Effects of the amyloid protein precursor of Alzheimer's disease and other ligands of the LDL receptor-related protein on neurite outgrowth from sympathetic neurons in culture

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Abstract The amyloid protein precursor (APP) of Alzheimer's disease can stimulate neurite outgrowth in vitro. The receptor responsible for this effect has not been identified. Kunitz protease inhibitor (KPI)-containing forms of APP bind to the low-density lipoprotein receptor-related protein (LRP). As LRP may regulate neurite outgrowth, we examined whether the effects of APP are mediated by LRP. Inhibitors of LRP decreased neurite outgrowth from chick sympathetic neurons. Most LRP ligands (α2-macroglobulin, lactoferrin, and lipoprotein lipase) stimulated outgrowth. However, in soluble form, the KPI-containing APP_{751} was a weak inhibitor of outgrowth. In substrate-bound form, both APP_{751} and APP_{695} (which does not bind to LRP) stimulated outgrowth. Thus the effect of substrate-bound APP on neurite outgrowth is not mediated by LRP.

Key words: Amyloid protein precursor; Heparin; Alzheimer; Apolipoprotein E; Low-density lipoprotein receptor-related protein

1. Introduction

In Alzheimer's disease (AD), amyloid plaques contain a protein, Aβ, derived from a larger β-amyloid precursor protein (APP) [1]. Differential splicing of APP mRNA produces major isoforms which either contain (APP_{751} and APP_{770}) or lack (APP_{695}) a Kunitz protease inhibitor (KPI) domain [2–4]. A number of studies have shown that APP can stimulate neurite outgrowth [5–7].

A recently discovered risk factor for late-onset AD is the ε4 allele of the apolipoprotein E (apoE) gene [8]. ApoE is important for neurite outgrowth, an effect that is mediated by the low-density lipoprotein receptor-related protein (LRP) [9–13]. KPI-containing forms of APP have also been shown to bind to LRP [14]. These findings raise the possibility that KPI-containing forms of APP may influence neurite outgrowth via LRP. In support of this hypothesis, Qiu et al. [15] found that KPI-containing forms of APP more potently stimulate neurite outgrowth than KPI-lacking forms, and Diaz-Nido et al. [16] found that a peptide homologous to the KPI domain induced neurite outgrowth.

The aim of this study was to examine the effect of various LRP ligands, including APP_{751}, on neurite outgrowth from chick sympathetic neurons. We show that the effect of APP in stimulating neurite outgrowth is not mediated via LRP.

2. Materials and methods

2.1. Materials

The 39-kDa receptor-associated protein (RAP) was prepared as described by Williams et al. [17]. Affinity-purified anti-LRP antisera R777 and R2999 were prepared as described by Kounnas et al. [18]. Anti-LRP antiserum R704 was raised against cytoplasmic LRP residues 4513–4524 conjugated to keyhole limpet haemocyanin, purified over a Sepharose-peptide column, and heated at 56°C for 60 min prior to use. A peptide homologous to residues 136–166 of apoE3/E4 (Ac-RLASHLRKLRKRLLRDADDLQKRLAVYQAGA-NH_2) was synthesized by Chiron-Mimotopes (Clayton, Australia). Putrescine, nerve growth factor (2.5 S) and progesterone were from Sigma-Aldrich Pty. Ltd. (Castle Hill, Australia). Insulin-transferrin-selenium-X growth supplement was from Gibco-BRL (Grand Island, NY). Poly-L-lysine (molecular weight 144 000) and α2-macroglobulin (α2M) were from ICN Biomedicals Inc. (Seven Hills, Australia). α2M was activated by incubation with 400 mM methylamine and then desalted against phosphate-buffered saline (PBS) [19]. Bovine lipoprotein lipase (LPL), prepared according to [20], was donated by Prof. W. Sawyer (University of Melbourne, Australia). Recombinant APP and its fragments expressed in *Pichia pastoris* were purified as described in Henry et al. [21]. APP/Factor IXa complexes were prepared as previously described [14].

2.2. Culture of chick sympathetic neurons

E12 chick sympathetic neurons were cultured on 24-well plastic dishes at 10 000 cells/well as described previously [22]. Compounds were added 2 h after plating to allow time for cells to attach. For experiments on the effect of substrate-bound proteins, wells were coated with 0.1 mg/ml polylysine followed by the protein of interest (10 μg/ml) [23]. Neurite length was measured by image-capture analysis as described [22]. Five fields in each of 4 wells per treatment group were examined. Routinely, 100–200 neurites were measured per group. Differences between groups were calculated by a one-way analysis of variance and a two-tailed Student's *t*-test.

3. Results

To examine whether LRP regulates neurite outgrowth from chick sympathetic neurons, the effect of LRP inhibitors was

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Abbreviations: α2M, α2-macroglobulin; Aβ, amyloid protein; AD, Alzheimer's disease; apoE, apolipoprotein E; APP, amyloid protein precursor; CHO, Chinese hamster ovary; KPI, Kunitz protease inhibitor; HSPG, heparan sulfate proteoglycans; LPL, lipoprotein lipase; LRP, low-density lipoprotein receptor-related protein; RAP, receptor-associated protein

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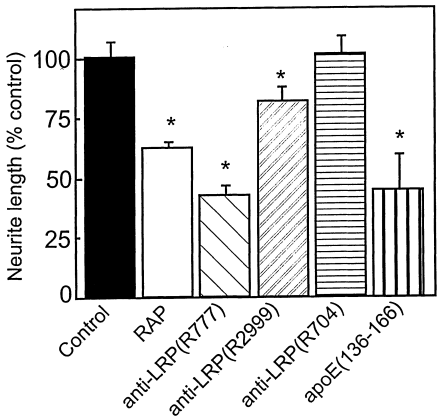

Fig. 1. Effect of LRP inhibitors on neurite length using isolated E12 chick sympathetic neurons. The figure shows quantitative image-capture analysis of neurite length [22]. Values are means ± S.E.M., with the mean control value = 100% (approximately 100 μm). Asterisks indicate significantly different from controls ($P < 0.05$) as determined by a Student's *t*-test. Cultures contained 350 μl of growth medium to which was added 5 μl PBS (Control), RAP (3 μg in 5 μl PBS), R777 anti-LRP antibody (1 μg in 5 μl PBS), R2999 anti-LRP antibody (1 μg in 5 μl PBS), R704 anti-LRP antibody (1 μg in 5 μl PBS) or $apoE_{136-166}$ peptide (25 μg in 5 μl).

studied (Fig. 1). The 39-kDa receptor-associated protein (RAP), two affinity-purified antibodies raised against the extracellular domain of LRP (R777 and R2999), and a peptide (apoE 136–166) homologous to the receptor-binding region of apoE [24–28] caused a significant ($P < 0.05$) reduction in neurite outgrowth. In contrast, a control antibody specific for the cytoplasmic domain of LRP (R704) did not inhibit neurite outgrowth.

LRP ligands were tested for their effects (Fig. 2). α2M (0.5–3 μg/ml or 0.7–3.9 nM) stimulated neurite outgrowth by 30–40%. This effect was still evident after activation with methylamine, which removes its protease inhibitor activity [19], thereby demonstrating that the effect was not due to inhibition of an endogenous protease. Similarly, lipoprotein lipase (LPL, 15–50 μg/ml or 300–1000 nM) and lactoferrin (3–30 μg/ml or 40–400 nM) also stimulated outgrowth. The concentrations of these ligands required to stimulate neurite outgrowth were similar to those reported previously as being necessary for binding to LRP [29,30].

To examine whether the neurite outgrowth-promoting effects of APP on chick sympathetic neurons are mediated by LRP, *P. pastoris*-expressed human APP_{695} and APP_{751} were tested for their effects (Figs. 2 and 3). APP_{751} produced a weak (20%) inhibition of neurite outgrowth at the highest concentration tested (7 μg/ml, ~80 nM). With the addition of Factor IXa, which promotes binding of APP to LRP [14], neurite length was 70% of control values. Soluble APP_{695} did not inhibit neurite outgrowth, supporting the view that the effect was mediated by LRP. In contrast, substrate-bound APP stimulated outgrowth (Fig. 3, right). There was no difference between the effect of APP_{695} and APP_{751}, indicating that this effect was not mediated via LRP.

To identify regions of APP which could be involved in neurite outgrowth promotion, we examined three fragments

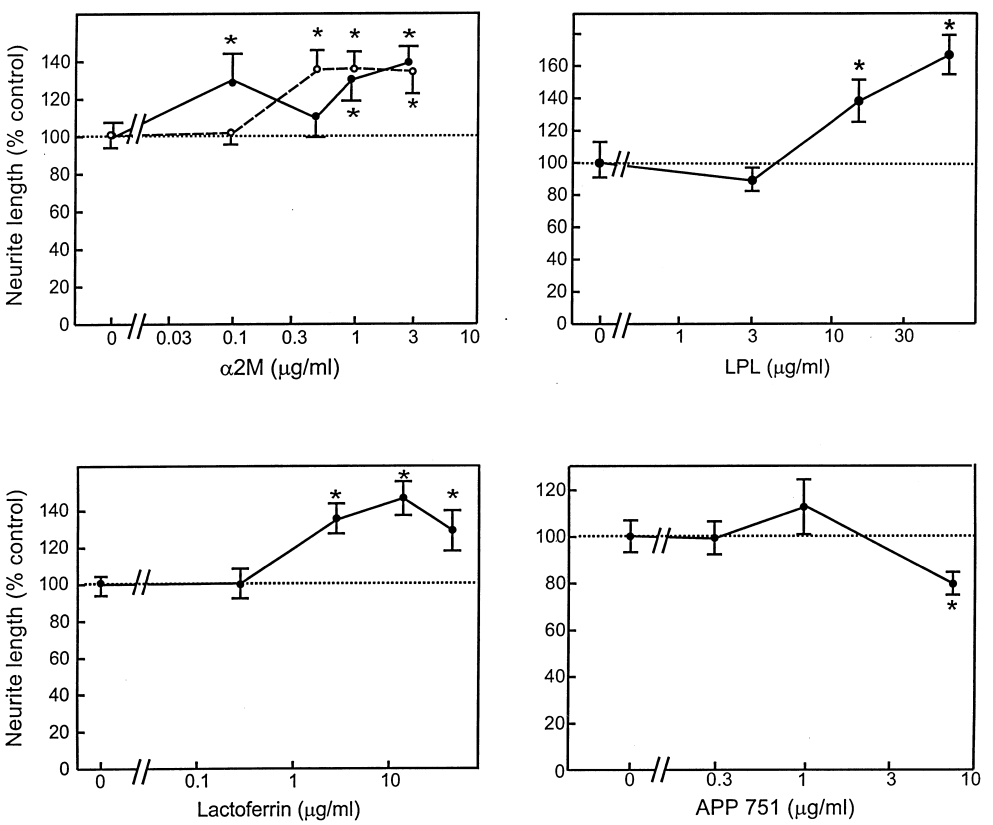

Fig. 2. Effect of LRP ligands on neurite length, using isolated E12 chick sympathetic neurons. Values are means ± S.E.M. One-way analysis of variance on all curves indicated a significant effect for each ligand ($P < 0.05$). Asterisks show values significantly different from controls as assessed by a Student's *t*-test. Cultures contained 350 μl of growth medium to which were added different concentrations of native α2M (●), activated α2M (○), lipoprotein lipase (LPL), lactoferrin, or APP_{751}.

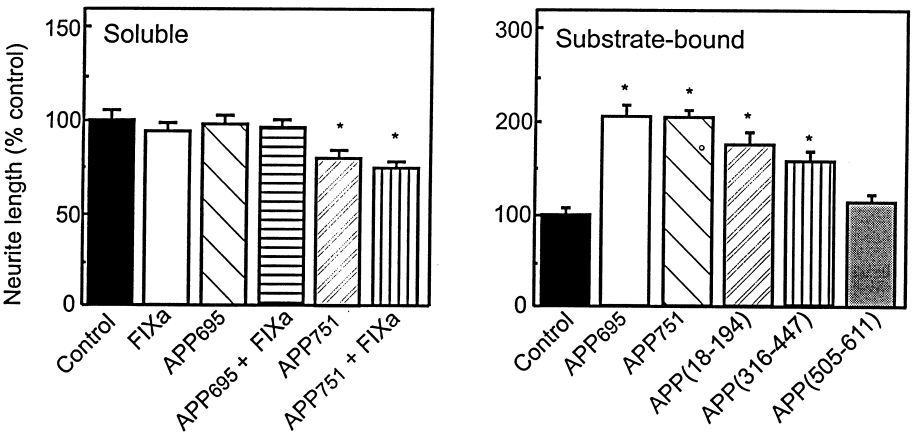

Fig. 3. Effect of APP isoforms and recombinant APP fragments on neurite length, using isolated E12 chick sympathetic neurons. Values are means ± S.E.M. Asterisk indicates significantly different from controls ($P < 0.05$) as assessed by a Student's *t*-test. For experiments using soluble APP, cultures contained 350 μl of growth medium to which was added PBS (Control), factor IXa (40 nM, 2.4 μg/ml), APP 695 alone (80 nM, 6.4 μg/ml), APP 751 alone (80 nM, 6.9 μg/ml), or both APP solutions preincubated with factor IXa (80:40 nM). For experiments to test the effect of substrate-bound APP and fragments, plates were coated at a protein concentration of 10 μg/ml.

of APP for their effects in substrate-bound form (Fig. 3, right). Neurite length was significantly increased by APP (18–194) and by APP (316–447), but APP (505–611) did not stimulate outgrowth.

4. Discussion

This study demonstrates that (i) several ligands of LRP (α2M, lactoferrin, LPL) can stimulate neurite outgrowth from chick sympathetic neurons; (ii) soluble APP_{751} is a weak inhibitor of neurite outgrowth in the same system; and (iii) the ability of substrate-bound APP to stimulate neurite outgrowth is not due to an interaction with LRP. Instead two domains in APP that were previously shown to interact with heparan sulfate proteoglycans (HSPGs) (APP 18–194, APP 336–447) [31–33], may contribute to the stimulatory effects of substrate-bound APP, suggesting the possibility that a cell-surface HSPG could be an APP receptor.

The effects of RAP, α2M and anti-LRP antibodies on neurite outgrowth are similar to those previously reported [13,19]. Other reports also indicate that LRP has important developmental functions [9–12,34]. The mechanism by which LRP ligands, and especially apoE, regulate neurite outgrowth is unclear. It has been suggested that the effects of apoE on neurite outgrowth are due to the increased availability of lipids for outgrowth [9,35]. Howeve,r such a mechanism does not explain why other LRP ligands can stimulate outgrowth.

The reasons why soluble APP_{751} differed from other LRP ligands is also unclear. APP_{751} may inhibit neurite outgrowth by an action which is independent of its binding to LRP. Alternatively, APP_{751} and RAP may interact with LRP at a site which is distinct from sites used by other ligands.

The role of LRP in the pathogenesis of AD needs to be clarified. Several LRP ligands have been found around amyloid plaques [36,37]. However, the role of these ligands in plaques is unknown. The observation that APP_{751} mRNA expression is higher around plaques [38] raises the possibility that interactions between APP and LRP could disturb the trophic environment of the plaque, contributing to the abnormal neuritic pathology.

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