure on APP phosphorylation in HEK293-wt and HEK293-APP695 cells. B, Densitometric quantification of HEK293-APP695 cells data shown in panel A. C, The time course for APP phosphorylation in cells exposed to 10 nM insulin for the indicated times. D, Quantification of HEK293-APP695 cell data shown in panel C. E, APP phosphorylation was induced by 10 and 100 nM insulin (24 h) in H4, SK-N-SH, SK-N-MC, and SH-SY5Y human neuronal cells. F and G, Quantification of T668 and Y682 APP phosphorylation data shown in panel E. H, Dose effect of insulin on T668 APP phosphorylation in nontransformed human HCN-2 cells incubated for 3 h with the hormone. I, Quantification of the data shown in panel H. J, Dose effect of insulin exposure on T668 APP phosphorylation in rat primary neurons incubated for 3 h. K, Quantification of the experiments shown in panel J. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* the untreated control. Ins, Insulin; wt, wild type.

TABLE 1. Primer sequences

Gene	GenBank accession no.	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
APP	NM_201414.2	TGCCCATTTCCAGAAAGCC	CGGCGGTCATTGAGCATGGC	242
BACE1	NM_012104.4	TGGAGGGCTTCTACGTTGTCTT	CCTGAACTCATCGTGCACATG	86
ADAM10	NM_001110.2	TCATGATGACTACTGTTTGGCCTAT	TCCTCCAGAGCTTCCTGAAGGT	103
Presenilin1	NM_000021.3	CCTGCACCGTTGTCCTACTTC	TGCCGTTCTCTATTGTCATTCTG	95
IDE	NM_004969.3	ATGTTCTTGCCAGGGAAATG	CAGAGTTTTGCAGCCATGAA	190
GSK3-β	NM_001146156.1	ATCACTGTAACATAGTCCGAT	ATAAATCACAGGGAGCGTCT	158
GAPDH	NM_002046.3	ACCCACTCCTCCACCTTTG	CTCTTGTGCTCTTGCTGGG	178

GAPDH, Glyceraldehyde phosphate-3-dehydrogenase.

Prism 7500 (PE Applied Biosystems, Foster City, CA) using SYBR Green PCR Master Mix (PE Applied Biosystems) according to the manufacturer's instructions. Amplification reactions were checked for the presence of nonspecific products by dissociation curve analysis. Relative quantitative determination of target gene levels was done by comparing ΔCt.

Aβ and sAPP measurements

Human Aβ-40 and Aβ-42 and human sAPPα and sAPPβ peptides were quantified in conditioned medium using commercial ELISA kits according to the manufacturer's instructions (Genetics Co., Schlieren, Switzerland; and Millipore Corp., Billerica, MA). sAPPα and sAPPβ peptides were quantified also by Western blot. Serum-starved HEK293-APP-695 cells were incubated with or without 10 nM insulin for 24 h for Aβ peptide and for 3 h for sAPP peptide measurements. Aβ peptides were measured also in solubilized cells.

Subcellular fractionation

Serum-starved HEK293-APP695 and HEK293-APPT668A cells were stimulated with insulin for 24 h and then resuspended in a hypotonic buffer [10 mM Tris (pH 8.0), 10 mM KCl, 2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture]) to allow cell swelling. Next, Nonidet P-40 was added to a final concentration of 0.4%. After centrifugation for 10 min at 1500 × *g* the supernatants were collected as the cytoplasmic fraction. The pellets containing nuclei were washed in hypotonic buffer, centrifugated for 10 min at 1500 × *g* and resuspended with a high-salt lysis buffer [50 mM Tris (pH 8.0), 5 mM EDTA (pH 8.0), 1% Nonidet P-40, 1% sodium deoxycholate, 0.025% sodium dodecyl sulfate, 400 mM NaCl, 2 mM phenylmethylsulfonyl fluoride and a protease inhibitor mixture]. The resuspended nuclei were centrifuged for 1 h at 22,000 × *g* and the supernatants collected. Equal amounts of proteins were separated using 10-20% SDS-PAGE, transferred onto polyvinylidene difluoride membranes and blotted with an anti-APP C-terminal antibody. Membranes were reblotted with anti-α-tubulin and antihistone-2B antibodies to confirm cell fraction purity.

Immunofluorescence studies

HEK293 cells were transiently cotransfected with either APP-695-green fluorescent protein (GFP) or APP-T668A-GFP cDNA and the cDNA of Fe65-Flag. Next, cells were serum starved, exposed to insulin for 24 h, fixed in PBS containing 4% paraformaldehyde for 15 min at room temperature, and then incubated with a blocking solution (normal goat serum 5%, Triton 0.3% in PBS) for 1 h. Fixed materials were incubated overnight at 4 C with the anti-Flag M2 antibody in PBS supplemented with 1% BSA (wt/vol) and 0.3% Triton X-100. After rinsing, cells were incubated in an Alexa Fluor-549 anti-Mouse IgG secondary antibody (Invitrogen, Monza MB, Italy) for 1 h at room temperature. Cells were finally counterstained with Hoechst 33258 to color the nuclei. Images were digitally acquired with epifluorescence microscopy using an Orca charge-coupled device Camera (Hamamatsu City, Shizuoka, Japan) and processed with Image-Pro Plus 4.0 software (Media Cybernetics, Silver Spring, MD).

Statistical analysis

All values are expressed as the mean ± SE. Differences between groups were analyzed using a paired two-tailed Student's *t* test with GraphPad Prism 5 statistical software. Differences were considered significant at $P < 0.05$.

Results

Insulin causes APP dephosphorylation at T668 in human neuronal cell lines and in HEK293 cells overexpressing APP-695

To assess the effect of insulin on APP processing, we first analyzed APP phosphorylation in both HEK293-wild type and HEK293-APP695 cells exposed to increasing concentrations of insulin. Phosphorylation at residues T668, Y682, S198, and S655 was measured by immunoblotting with specific antibodies. Under basal conditions (no insulin present) the antibodies used showed an APP phosphorylation relatively low at S198 and S655, more marked at T668, and very high at Y682 (Fig. 1, A and D). After exposure to insulin, we observed a dose-dependent decrease of T668 APP phosphorylation in both transfected and untransfected cells (Fig. 1, A and B). The other phosphorylation sites were less affected by insulin except for a slight increase in Y682 phosphorylation (Fig. 1, A and B). T668 dephosphorylation was already evident after exposure to 1 nM insulin and reached a maximum inhibition at 100 nM

(Fig. 1, A and B). APP-T668 dephosphorylation was already observed 30 min after cell exposure to 10 nM insulin and reached a maximum after 24 h (Fig. 1, C and D).

In H4, SK-N-SH, SK-N-MC, and SH-SY5Y cells exposed to insulin, the APP phosphorylation pattern at residues S198, S655, T668, and Y682 was similar to that observed in HEK-293 cells (Fig. 1, E–G).

To confirm the results obtained in tumor cells of neural origin, we evaluated the effect of insulin in a nontransformed human cell line from normal cortical neurons (HCN-2), and in four independent primary cultures of rat cortical neurons. After exposure to insulin we observed a dose-dependent decrease of T668 APP phosphorylation in both HCN-2 cells (Fig. 1, H and I) and in primary neurons (Fig. 1, J and K). Our data indicate that insulin significantly stimulates T668 dephosphorylation and, to a lesser extent, Y682 phosphorylation.

The signaling pathway for T668 APP dephosphorylation induced by insulin

Insulin can activate the intracellular signaling pathways via its own receptor (IR) but also via the cognate receptor for IGF-I (IGF-IR) and the IR/IGF-IR hybrid receptors (HRs) (30). We measured cell receptor content (Table 2) and found that HR was the prevalent receptor type in the human neuronal cells studied except SH-SY5Y, which also expressed low levels of IR (the second most abundant receptor in all cell lines). IR-A was the predominant IR isoform (65-85%) in all neuronal cells. In wild-type and APP-695 transfected HEK293 cells, the IGF-IR content was nearly 4 times higher than the IR content.

Similar to insulin, 10 nM IGF-I or IGF-II induced APP-T668 dephosphorylation (Fig. 2A). To identify the receptor type involved in APP-T668 dephosphorylation by insulin, we used specific antibodies that blocked either the IR (MA-10) or the IGF-IR (αIR3). IR blockade with MA-10 markedly inhibited APP-T668 dephosphorylation induced by insulin, but it only partially inhibited the effect of IGF-I. Conversely, blockade with αIR3 markedly inhibited the effect of IGF-I but only mildly inhibited the effect of insulin (Fig. 2, B and C). Therefore, both insulin and IGF-1 cause APP-T668 dephosphorylation, mainly through their specific receptors.

Insulin activates two major intracellular pathways, including the PI3K/AKT pathway, which produces predominant metabolic effects, and the MAPK/ERK pathway, which produces predominant mitogenic effects (31). In conditions of insulin resistance, the metabolic pathway can be impaired more than the mitogenic pathway (32). To evaluate which intracellular pathway was involved in the insulin-induced APP-T668 dephosphorylation, HEK293-APP695 cells were incubated with insulin in the presence or absence of several kinase inhibitors, including NVP-AEW541 (IR and IGF-IR tyrosine kinase inhibitor), PD98059 [MAPK kinase (MEK)-1 inhibitor], and LY294002 (phosphatidyl-inositol-3-kinase, PI3K inhibitor). Cell treatment with LY294002 (10 μM) not only completely blocked the effect of insulin on APP dephosphorylation at T668 (Fig. 2, D and E) but also increased APP-T668 phosphorylation under basal conditions. In contrast, PD98059 treatment (50 μM) did not alter the effects of insulin (Fig. 2, D and E). As expected, IR and IGF-IR inhibition by NVP-AEW541 (10 μM) blunted both AKT and ERK phosphorylation and the insulin-induced APP dephosphorylation at T668 (Fig. 2, D and E).

Cell exposure to insulin activates a phosphorylation cascade involving many kinases of the PI3-kinase/AKT pathway, including Gsk-3β. This enzyme plays a major role on APP phosphorylation at T668. Other kinases, such as JNK (8), Cdk5 (5), and Cdc2 (33), are also able to phosphorylate APP-T668. Insulin causes Gsk-3β phosphorylation at S9 reducing its enzymatic activity. As a consequence of reduced Gsk-3β activity, the constitutive APP-T668 phosphorylation (7) is decreased after cell exposure to insulin.

To evaluate whether the effect of insulin on APP-T688 dephosphorylation was mediated only by Gsk-3β or also by other kinases, we studied insulin effect on different kinases and the influence of specific kinase inhibitors on APP-T668 phosphorylation.

When HEK293-APP695 cells were exposed to insulin, Gsk-3β phosphorylation was clearly increased and JNK phosphorylation (T183/Y185) was slightly decreased, resulting in a decrease in the enzymatic activity of both kinases (Fig. 2, F and G). Cdk5 and Cdc2 were not affected by the cells' exposure to insulin. Next, cells were exposed to the specific kinase inhibitors SB216763 (Gsk-3β inhibitor), SP600125 (JNK inhibitor), olomoucine (Cdc2 inhibitor), and kenpaullone (Cdk5 inhibitor). The effect of insulin on APP dephosphorylation at T668 was mimicked by SB216763 and, to a lesser extent, by SP600125 and

TABLE 2. IR, IGF-IR, and HR content and IR-A relative abundance

Cells	IR[a]	IGF-IR[a]	HR[a]	IR-A[b] (%)
H4	2.4	1.9	5.2	67
SK-N-SH	1.8	0.8	3.6	73
SK-N-MC	5.6	3.6	12.4	85
SH-SY5Y	1.8	11.2	8.4	65
HEK-293	2.3	8.5	12.3	100

[a] Nanograms/100 μg protein (ELISA); [b] RT-PCR.

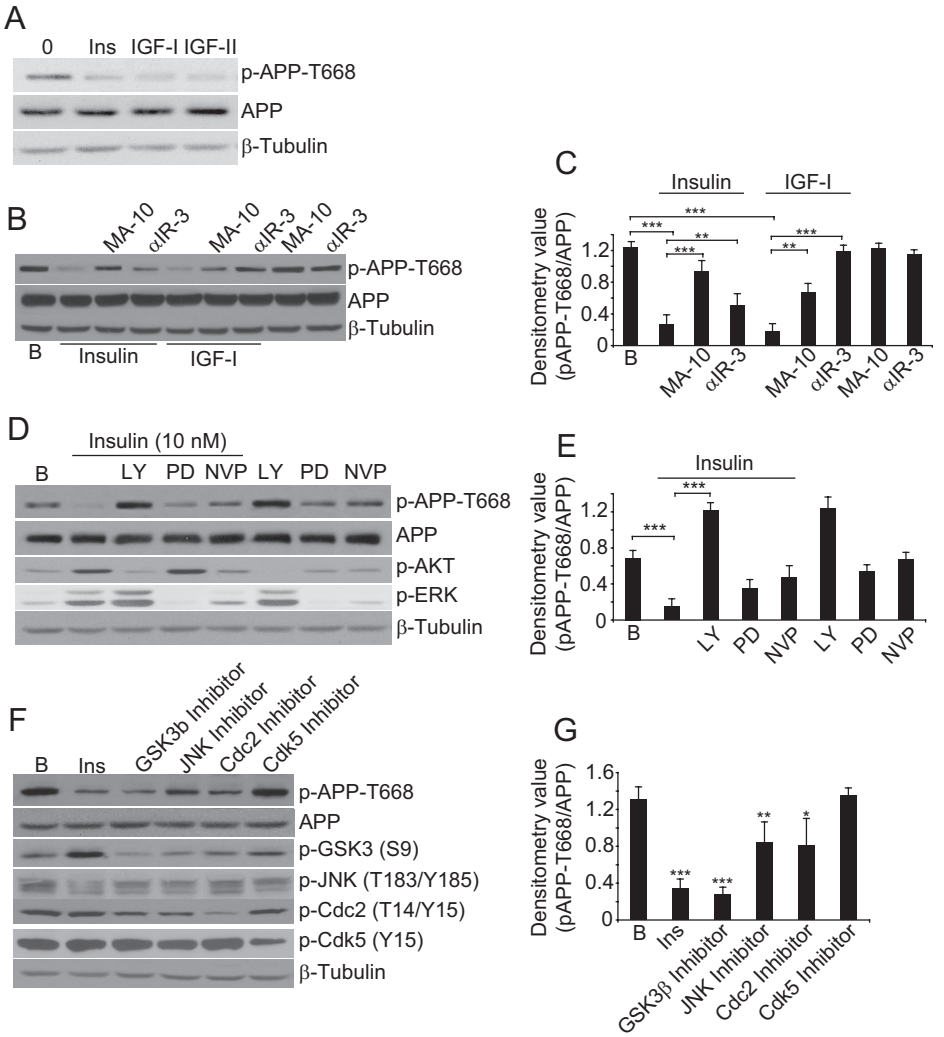

FIG. 2. The insulin-signaling pathway for inducing APP-T668 dephosphorylation. A, Serum starved HEK293-APP695 cells were incubated in the presence or absence of 10 nM insulin, IGF-I, or IGF-II. T688 APP phosphorylation was evaluated after 24 h. B, Serum-starved HEK293-APP695 cells were preincubated for 45 min in the presence or absence of 50 nM blocking antibodies specific for either the IR (MA-10) or the IGF-IR (αIR3). Next, cells were stimulated with 10 nM insulin or IGF-I for 30 min. Cell lysates were separated by SDS-PAGE and immunoblotted with an anti-p-APP (T668) antibody. Membranes were reblotted with an anti-APP antibody and an anti-β-tubulin antibody to control for protein loading. A representative result from three independent experiments is shown. C, Densitometric quantification of the data shown in panel B. D, T668 APP phosphorylation was evaluated in serum-starved HEK293-APP695 cells before and after exposure to insulin. To inhibit either the AKT or the ERK pathway, cells were preincubated for 45 min in the presence or the absence of 10 μM LY294002 (PI3K inhibitor), 50 μM PD98059 (MEK-1 inhibitor), or 10 μM NVP-AEW541 (IR and IGF-IR inhibitor). E, Quantification of the data shown in panel D. F, The effect of insulin on various kinases known to influence T668 APP phosphorylation. Cells were exposed to insulin and 10 μM of either SB216763 (Gsk-3β inhibitor), SP600125 (JNK inhibitor), Olomoucine (Cdc2 inhibitor), or Kenpaullone (Cdk5 inhibitor) for 45 min to evaluate the effect of kinase inhibition on T688 APP phosphorylation. Representative results from three independent experiments are shown. G, Quantification of the data shown in panel F. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* the untreated control unless otherwise indicated. Ins, Insulin; Ly, LY294002; NVP, NVP-AEW541; PD, PD98059.

Olomoucine. Kenpaullone had no effect in our model. Immunoblots using the antiphosphokinase-specific antibodies indicated that the inhibitors used specifically inhibited the kinases (Fig. 2, F and G).

Therefore, insulin acts mainly via its own receptor, activates the PI3K/Akt pathway, phosphorylates Gsk-3β, and slightly dephosphorylates JNK, reducing the activity of these kinases on APP-T668 phosphorylation.

Insulin favors nonamyloidogenic APP processing and stimulates Aβ extrusion from cells into the medium

Aβ accumulation in neuronal cells depends on the amount of the protein that is produced through the β-secretase amyloidogenic pathway and the amount released outside the cell. To investigate the effect of insulin on Aβ accumulation, we first evaluated whether insulin

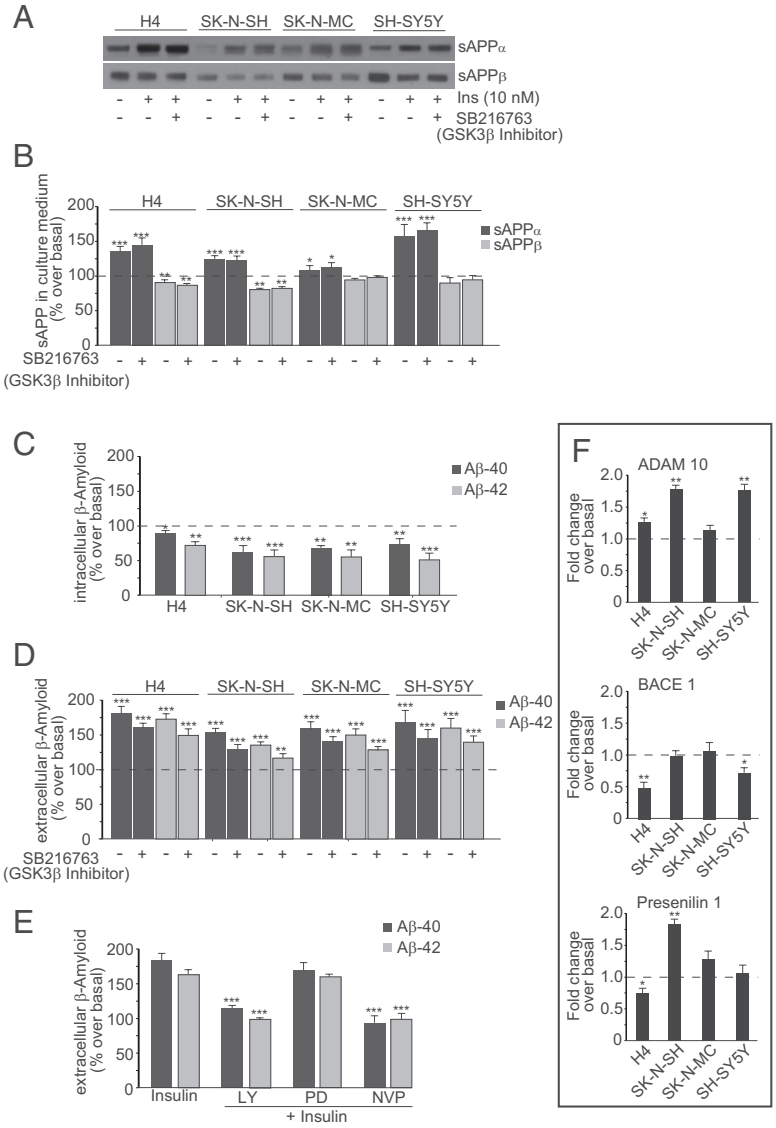

FIG. 3. Insulin influences APP processing and increases β-amyloid secretion in human neuronal cells. Serum-starved human neuronal cells were incubated in the presence or absence of insulin, and sAPP-α and -β and β-amyloid fragments 1-40 and 1-42 were measured by specific ELISAs and SDS-PAGE. Basal values (not exposed to insulin) are indicated as 100 (*dotted line*), and *columns* indicate average values after cell exposure to insulin. sAPP-α and -β were measured in the culture medium by SDS-PAGE (A) or ELISA (B). Intracellular Aβ40 and Aβ42 were measured in the cell lysates (C), and extracellular Aβ40 and Aβ42 were measured in the culture medium (D). E, The extracellular Aβ40 and Aβ42 concentrations in the culture medium of HEK293-APP695 cells preincubated for 45 min in the presence or absence of 10 mM LY294002 (PI3K inhibitor), 50 mM PD98059 (MEK-1 inhibitor), or 10 mM NVP-AEW541 (IR and IGF-IR inhibitor) and then incubated with insulin for 24 h. Protein values from the conditioned medium were normalized according to the total protein content. F, As indicated in *Materials and Methods*, the mRNA for three secretase enzymes was measured after neuronal cell exposure to 10 nM insulin for 8 h. Relative mRNA amounts were normalized to the abundance of GAPDH mRNA. Data are expressed as the mean ± SEM of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* basal values.

influences the amyloidogenic *vs.* nonamyloidogenic pathway of APP processing.

After insulin treatment, sAPPα was significantly increased in the conditioned medium of all cell lines (as previously reported in SH-SY5Y cells) (34), whereas sAPPβ was significantly decreased in H4 and SK-N-SH cells (Fig. 3, A and B). These results indicate that insulin may reduce Aβ production by favoring APP processing via the nonamyloidogenic sAPPα pathway. The presence of the Gsk-3β inhibitor SB216763 (10 μM) did not influence insulin-induced APP processing, indicating that this effect of insulin is not mediated by Gsk-3β (Fig. 3B).

To investigate the mechanisms underlying insulin-stimulated α-secretase activity, we studied the effect of insulin on the expression of secretase encoding genes. After cells were exposed to 10 nM insulin for 8 h, ADAM10 (α-secretase) mRNA was significantly increased in all cell types except SK-N-MC, β-secretase (BACE1) mRNA was significantly decreased in H4 and SH-SY-5Y cells, and presenilin-1 (a component of γ-secretase) mRNA responded to insulin in a heterogeneous way. More specifically, presenilin-1 mRNA was significantly decreased in H4 cells, significantly increased in SK-N-SH, and did not change in the other two neuronal cell lines (Fig. 3F). These data suggest that, at least in some human neuronal cells, insulin favors the nonamyloidogenic processing of APP by stimulating α-secretase and/or inhibiting β-secretase transcription.

We next measured the effect of insulin on the intracellular accumulation of the detrimental Aβ. After insulin exposure intracellular levels of both Aβ-40 and Aβ-42 decreased in all neuronal cells (Fig. 3C) confirming the data reported by Gasparini *et al.* (35) in N2a neuroblastoma cells transfected with human APP-695. In contrast, both peptides increased in the culture medium (Fig. 3D), suggesting that insulin reduces the intracellular accumulation of Aβ also by promoting its release into the culture medium. To analyze the mechanism of insulin-induced release of Aβ we preincubated HEK293-APP695 cells with LY294002, PD98059, or NVP-AEW541 to selectively inhibit the major insulin-signaling pathways. Data indicated

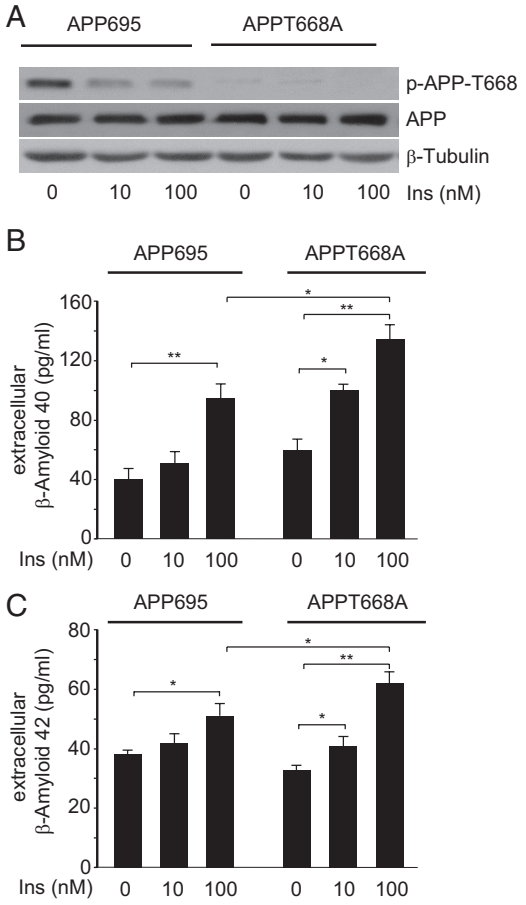

FIG. 4. Insulin-induced β-amyloid production is not dependent on APP-T668 phosphorylation. Serum-starved HEK293 cells, stably transfected with cDNA encoding either the human APP695 or the nonphosphorylatable APPT668A were incubated with 10 or 100 nM insulin for 24 h. A, Cell lysates were separated by SDS-PAGE and immunoblotted with an anti-p-APP (T668) antibody. Reblotting with an anti-APP antibody and an anti-β-tubulin antibody was completed to control for protein loading. A representative result of three independent experiments is shown. B and C, Both Aβ40 and Aβ42 levels progressively increased in the culture medium of cells transfected with APP695 or APP668A after exposure to increasing insulin concentrations. The mean values of three separate experiments are shown. *, $P < 0.05$; **, $P < 0.01$ *vs.* the untreated control.

that both IR and PI3K blockage completely prevented the insulin-induced increase of extracellular Aβ-40 and Aβ-42 whereas ERK inhibition had no effect (Fig. 3E).

When cells were exposed to insulin in the presence of 10 μM Gsk-3β inhibitor, SB216763, extracellular Aβ was only slightly reduced in comparison with cells treated with only insulin: differences were not statistically significant. This result indicates that the effect of insulin on Aβ secretion is, at least in part, independent from insulin-induced phosphorylation of Gsk-3β (Fig. 3D).

To assess whether insulin-induced APP-T688 dephosphorylation, which is mediated via the activation of PI3K/Gsk-3β pathway, could be linked to insulin-stimulated Aβ secretion, we measured Aβ levels in the culture medium of HEK293 cells transfected with an APP-695 mutant (APP-T668A) that cannot be phosphorylated at position 668. In comparison with HEK 293-APP695 cells, APP phosphorylation was not observed in HEK293 cells expressing similar amounts of APP-T668A (Fig. 4A). When HEK-APP-695 and HEK-APP-T668A cells were exposed to insulin for 24 h, a similar dose-dependent increase of both Aβ fragments was detected (Fig. 4, B and C), suggesting that insulin-stimulated Aβ secretion occurs via PI3K activation but is independent from Gsk-3β and the phosphorylation state of APP-T668.

Insulin inhibits the translocation of AICD into the nucleus and affects gene transcription

Phosphorylation at T668 may be required for the APP intracellular fragment (AICD) translocation into the nucleus (11). We evaluated whether insulin-dependent APP-T668 dephosphorylation influenced AICD subcellular localization. For this purpose, HEK293 cells were transfected with either the wild-type (APP695) or the nonphosphorylatable mutated APP-T668A. Cells were incubated for 24 h with 10 nM insulin, after which AICD was measured in the cytosolic and nuclear fractions using Western blot analysis. After exposure to insulin, AICD levels decreased in the nuclear fraction but not in the total cell lysates or the cytosolic fraction of HEK293-APP695 cells. In contrast, in HEK293 cells expressing the mutated APP, AICD was detected only in the cytosolic fraction both before and after exposure to insulin (Fig. 5A). Cell fraction purity was assessed by blotting the cell lysate with antibodies against marker proteins for either the cytosolic (α-tubulin) or the nuclear fraction (histone H2B).

To confirm the inhibitory effect of insulin on AICD nuclear translocation, we investigated the subcellular localization of AICD by a different method. HEK293 cells were transiently cotransfected with plasmids encoding for either APP695-GFP or APPT668A-GFP and the plasmid encoding for Fe65-flag. Fe65 is a scaffolding/adaptor protein that protects AICD from rapid degradation (36) and is important for its nuclear translocation (13, 36). Using immunofluorescence microscopy, AICD was detected in the nucleus of HEK293-APP695-GFP cells before exposure to insulin, but disappeared from the nucleus after cells were exposed to insulin for 24 h. In contrast, no AICD was observed in the nucleus of cells carrying the mutated APP, both before and after insulin treatment (Fig. 5B).

These data indicate that insulin inhibits AICD translocation into the nucleus and that this effect depends on APP-T668 dephosphorylation.

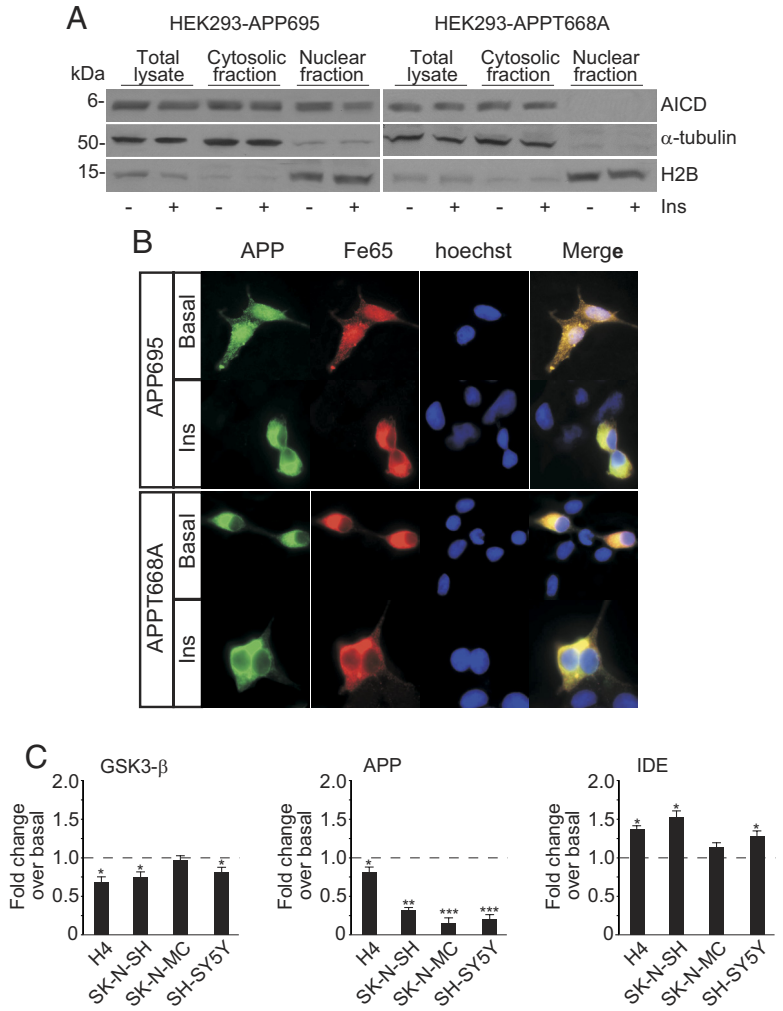

FIG. 5. Insulin inhibits AICD translocation to the nucleus via APP-T668 dephosphorylation and activates gene expression in neuronal cells. A, Serum-starved HEK293 cells were stably transfected with the plasmids encoding for either APP695 or APPT668A. Next, cells were incubated with 10 nM insulin for 24 h. After cell fractionation, subcellular APP localization in the cytosolic and the nuclear fractions was measured by SDS-PAGE and immunoblotting with an anti-AICD antibody. Cell fraction purity was determined by reblotting with an anti-α-tubulin antibody (cytoplasm) or with an anti-H2B antibody (nucleus). A representative result from three independent experiments is shown. B, Immunofluorescence analyses were carried out in serum-starved HEK293 cells, transiently cotransfected with the plasmids encoding for either APP695-GFP or APPT668A-GFP and the plasmid encoding for Fe65-Flag before and after treatment with 10 nM insulin for 24 h. An anti-flag antibody was used to detect Fe65 and dyed with a goat antimouse antibody labeled with Alexa Fluor 594 (*red*). Nuclei were stained with Hoechst (*blue*). C, The effects of insulin on gene expression. Serum-starved human neuronal cells were incubated in the presence or absence of insulin (10 nM) for 8 h. The mRNA for the indicated genes was measured by quantitative real-time PCR. Relative mRNA amounts were normalized to the abundance of glyceraldehyde 3-phosphate dehydrogenase mRNA. The results are presented as the fold increase of untreated cells that were indicated as 1 (*dotted line*). *Columns* indicate the mean of three separate experiments; *bar*, SD; *, $P < 0.05$; **, $P < 0.01$ *vs.* the untreated control. Ins, Insulin.

Once inside the nucleus, AICD has transcriptional activity (10, 36, 37). Combined with the adaptor protein Fe65 and the histone acetyltransferase Tip60, the AICD ternary complex colocalizes with active transcription sites and regulates the transcription of many genes that are involved in the pathogenesis of AD, including APP, Gsk-3β (12-14), BACE1 (12), and the Aβ protease insulin degrading enzyme (IDE) (38).

Quantitative real-time PCR analysis revealed that exposure to insulin (10 nM for 8 h) significantly decreased APP mRNA in all neuronal cells and significantly decreased Gsk-3β mRNA and increased IDE mRNA in all cell types except SK-N-MC (Fig. 5C). The data suggest that insulin, by inhibiting AICD nuclear translocation, influences the transcription of many genes directly or indirectly involved in Aβ production and accumulation. These multiple effects of insulin are variable in terms of quantity and quality in different neuronal cell lines but are always antiamyloidogenic.

Discussion

Numerous clinical and experimental studies indicate that reduced insulin action may play a role in the pathogenesis of AD. The association of AD with insulin resistance (39), type 2 diabetes (16-18, 40, 41), reduced insulin concentration in the cerebrospinal fluid (23, 39), and cognition improvement after intranasal insulin administration (23, 24, 39) are some of the clinical evidence suggesting that a defective insulin action may contribute to the pathogenesis of AD. Studies in experimental animals (42-44) and in cultured cells (45, 46) confirm that insulin-signaling impairment leads to abnormal APP processing and Aβ accumulation; the underlying molecular mechanisms, however, remain largely unknown.

We carried out a comprehensive *in vitro* study highlighting the sequence of molecular steps for APP processing and Aβ accumulation that are influenced by insulin (Fig. 6).

We demonstrate that insulin and IGF-I, although causing similar APP-T688 dephosphorylation, mainly act via their own receptor, with minimal cross-activity via the receptor of the cognate ligand, a possible consequence of the different binding affinity for the other receptor. We

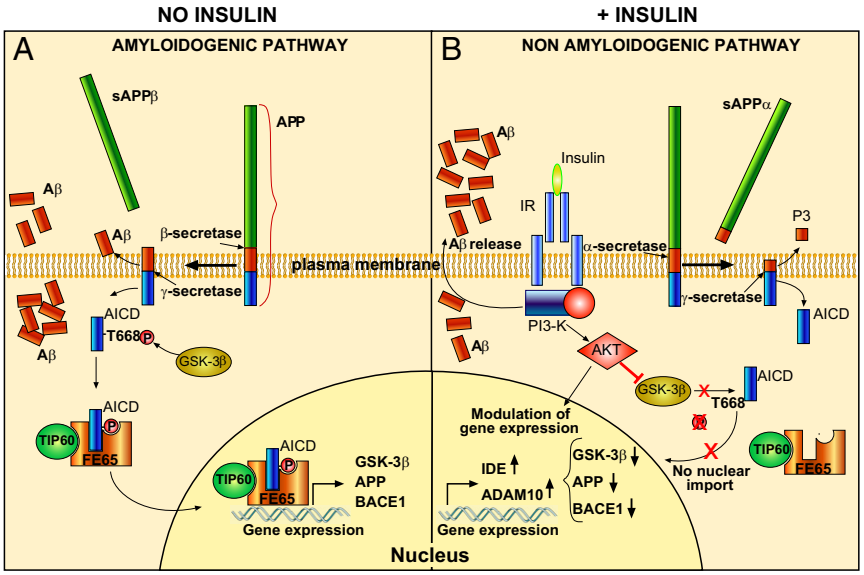

FIG. 6. *Schematic diagram* of APP processing in the absence (A) or the presence (B) of insulin. In the amyloidogenic pathway (A) subsequent cleavages of APP by β- and γ-secretases generate a long fragment (sAPPβ) secreted in the medium and a C-terminal fragment that includes AICD and Aβ. AICD phosphorylated at T668 by GSK3β interacts with Fe65 and Tip60, and this protein complex is imported inside the nucleus where it activates transcription of target genes involved in the amyloidogenic pathway (GSK3β, APP, BACE1). Conversely, insulin activates the nonamyloidogenic pathway (B). Through its own receptor and the PI3K/Akt pathway insulin promotes the nonamyloidogenic effect by different mechanisms: it inhibits GSK-3β activity on the phosphorylation of APP at T688. As a consequence, AICD nuclear import and its transcription activity of proamyloidogenic factors are inhibited. Moreover, the α-secretase pathway (that does not produce Aβ) is favored and the cell extrusion of Aβ is increased.

also demonstrate that, in the neuronal cells studied, the IR/IGF-IR hybrid receptors are the most represented of the insulin receptor family. Although insulin receptors are ubiquitously expressed in the brain, they are found at higher density in certain areas (*i.e.* hippocampus) (47). The presence of other receptors able to be activated by insulin raises the question of different responsiveness to insulin in different neuronal cells on the basis of different receptor expression (in terms of both quality and quantity).

After binding and activating the insulin receptor, insulin promotes APP-T688 dephosphorylation via the PI3K/Akt pathway, as indicated by the full inhibition of this effect by LY294002, a specific PI3K inhibitor.

Insulin-induced APP phosphorylation at T688 is rapid (occurs after few minutes) and prolonged (lasts more than 24 h). The effect is also dose dependent as is insulin's effect on APP-Y682 phosphorylation, which is also rapid but of shorter duration (peak at 30 min). The biological significance of insulin-induced Y682 phosphorylation is not known at present.

Insulin is known to deactivate via the PI3K/Akt pathway Gsk-3β (48). Because the effect of insulin on APP-T688 dephosphorylation is fully mimicked by Gsk-3β inhibition, it is likely that most of insulin effect on APP-T688 dephosphorylation is mediated by Gsk3β inhibition. However, other kinases (namely JNK) may contribute.

Insulin-induced dephosphorylation of APP at T668 inhibits the translocation of its intracellular fragment (AICD) from the cytoplasm to the nucleus, thus preventing its transcriptional activity. These findings, obtained using two independent methods (cell fractionation and immunofluorescence), indicate that AICD transcriptional activity is a key step for the antiamyloidogenic effect of insulin. In fact, by inhibiting AICD nuclear activity, insulin reduces the transcription of genes encoding pro-amyloidogenic enzymes, such as Gsk-3β and BACE1, and increases the transcription of antiamyloidogenic enzymes such as ADAM10 (α-secretase) and IDE, a protease that degrades Aβ (49). The mRNA of APP (the precursor of Aβ peptides) is also reduced after neuronal cell exposure to insulin.

The role of insulin, however, is not restricted to this cascade of antiamyloidogenic events mainly mediated by dephosphorylation of Gsk-3β and of APP-668T. Insulin also decreases the accumulation of the neurotoxic Aβ peptides inside cells by favoring, in a dose-dependent manner, the extrusion of the Aβ peptides into the extracellular compartment, where they can be degraded by membrane-associated and secreted IDE (50). This property of insulin is mediated by the PI3K pathway but is independent of Gsk-3β activity and occurs also in cells expressing the nonphosphorylatable APP-T668A mutant. Therefore, insulin influences Aβ production and clearance in several ways that are both dependent on and independent of Gsk-3β. Moreover, by favoring α-secretase activity, insulin stimulates the release of the soluble sAPPα extracellular fragment with neurotrophic activity (34). All of these mechanisms can possibly exert a neuroprotective action by reducing Aβ accumulation and aggregation in the Aβ plaques typical of AD.

Our study has some limitations. The use of transformed cells in most experiments may only partially reflect what occurs after insulin stimulation of normal neuronal cells. To avoid cell-specific effects we used a panel of human

neuronal cells of different origin and also confirmed the effect of insulin on APP dephosphorylation at T668 both in a human cortical neuronal cell line (HCN-2) and in primary cultures of rat cortical neurons. In the four neuronal cell lines studied, however, even maintaining identical experimental conditions, the responses to insulin were similar but not identical and, occasionally, different. Cell-specific intracellular mechanisms can influence the overall cell response as occurs in different animal models because of species specificity. The different models used and different protocols could also explain some discrepancies with previous studies. For instance, Gasparini *et al.* (35) found that the decrease of intracellular Aβ stimulated by insulin in N2a murine neuroblastoma cells occurs via the MAPK pathway activation. Different species, different cell types, the use of different signaling pathway inhibitors with different selectivity (LY294020 in our study *vs.* wortmannin), and differences in insulin concentration (10 nM in our study *vs.* 1,000 nM, which entails an important spillover on the IGF-IR) can explain the discrepancy.

Finally, our study is focused on the *in vitro* mechanisms regulating APP processing by insulin. It does not provide, therefore, evidence of either Aβ aggregation in plaques or signs of neurodegeneration. Therefore, the link between our experimental observations and the pathological brain characteristics of AD is indirect.

AD is recognized to be heterogeneous in nature (51). Abnormally processed APP with insoluble Aβ plaque production and neurofibrillary tangles due to τ-protein hyperphosphorylation are probably the end result of several etiopathogenetic factors, in analogy with hyperglycemia that can result from different types of diabetes. Our study elucidates different mechanisms that, by involving an impaired insulin signaling, can promote Aβ production and accumulation, a hallmark of AD. Results support the possibility that a defective insulin signaling may favor the accumulation of APP byproducts with ensuing neurodegeneration. This may be the case in diabetic and insulin-resistant patients and can explain why AD is more frequent in these subjects. However, the role of insulin deficiency in the individual AD patient and how insulin interacts with other genetic and acquired factors favoring the disease are is still nuclear. Defining the mechanisms involved in the link between insulin-resistance syndromes (*e.g.* diabetes, obesity) and AD may contribute to identify minimal cognitive impairment and AD patients that may benefit from intranasal insulin administration in terms of stopping progression or promoting recovery of their cognitive impairment (27).

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