IGF-1-induced Processing of the Amyloid Precursor Protein Family Is Mediated by Different Signaling Pathways*

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The mammalian amyloid precursor protein (APP) protein family consists of the APP and the amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2). The neurotoxic amyloid β-peptide (Aβ) originates from APP, which is the only member of this protein family implicated in Alzheimer disease. However, the three homologous proteins have been proposed to be processed in similar ways and to have essential and overlapping functions. Therefore, it is also important to take into account the effects on the processing and function of the APP-like proteins in the development of therapeutic drugs aimed at decreasing the production of Aβ. Insulin and insulin-like growth factor-1 (IGF-1) have been shown to regulate APP processing and the levels of Aβ in the brain. In the present study, we show that IGF-1 increases α-secretase processing of endogenous APP and also increases ectodomain shedding of APLP1 and APLP2 in human SH-SY5Y neuroblastoma cells. We also investigated the role of different IGF-1-induced signaling pathways, using specific inhibitors for phosphatidylinositol 3-kinase and mitogen-activated protein kinase (MAPK). Our results indicate that phosphatidylinositol 3-kinase is involved in ectodomain shedding of APP, and APLP1, but not APLP2, and that MAPK is involved only in the ectodomain shedding of APLP1.

The amyloid β-peptide (Aβ)2 is the major constituent of senile plaques found in brains of patients suffering from Alzheimer disease (AD) (1, 2). This neurotoxic peptide originates from the amyloid precursor protein (APP) (3). APP together with its paralogues, the amyloid precursor-like proteins 1 and 2 (APLP1 and APLP2), comprise the mammalian APP protein family. Although APP has been thoroughly studied, its physiological function has not yet been established, and even less is known about the two homologous proteins. Double knock-out studies in mice have indicated that APP family proteins are essential for survival and that they possess partially redundant functions (4). APLP2 was suggested to play a more important role since APLP1−/−/APP−/− mice are viable and apparently normal, whereas APLP2−/−/APP−/− or APLP2−/−/APLP1−/− mice are perinatally lethal. However, gene silencing analysis in an in vitro study suggested that APLP1 rather than APLP2 may be more important for survival and differentiation (5). Earlier studies showed that the human APP gene could rescue the Drosophila homologue APPL deletion mutants, again indicating functional redundancy between the proteins in this family (6). In addition, deficiency in neuronal repair mechanisms after brain injury was observed in Drosophila after deletion of the gene coding for APPL, and both human APP and APPL could induce axonal aborization (7). Previously, we have shown that all members of the APP protein family are up-regulated in parallel with neurite outgrowth in human neuroblastoma SH-SY5Y cells subjected to retinoic acid (RA) (8). Functions during neurogenesis are also supported by in vivo studies in mice showing increased expression of the APP protein family during embryogenesis (9).

All APP family members are type 1 membrane proteins with a single membrane-spanning domain, a large exoplasmic N-terminal domain and a short cytoplasmic C-terminal domain (3, 10–13). In fact, the APP protein family is believed to function as cell surface receptors that signal through proteolytic processing followed by nuclear translocation of the cytoplasmic domain (APP intracellular domain or APP-like intracellular domain 1 or 2) (14–16). The processing of APP has been shown to be complex and to involve several different cleavage sites and proteolytic enzymes (reviewed in Refs. 17 and 18). Cleavage of APP at the β-secretase site leads to secretion of the large N-terminal domain (sAPPβ) and formation of a 99-amino-acid-long C-terminal membrane-bound fragment (C99). Subsequent cleavage by γ-secretase results in the parallel formation of Aβ and APP intracellular domain. However, when APP is proteolytically processed at the α-secretase site, Aβ formation is precluded. Instead, sAPPα together with an 83-amino-acid-long C-terminal membrane-bound fragment (C83) are produced. Further cleavage of C83 by γ-secretase leads to the production of APP intracellular domain and a smaller secreted peptide (p3). APLP1 and APLP2 have been shown to be proteolytically processed in similar ways (19–22) and to secrete their ectodomains (sAPLP1 and sAPLP2, respectively) into the surrounding environment (11, 23).

In addition to the receptor function attributed to the full-length proteins, the secreted fragments of the APP family proteins have been proposed to play physiological roles. sAPP has been suggested to be involved in cell survival and neurite out-
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growth as well as to modulate neuronal excitability, synaptic plasticity, and synaptogenesis (reviewed in Ref. 24). sAPLP2 has also been shown to promote neurite outgrowth (25). Previously, we showed that the processing of APP, APLP1, and APLP2 increased in response to RA and brain-derived neurotrophic factor (26). In addition, our results suggested that RA and brain-derived neurotrophic factor shifted APP processing toward the α-secretase pathway since secretion of sAPPα increased, whereas the levels of the membrane-bound C-terminal fragment C99 decreased. Under these conditions, no secretion of sAPLP1 could be detected. Growth hormones such as nerve and epidermal growth factors have also been shown to lead to secretion of sAPPα (27) and sAPLP2 (23, 28). Furthermore, it was demonstrated that insulin increased extracellular levels of Aβ and sAPPα and at the same time decreased intracellular levels of Aβ (29). Interestingly, it has become evident in recent years that insulin and insulin-like growth factor-1 (IGF-1) signaling, besides regulating the energy balance of the brain, also modulates cognitive processes such as learning and memory as well as neuroprotection (reviewed in Ref. 30). In an AD transgenic mice model, IGF-1 was demonstrated to induce Aβ clearance from the brain by up-regulating levels of transport proteins such as albumin and transthyretin (31). IGF-1 treatment also resulted in enhanced cognitive performance, increased levels of synaptic proteins, and reduced astrogliosis associated with plaques (32). In addition, a positive correlation between Mini-Mental State Examination scores and serum IGF-1 concentrations in AD has been reported, and it was suggested that lower levels of IGF-1 might lead to cognitive impairment (33). These findings suggest components of the insulin/IGF-1 signaling pathways as potential therapeutic targets in AD.

Many studies on AD are focused on finding means to inhibit the production and accumulation of Aβ. However, based on the proposed essential functions of the APP family, it is of importance to further elucidate how the processing of APP and the other members of the APP protein family is regulated. In this study, we show that exposure of human neuroblastoma cells to IGF-1 results in increased secretion of sAPPα, sAPLP1, and sAPLP2 as well as decreased production of Aβ. In addition, using specific inhibitors for signaling pathways activated by IGF-1, we found that phosphatidylinositol 3-kinase (PI3-K), cyclin-dependent kinase 5 (cdk5), and mitogen-activated protein kinase (MAPK) have different impact on ectodomain shedding of the three homologous proteins.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**—SH-SY5Y human neuroblastoma cells (American Type Culture Collection) were routinely maintained in minimum essential medium with Earl’s salts, 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 1% l-glutamine, and 1% non-essential amino acids as described previously (34). Cells were seeded at a density of 25,000 cells/cm² in Nunc 60-mm dishes. At 24 h as well as 4 days after seeding, the medium was changed to serum-free culture medium (Dulbecco’s modified Eagle’s medium:F12 with the addition of N2 supplements, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% l-glutamine). Cells were grown in 4 ml of serum-free culture medium for 6 days and then incubated for 45 min with 5 ml of serum-free culture medium devoid of insulin before treatment. Subsequently, cells were treated with 1 nm, 10 nm, or 1 μM insulin (Sigma-Aldrich) or 1, 5 or 10 nm IGF-1 (Sigma-Aldrich) for 18 h in 2 ml of serum- and insulin-free culture medium. For inhibition studies, 1 or 10 μM LY 294002 (Merck Biosciences), a specific PI3-K inhibitor, 5 or 20 μM PD 98059 (Merck Biosciences), a specific MAPK kinase (MEK) inhibitor, or 5 μM roscovitine (Sigma-Aldrich), a specific cdk5 inhibitor, was added to the cells during the washing step. The medium was then changed to serum- and insulin-free culture medium with 10 nm IGF-1 in the presence or absence of inhibitors. All cell culture reagents were purchased from Invitrogen unless otherwise indicated.

**Western Blot Assay**—Conditioned medium (2 ml) was collected and supplemented with Complete protease inhibitor mixture (Roche Applied Science) and concentrated as described previously (26). Cells were harvested and analyzed by Western blot assay as described previously (26). Concentrated culture medium from an equal number of cells (as determined by protein content in the cell lysate and corresponding to ~200 μl of conditioned medium) from each culture was loaded on a 7.5% Tris-glycine polyacrylamide gel. The concentrations of antibodies used were chosen to ensure relative quantitative measurements. Primary antibody concentrations were as follows: 1:4000 for 6E10 (directed against Aβ1–17), and CT11 (directed against the C-terminal 11 amino acids of APLP1), 1:3000 for 42464 (directed against amino acids 499–557 of APLP1, cf. 11), and 1:7500 for D2II (directed against full-length APLP2), and subsequently, 1:5000 for horseradish peroxidase-coupled anti-mouse IgG, anti-rabbit IgG, and protein A. Only bands that could not be observed in the absence of primary antibodies were quantified. All Western blotting reagents were from GE Healthcare or Bio-Rad Laboratories except CT11 and D2II, which were from Calbiochem, 6E10, which was from Signet Laboratories, and protein A, which was from Sigma-Aldrich.

**ELISA**—Conditioned medium and cell lysate were analyzed for Aβ40 in a high sensitivity sandwich ELISA according to the manufacturer (The Genetics Company Inc.). The Aβ concentration was normalized to the amount of cells in each culture (as determined by protein content in the cell lysate).

**Statistical Analysis**—Statistical analysis was performed using analysis of variance followed by Tukey-Kramer multiple comparison test. Data are presented as mean ± S.E. unless otherwise stated.

**RESULTS**

**Increased Ectodomain Shedding of APP and APLP1 in Response to IGF-1**—Human neuroblastoma SH-SY5Y cells were cultured for 18 h in the absence or presence of different concentrations of insulin. Conditioned media from the cultures were analyzed by Western blot using antibodies 6E10 (directed against Aβ1–17) and 42464 (directed against amino acids 499–557 of APLP1). The results showed that treatment with 1 μM insulin led to a significantly increased secretion of both sAPPα and sAPLP1 (Fig. 1A). Since insulin at this high concentration can activate both insulin and IGF-1 receptors, the effect of different concentrations of IGF-1 on the secretion of sAPPα and
sAPP1 was also investigated. IGF-1 treatment led to an ~10-fold increase in the release of sAPPα as well as sAPLP1 as compared with non-treated control cells (Fig. 1B). In contrast to the effects of insulin, significant effects were observed at low concentrations of IGF-1 (~1 nM). IGF-1 treatment for 18 h did not affect the steady-state levels of the corresponding membrane-bound full-length proteins as shown by Western blot analysis of the cell lysate using antibody CT11 and 6E10 for APPL1 and sAPPα, respectively, are shown below each graph. The levels of the corresponding full-length proteins APPL1 and APP are not affected by either insulin (C) or IGF-1 (D) as shown by Western blot using antibody CT11 and 6E10 for APPL1 and APP, respectively.

**TABLE 1**

| Treatment | APPL1 | APP | APPL2 |
|-----------|-------|-----|-------|
| Mean ± S.D. | n | Mean ± S.D. | n | Mean ± S.D. | n |
| Control | 70 ± 10 | 4 | 93 ± 10 | 4 | 88 ± 17 | 4 |
| IGF-1 | 100 ± 15 | 4 | 100 ± 15 | 4 | 100 ± 15 | 4 |
| LY10 | 89 ± 16 | 4 | 91 ± 22 | 4 | 107 ± 39 | 3 |
| PD20 | 92 ± 18 | 4 | 95 ± 24 | 4 | 96 ± 33 | 4 |
| RO55 | 106 ± 12 | 4 | 88 ± 15 | 4 | 106 ± 15 | 4 |

**FIGURE 1.** Secretion of sAPPα and sAPLP1 increases in response to high concentrations of insulin and low concentrations of IGF-1. A and B, relative abundance of sAPPα (open bars) and sAPLP1 (closed bars) in culture medium from SH-SYSY cells treated with insulin (A) and IGF-1 (B) for 18 h as indicated. Data represent mean ± S.E. from 9–10 cultures derived from 4 independent experiments. *, p < 0.05, **, p < 0.01, ***, p < 0.001, significantly different from non-treated control. Representative Western blot analyses, using antibody 42464 and 6E10 for sAPLP1 and sAPPα, respectively, are shown below each graph. The levels of the corresponding full-length proteins APPL1 and APP are not affected by either insulin (C) or IGF-1 (D) as shown by Western blot using antibody CT11 and 6E10 for APPL1 and APP, respectively.

PI3-K, MAPK, and cdk5 Are Involved in IGF-1-induced Secretion of sAPLP1—To investigate the mechanisms that may be involved in the effects of IGF-1 on APPL1 ectodomain shedding, inhibitors for different signaling pathways were used. 10 μM specific PI3-K inhibitor LY 294002 blocked the IGF-1-induced secretion by ~90% (Fig. 2A). Furthermore, 20 μM specific MEK inhibitor PD 98059, and 5 μM specific cdk5 inhibitor roscovitine reduced the IGF-1-induced secretion of
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FIGURE 2. IGF-1-induced secretion of sAPPα and sAPLP1 is blocked by the PI3-K-specific inhibitor LY 294002, whereas the MEK-specific inhibitor PD 98059 selectively blocks secretion of sAPLP1. A and B, relative abundance of sAPLP1 (A) and sAPPα (B) in culture medium from SH-SY5Y cells treated with 10 nM IGF-1 in the presence or absence of 1 and 10 μM LY 294002 (LY1 and LY10, respectively) or 5 and 20 μM PD 98059 (PD5 and PD20, respectively) for 18 h as indicated. The expression levels of APLP1 and APP (A and B, lower panels, respectively) are not affected. Data represent mean ± S.E. from 9–24 cultures derived from 4–7 independent experiments. **, p < 0.01, significantly different from cells treated with IGF-1. Note: Control (C) versus LY10 is not significantly different, whereas control versus 20 μM PD 98059 is significantly different (p < 0.01) for both sAPLP1 and sAPPα. Representative Western blots analyses using antibody 42464 for sAPLP1, CT11 for APLP1, and 6E10 for sAPPα and APP are shown below each graph.

sAPLP1 by ~60 and 40%, respectively (Figs. 2A and 3). Treatment for 18 h with either of the inhibitors did not affect steady-state levels of the membrane-bound APLP1 (Fig. 2, lower panel, and Table 1).

PI3-K and cdk5, but Not MAPK, Are Involved in IGF-1-induced Secretion of sAPPα—The levels of sAPPα in conditioned media from cells treated with IGF-1 in the presence or absence of LY 294002, PD 98059, or roscovitine were also analyzed. In cells treated with IGF-1 in the presence of 10 μM LY 294002 or 5 μM roscovitine, secretion of sAPPα was reduced by ~80% (Figs. 2B and 3). On the contrary, 20 μM specific MEK inhibitor had no effect on the IGF-1-induced secretion of sAPPα (Fig. 2B). Treatment for 18 h with inhibitors had no effect on the steady-state levels of the membrane-bound APP (Fig. 2, lower panel, and Table 1).

Increased Ectodomain Shedding of APLP2 in Response to IGF-1 Does Not Involve PI3-K or MAPK—The effect of IGF-1 on the third member of the mammalian APP protein family was also investigated. Western blot analysis using antibodies DTII (directed against full-length APLP2) showed that IGF-1 significantly increased the secretion of sAPLP2 and CS GAG sAPLP2 (i.e. chondroitin sulfate glycosaminoglycan-modified sAPLP2) (Fig. 4). The increase of sAPLP2 secretion in response to 10 nM IGF-1 was not as dramatic as for APP and APLP1, ~3-fold as compared with ~10-fold (cf. Figs. 1 and 2). Furthermore, neither the PI3-K inhibitor nor the MEK inhibitor affected the IGF-1-induced secretion of sAPLP2 (Fig. 4). As a control, the polyvinylidene difluoride membranes were reprobed with antibody 6E10 (recognizing sAPPα) to show that in the same samples, a clear effect of the PI3-K inhibitor on the levels of sAPPα in the conditioned media could be observed (Fig. 4, B and C, upper panels).

Decreased Production of Aβ40 in Response to IGF-1—To further investigate the mechanisms behind IGF-1-induced processing of APP, conditioned medium and cell lysate were analyzed for Aβ40 by a high sensitive sandwich ELISA (Table 2). After treatment with IGF-1, the levels of both intra- and extracellular Aβ40 were decreased by ~30%. Effects of inhibitors for different signaling pathways were also analyzed, showing that in the presence of 10 μM LY 294002, the IGF-1-induced decrease in Aβ40 levels was reversed. However, as for the IGF-1-induced effects on sAPPα, 20 μM specific MEK inhibitor had no effect.

DISCUSSION

Previous studies have shown that processing of APP and secretion of sAPP in response to insulin (35). In the concentration range used (10 nM–1 μM), insulin can activate both the insulin and the IGF-1 receptor. The affinity of insulin and IGF-1 to their receptors lies within the range of 0.1–10 nM, whereas 100–500-fold higher concentrations are required for cross-activation of the receptors (reviewed in Ref. 36). The human neuroblastoma SH-SY5Y cells, used in the present study, have previously been shown to express functional insulin and IGF-1 receptors (37, 38). Our results suggest that the increased levels of sAPPα and sAPLP1 in response to either
IGF-1 or insulin were mediated by activation of IGF-1 receptors. Activation of insulin and IGF-1 receptors triggers two major signaling pathways, the PI3-K or MAPK cascades that ultimately lead to proliferation, differentiation, or survival (reviewed in Ref. 39). It has previously been shown that high concentrations of insulin regulate sAPP release via a PI3-K-dependent signaling pathway (35). We observed that a specific PI3-K inhibitor totally abolished the increase in sAPPα levels in response to IGF-1. In contrast, a specific MEK inhibitor did not significantly reduce the elevated levels of sAPPα, excluding the involvement of the MAPK signaling cascade. Previously, we have shown that RA increases the secretion of sAPPα concomitant with neurite outgrowth (8, 26). PI3-K signaling has been shown to be required for RA-induced differentiation of SH-SY5Y cells (40). It can be speculated that effects on sAPPα secretion and neurite outgrowth are mediated by the same signaling pathways. However, both PI3-K and MAPK were shown to be involved in IGF-1-induced neurite outgrowth in SH-SY5Y cells (38). In addition, it has also been shown that RA increases the expression of cdk5 and p35 as well as stimulates neurite outgrowth through the MAPK pathway in another human neuroblastoma cell line (41). Here we observed that a specific cdk5 inhibitor completely blocked the IGF-1-induced secretion of sAPPα, suggesting that cdk5 plays a central role in the IGF-1-induced processing of APP. Indeed, several other studies have indicated a connection between cdk5 and APP. On one hand, a novel neuroprotective function of sAPP in preventing tau hyperphosphorylation via suppression of overactivation of cdk5 has been suggested (42). On the other hand, cdk5 has been suggested to be involved in the regulation of APP processing. Activation of cdk5 by overexpression of p25 or p35 in SH-SY5Y cells led to phosphorylation of APP at Thr-668 (43). The same study also showed that activation of cdk5 in cells overexpressing APP with the Swedish mutation resulted in higher levels of secreted Aβ, sAPPβ, and sAPPα without affecting the steady-state levels of APP. The same group also reported that inhibition of cdk5 by roscovitine led to increased hippocampal levels of Aβ in an AD mice model (44). Thus, the role of cdk5 in Aβ production and accumulation is not completely clear.

Enhanced production, aberrant catabolism, or abnormal transport of Aβ across the blood-brain barrier are processes that all could contribute to increased levels of Aβ inside the brain. Several enzymes have the capability to cleave Aβ, and neprilysin has been suggested to play the most important role (reviewed in Ref. 45). Another physiologically relevant Aβ-degrading enzyme is insulin-degrading enzyme (46). As substrates, insulin and Aβ will compete for insulin-degrading enzyme. It has been speculated that age-related reduction of IGF-1 signaling leads to decreased transport of Aβ across the blood-brain barrier. The resulting elevated levels of Aβ in the brain may in turn lead to antagonized insulin receptor binding (loss of insulin sensitivity). As a consequence, increased insulin levels may follow and interfere with Aβ degradation and further contribute to increased Aβ load in the brain (30). In our study, we showed that IGF-1 led to a reduction in intra- as well as extracellular levels of Aβ40 and that the PI3-K inhibitor totally reversed these effects. On the other hand, PD 98059 had no effect on secreted Aβ40 levels, again indicating that MAPK is not involved in IGF-1-induced processing of APP. The levels of secreted Aβ42, considered to be the most toxic form, were close to, or below, the detection limit, and accurate measurements could not be obtained (data not shown). However, the effects on Aβ42 secretion in response to the various treatments were similar to the effects on Aβ40, and, likewise, the presence of the PI3-K inhibitor resulted in significantly increased levels of Aβ42 ($p < 0.01, n = 6$). Together, our results clearly indicate that IGF-1 treatment leads to a shift in APP processing toward the α-secretase pathway instead of the amyloidogenic pathway.

Previously, it has been proposed that the members of the APP family are processed in a similar way (19–22). In this study, a cell line with endogenous expression of all three proteins in the mammalian APP family was used. We observed that processing of APP and APLP1 and secretion of sAPPα and sAPLP1 were increased to the same extent in response to insulin or IGF-1. As for sAPPα, the IGF-1-induced increase in sAPLP1 returned to control levels after treatment with LY 294002 and was partly inhibited by roscovitine. Interestingly, and in contrast to sAPPα, PD 98059 inhibited the IGF-1-induced sAPLP1 release. APP and APLP2 are considered to be the most closely
related members of the APP family. Contradictorily, we show that the PI3-K inhibitor that totally blocked the sAPP secretion did not have any effect on the secretion of sAPLP2 or CS GAG sAPLP2. However, similar to sAPPα, and in contrast to sAPLP1, the MEK inhibitor did not affect the IGF-1-induced secretion of sAPLP2. A previous study from our group also indicated differences in the regulation of the processing of the APP protein family (8). These data suggested that a PKC inhibitor, curcumin, had no effect on APLP1, blocked APLP2, and partly blocked APP ectodomain shedding. The results from our two studies indicate that the regulation of APLP1 and APLP2 is more different from each other than from APP.

In conclusion, in the present study, we show that ectodomain shedding of the APP family is induced by IGF-1. However, the effect of inhibitors specific for different signaling pathways indicates that the mechanisms behind the IGF-1-induced processing of the different APP family proteins are not identical. We propose that PI3-K is involved in ectodomain shedding of APP and APLP1, but not APLP2, and that MAPK is involved only in the ectodomain shedding of APLP1. In addition, cdk5 was shown to be involved in the processing of both APP and APLP1. Proteolytic processing of APP and APLPs may be modulated by regulation of enzyme activity, by specific post-translational modifications of the substrate, or by altered subcellular localization of the substrate or the enzyme. More studies are needed to put together all the clues about the processing and the signaling pathways involved. This will lead to a more complete understanding of the physiological function of the APP protein family.

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