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Authors
Heredia, Lorena
Helguera, Pablo
de Olmos, Soledad
et al.

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Phosphorylation of Actin-Depolymerizing Factor/Cofilin by LIM-Kinase Mediates Amyloid β-Induced Degeneration: A Potential Mechanism of Neuronal Dystrophy in Alzheimer’s Disease

Lorena Heredia,1 Pablo Helguera,4 Soledad de Olmos,2 Gabriela Kedikian,1 Francisco Solá Vigo,1 Frank LaFerla,4 Matthias Staufenbiel,3 José de Olmos, Jorge Busciglio, Alfredo Cáceres,3 and Alfredo Lorenzo1

Laboratories of 1 Experimental Neuropathology, 2 Neuroanatomy, and 3 Cell Biology, Instituto de Investigación Médica “Mercedes y Martín Ferreyra” (Consejo Nacional de Investigaciones Científicas y Técnicas), 5000 Córdoba, Argentina, 4 Department of Neurobiology and Behavior, University of California, Irvine, California 92697-4550, and 5 Novartis Institute for BioMedical Research Basel, Inc., CH-4002 Basel, Switzerland

Deposition of fibrillar amyloid β (fAβ) plays a critical role in Alzheimer’s disease (AD). We have shown recently that fAβ-induced dystrophy requires the activation of focal adhesion proteins and the formation of aberrant focal adhesion structures, suggesting the activation of a mechanism of maladaptive plasticity in AD. Focal adhesions are actin-based structures that provide a structural link between the extracellular matrix and the cytoskeleton. To gain additional insight in the molecular mechanism of neuronal degeneration in AD, here we explored the involvement of LIM kinase 1 (LIMK1), actin-depolymerizing factor (ADF), and cofilin in Aβ-induced dystrophy. ADF/cofilin are actin-binding proteins that play a central role in actin filament dynamics, and LIMK1 is the kinase that phosphorylates and thereby inhibits ADF/cofilin. Our data indicate that treatment of hippocampal neurons with fAβ increases the level of Ser3-phosphorylated ADF/cofilin and Thr508-phosphorylated LIMK1 (P-LIMK1), accompanied by a dramatic remodeling of actin filaments, neuritic dystrophy, and neuronal cell death. A synthetic peptide, S3 peptide, which acts as a specific competitor for ADF/cofilin phosphorylation by LIMK1, inhibited fAβ-induced ADF/cofilin phosphorylation, preventing actin filament remodeling and neuronal degeneration, indicating the involvement of LIMK1 in Aβ-induced neuronal degeneration in vitro. Immunofluorescence analysis of AD brain showed a significant increase in the number of P-LIMK1-positive neurons in areas affected with AD pathology. P-LIMK1-positive neurons also showed early signs of AD pathology, such as intracellular Aβ and pretangle phosphorylated tau. Thus, LIMK1 activation may play a key role in AD pathology.

Key words: amyloid β; Aβ; LIMK1; ADF/cofilin; Alzheimer’s disease; dystrophic neuritis; neuronal neurodegeneration

Introduction

Dystrophic neurites are markedly distorted axons and dendrites that characterize Alzheimer’s pathology (Benzing et al., 1993), playing an important role in cognitive impairment in the disease (McKee et al., 1991; Spires and Hyman, 2004). Dystrophic neurites typically associate with senile plaques, the hallmark lesions of Alzheimer’s disease (AD). Amyloid β (Aβ), a 40–43 amino acid derivative of the metabolism of amyloid β precursor protein (AβPP), is the main constituent of senile plaques (Hardy and Selkoe, 2002). Aβ aggregation is required for senile plaque formation and renders Aβ neurotoxic (Fike et al., 1991; Lorenzo and Yankner, 1994; Lambert et al., 1998). Aβ neurotoxicity induces neuronal dystrophy characterized by aberrant neuritic morphology, sprouting, and breakdown (Busciglio et al., 1992; Grace et al., 2002). Much effort was devoted to understand the mechanism that links Aβ deposition to tau pathology as an attempt to define the events that lead to neurofibrillary tangle formation in AD (Busciglio et al., 1995; Lee et al., 2000; Götz et al., 2001; Oddo et al., 2003), whereas less attention was put on upstream events that may trigger the dystrophic process. We have shown recently that such early events may include the clustering of AβPP and integrin receptors at the cell surface around Aβ fibrils, the activation of focal adhesion proteins, and the formation of aberrant focal adhesion structures (Grace and Busciglio, 2003; Heredia et al., 2004), suggesting that Aβ deposition triggers a pathological mechanism of plasticity leading to neuronal dystrophy.

Focal adhesions provide a structural link between the extracellular matrix and the actin cytoskeleton. Actin filaments are
essential components of the adhesive complex, and its dynamic reorganization provides the initial force to adapt neuronal morphology to its surrounding. Actin-depolymerizing factor (ADF) and cofilin are actin-binding proteins that critically control actin filament dynamics and reorganization by severing and depolymerizing actin filaments (Bamburg and Wiggan, 2002). LIM kinase 1 (LIMK1) phosphorylates and thereby inhibits ADF/cofilin (Arber et al., 1998). LIMK1 is widely expressed in tissues, including the nervous system (Proschel et al., 1995; Foletta et al., 2004), and its activity modulates neuritogenesis and synaptic plasticity (Meng et al., 2002; Endo et al., 2003; Rosso et al., 2004). LIMK1 is a key downstream effector of the Rho family of small GTPases and is activated by phosphorylation on Thr508 by Rho kinase ROCK, or by Rac-Cdc42 p21-activated kinase (PAK) (Edwards et al., 1999). Recent observations showed that a multiprotein complex containing LIMK1 and its kinase activator PAK can be recruited into the focal adhesion complex by p95PKL-mediated binding to Paxillin (Turner, 2000; Chen et al., 2005), raising the possibility that LIMK1 may be required for Aβ-induced actin filament remodeling in focal adhesions. To gain additional insight into the molecular mechanism that leads to neuronal dystrophy in AD, we analyzed the involvement of LIMK1, ADF/cofilin, and actin cytoskeleton in Aβ-induced neurotic dystrophy. Our results indicate that Aβ degeneration requires the activation of LIMK1 and the rearrangement of actin cytoskeleton; in addition, high levels of Thr508-phosphorylated LIMK-1 (P-LIMK1) were found in AD brain, suggesting the involvement of LIMK1 in AD pathology.

Materials and Methods

Human tissue, histological processing, and immunofluorescence. Human brain tissue samples from the entorhinal cortex of seven neuropathologically confirmed AD cases and three age-matched controls obtained from the Institute for Brain Aging and Dementia Tissue Repositories at the University of California, Irvine, were included in this study. Tissue sections from human brain were processed for immunofluorescence as described previously (Head et al., 2002). For specimen examination and imaging, an Axiovert 200 inverted microscope (Zeiss, Jena, Germany) was used. Fluorescent images were captured with a digital camera (Zeiss) and processed using AxioVision (Zeiss) as described previously (Helguera et al., 2005).

The following antibodies were used: polyclonal anti-P-LIMK (1:50; Cell Signaling Technology, Beverly, MA), monoclonal anti-human PHF-1 Tau (1:500; Pierce, Rockford, IL), monoclonal anti-Aβ clone 6E10 (1:100; Senetek, Napa, CA), and mouse monoclonal anti-Aβ42, which specifically recognizes the C terminus of Aβ4-42 (Busciglio et al., 2002; Helguera et al., 2005).

Neuronal cultures. Rat cortical and hippocampal cultures were established from embryonic day 18–19 fetuses as described previously (Lorenzo and Yankner, 1994). High-density cultures (2500 cells/mm²) were used for biochemical analysis; low-density cultures (100 cells/mm²) were used for immunocytochemistry. The cells were plated in DMEM (Invitrogen, Gaithersburg, MD) plus 10% horse serum (Hyclone, Logan, UT) on poly-l-lysine (1 mg/ml)–coated dishes or coverslips; after 2 h, the medium was replaced with glia-conditioned DMEM plus N2 and B27 supplements (Invitrogen) medium. The cultures were maintained at 37°C in a 5% CO₂ humidified atmosphere.

Peptides and treatments. Synthetic Aβ1–40 (Biopeptide, San Diego, CA) was dissolved in sterile double-distilled water to a concentration of 1 mM, incubated for 3 d at 37°C, and further diluted in PBS to 0.5 mM to allow fibril formation. Aβ25–35 was dissolved in sterile double-distilled water to a concentration of 1 mM, further diluted in PBS to 0.5 mM, and incubated for 1 d at 37°C. These Aβ preparations are mainly composed of amyloid fibrils, as shown by electron microscopy and Congo red birefringence (Lorenzo and Yankner, 1994). Nonfibrillar, soluble Aβ (sAβ) peptides were added to the medium without preincubation. S3 peptide (Biopeptide) was dissolved in sterile double-distilled water to a concentration of 2.5 mM and added to the cultures to the indicated final concentrations.

Western blot. Cultures were harvested in radioimmunoprecipitation assay buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 mM NaCl, and 0.05 mM Tris-HCl, pH 7.2) with protease inhibitors (Complete mini; Roche, Indianapolis, IN) at 4°C. The cell homogenates were then diluted in Laemmli sample buffer and incubated at 95°C for 5 min. Protein homogenates were separated on standard 8 or 12% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. After blocking with 5% milk or 5% bovine serum albumin in PBS with 0.05% Tween 20, the membranes were incubated overnight at 4°C with primary antibodies, followed by an HRP-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). The following primary antibodies were used: monoclonal anti-tubulin βIII (1:1000; Chemicon, Temecula, CA), polyclonal anti-Ser3 phosphorylated cofilin 4317 (1:2000; kindly provided by Dr. J. Bamburg, Colorado State University, Fort Collins, CO), polyclonal anti-P-LIMK (1:100; Cell Signaling Technology), a polyclonal antibody against LIMK1 (1:100; Santa Cruz Biotechnology, Santa Cruz, CA), and a polyclonal antibody against non-phosphorylated cofilin (1:100; Santa Cruz Biotechnology). Protein bands were visualized by chemiluminescence. Semi-quantitative analysis was performed using Scion image software; values were expressed as percentage of control samples. Each experimental condition was performed in triplicate, and experiments were replicated two to five separate times.

Immunofluorescence. Cell cultures were fixed in 4% paraformaldehyde and 0.12 mM sucrose in PBS for 30 min at 37°C, permeabilized for 5–7 min with 0.2% Triton X-100 in PBS, and blocked for 40 min in 5% normal horse serum. The cells were then incubated for 2 h at room temperature with primary antibodies, followed by incubation with Alexa-conjugated secondary antibodies (Invitrogen, Eugene, OR). The following antibodies were used: monoclonal anti-neuronal-specific tubulin βIII (1:500; Chemicon), monoclonal anti-Aβ clone 6F3/5D (1:500; Dako), polyclonal anti-Ser3 phosphorylated cofilin 4321 (1:200; kindly provided by Dr. J. Bamburg), monoclonal anti-microtubule-associate protein 2 (MAP2) clone AP20 (1:200; Sigma, St. Louis, MO), and polyclonal anti-P-LIMK (1:200; Cell Signaling Technology). Actin filaments were labeled with rhodamine–phalloidin (Invitrogen). To label Aβ fibrils, a mixture (10:1 ratio) of unlabeled Aβ40 and biotinylated Aβ41–40 were used; after fixation, the cultures were incubated with Neutravidin Alexa530 (Invitrogen). To assess fluorescence intensity, nonsaturating digital images were acquired from control and Aβ-treated cultures under the same conditions using a Zeiss Pascal confocal microscope. Image scans were performed using MetaMorph (Universal Imaging, West Chester, PA) controlled by a host IBM-AT computer.

Expression plasmids and transfections. Expression plasmids for full-length wild-type LIMK1 and the LIMK1-kinase dead mutant were described previously (Rosso et al., 2004). Transient transfection of cultured neurons was performed in rat hippocampal cultures that were grown in DMEM plus N2 and B27 supplements (Invitrogen) in a 96-well multwell culture plate at high density (80,000 cells/well). Cultures were cotransfected with green fluorescent protein (GFP; 0.04 µg/well), and LIMK1 wild type (0.06 µg/well) or LIMK1-kinase dead (0.06 µg/well), by using Lipofectamine 2000 (0.4 µl/well; Invitrogen) in OptiMEM (8 µl/well; Invitrogen) transfection medium. After 3 h, the medium was replaced with DMEM plus N2 and B27 supplement (Invitrogen) medium plus the respective treatments. At the indicated time, the cultures were fixed with 4% paraformaldehyde and 0.2% sucrose and analyzed by fluorescence microscopy.

Morphometric analysis. Individual neurons were characterized as dystrophic according to defined morphological features (Grace et al., 2002; Grace and Busciglio, 2003). Briefly, dystrophic neurons were characterized by obvious aberrant morphology of neurites, such as acute angles, loops, or processes growing upward. Dendritic length was determined in MAP2-immunolabeled cultures; random images of individual neurons
were taken at 40× magnification, and 80 neurons were analyzed using MetaMorph software.

Assessment of neuronal viability. To score neuronal viability in non-transfected cultures, the number of healthy nuclei per unit area after Hoechst staining was assessed using a combination of fluorescent illumination and phase contrast, which allowed the identification of individual neurons that were unequivocally healthy on the basis of its nuclear appearance. In GFP/LIMK1-cotransfected cultures, neuronal viability was assessed by counting GFP-expressing neurons per unit area as described previously (Arrasate et al., 2004).

Statistics. All experiments were performed on triplicate samples and replicated at least three times. Statistical comparisons were performed using Student’s t test or ANOVA, followed by post hoc comparisons. Data are expressed as the percentage of the mean ± SEM with respect to the vehicle-treated control.

Results

Aβ fibrils induce neuronal dystrophy and increase phosphorylation of ADF/cofilin and LIMK1

Rat hippocampal neurons in cultures show an extensive neurite network, with a smooth and healthy appearance of neurites as evidenced by tubulin class III immunostaining (Fig. 1A). Treatment with 20 μM fibrillar Aβ (fAβ) for 24 h resulted in neuronal dystrophy. Dystrophic neurites exhibit several abnormal morphological features including deformation and contortion of neurites, aberrant outgrowth of neuritic processes from the cell body, and a dramatic reduction in the axonal network (Fig. 1B) (Busciglio et al., 1992; Grace et al., 2002; Heredia et al., 2004).

Neuronal dystrophy is accompanied by a dramatic reorganization of actin filaments, as evidenced by rhodamine–phalloidin labeling. In control cultures, actin filaments are mainly observed at the tips of the neurites and in delicate filopodia extending at the sides of neuritic shafts (Fig. 1A, C). In contrast, in fAβ-treated cultures, dense phalloidin staining is found in dystrophic neurites in the proximity of fAβ deposits (Fig. 1C, D), indicating that fAβ deposition is linked to altered organization of actin filaments and neuronal dystrophy. Therefore, to address whether ADF/cofilin activity is altered by Aβ, we used an antibody that specifically recognizes inactive, Ser3-phosphorylated-ADF/cofilin (P-cofilin). Rat hippocampal cultures were treated with vehicle or with fAβ and analyzed by immunofluorescence. Triple-fluorescence confocal microscopy showed that fAβ increases P-cofilin fluorescence intensity in hippocampal cultures (Fig. 2A). This increase was associated with a dramatic remodeling of actin filaments and with neuritic dystrophy, as evidenced by rhodamine–phalloidin and tubulin class III labeling, respectively (Fig. 2A). To confirm that the increase in the P-cofilin level is attributable to the toxic fibrillar form of Aβ, cultures were treated with vehicle (control), soluble monomeric Aβ (sAβ) or fAβ for 24 h and analyzed by Western blot. A significant increase in the P-cofilin level was confirmed in fAβ-treated cultures (ANOVA; F_{(2,5)} = 10.44; p < 0.016, LSD post hoc test) (Fig. 2B); in contrast, the level of P-cofilin was not altered by sAβ, indicating that ADF/cofilin phosphorylation depends on the state of aggregation of Aβ. It was shown previously that Aβ may stimulate neurite outgrowth through the cell adhesion domain RHDS spanning residues 5–8 of the Aβ sequence (Ghisio et al., 1992; Saporto-Irwin and Van Nostrand, 1995). To determine the role of the RHDS domain in Aβ-induced neuronal dystrophy, we analyzed the effect of Aβ25–35 peptide, which lacks the RHDS domain but can form fibrils and induce neurotoxicity (Yankner et al., 1990; Hughes et al., 2000). A time-course analysis of P-cofilin showed that Aβ25–35 induces a persistent increase in Ser3 phosphorylation of ADF/cofilin over the period analyzed (ANOVA; F_{(3,11)} = 7.29; p < 0.005, LSD post hoc test) (Fig. 2C). These experiments indicate that fAβ-induced neuronal dystrophy is associated with a persistent inactivation of ADF/cofilin and a rearrangement of actin filaments.

LIM kinases can induce inactivation of ADF/cofilin by phosphorylation at its Ser3 (Arber et al., 1998). LIMK1, a LIMK family member predominantly expressed in the nervous system, is activated by phosphorylation at Thr508 (Edwards et al., 1999). To explore whether LIMK1 activity is altered by fAβ, we used an antibody that selectively recognizes LIMK1 phosphorylated at Thr508. Treatment of hippocampal cultures with fAβ resulted in an increased level of P-LIMK1 as evidenced by immunofluorescence (Fig. 3A). Densitometric determination using MetaMorph software showed a significant 30 ± 10.2% increase in P-LIMK1 in the soma (ANOVA; F_{(1,29)} = 6.16; p = 0.019) and a 35 ± 6.2% increase in P-LIMK1 in proximal neurites (ANOVA; F_{(1,29)} = 18.51; p < 0.001) compared with vehicle-treated control cultures. Western blot showed that increased levels of P-LIMK1 were not associated with an overall increase in LIMK1 levels (Fig. 3B). Together, these experiments show that Aβ fibril deposition increases the activity of LIMK1 in hippocampal cultures.

ADF/cofilin phosphorylation by LIMK1 mediates fAβ-induced neuronal dystrophy

To determine whether inactivation of ADF/cofilin by endogenous LIMK1 is required for fAβ-induced neuronal dystrophy, a
synthetic peptide, S3 peptide, which contains the unique Ser3 phosphorylation site of ADF/cofilin and a penetrating sequence to allow neuronal internalization of the peptide from the culture medium, was used as a specific competitor substrate for active LIMK1 (Fig. 4A). This peptide has been successfully used to prevent LIMK1-mediated phosphorylation of ADF/cofilin after semaphorin 3A treatment (Aizawa et al., 2001). As expected, the addition of the S3 peptide to hippocampal cultures reduces the endogenous levels of P-cofilin in a time- and dose-dependent manner (Fig. 4B). More importantly, Western blot and densitometric determinations showed that S3 peptide significantly abolished the increase in P-cofilin induced by fAβ/H9252 (Fig. 4C) (ANOVA; $F_{(4,10)} = 15.34; p < 0.0003$). Besides, immunofluorescence analysis also showed that S3 peptide dramatically reduced the increase in P-cofilin induced by fAβ/H9252 after 6 h of treatment (Fig. 4D). These experiments indicate that S3 peptide efficiently inhibits Aβ-induced ADF/cofilin phosphorylation.

To determine the role of ADF/cofilin activity on Aβ-induced neuronal dystrophy, cultures were treated for 24 h with vehicle or 20 μM fAβ/H9252, with or without S3 peptide (7.5 μg/ml); neuritic morphology was analyzed after tubulin class III immunolabeling. Treatment with fAβ caused a striking loss of axonal and dendritic processes as evidenced by a dramatic reduction in the neuritic network in the culture (Fig. 5); this reduction was prevented by S3 peptide (Fig. 5). An accurate determination in the reduction in axonal and dendritic length induced by fAβ in these cultures was
logical abnormalities induced by fA peptide alone did not alter dendritic length, but it significantly inhibited the increase in P-cofilin induced by fA peptide, whereas S3 peptide alone did not alter neuronal viability in an appreciable manner (ANOVA; $F_{(3,36)} = 23.32; p < 0.001$, LSD post hoc test). These data suggest that LIMK1-mediated phosphorylation of ADF/cofilin is also involved in Aβ-induced neuronal death. To further confirm the involvement of LIMK1 in Aβ-induced neuronal death by an independent method, rat hippocampal cultures were cotransfected with the GFP and with either full-length wild-type LIMK1 (wt-LIMK1) or full-length LIMK1 carrying a point mutation at 360th Lys by Met that abolishes its kinase activity (kd-LIMK1) (Sumi et al., 1999). Immunofluorescence analysis revealed a high degree (>80%) of GFP–LIMK1 cotransfection. In addition, we observed that overexpression of wt-LIMK1 was accompanied by increased staining for F-actin, whereas overexpression of kd-LIMK1 resulted in faint staining with rhodamine–phalloidin, consistent with a previous study (Rosso et al., 2004). We found that treatment with fA peptide resulted in a 55 ± 6% reduction in neuronal viability in GFP/wt-LIMK1-cotransfected cultures. Neuronal viability was only slightly decreased to 95 ± 6% in cultures treated with fA peptide and S3 peptide (Fig. 8).}

not possible because of the extensive development of neurites in control and S3 peptide–treated cultures. To overcome this difficulty, dendritic length was measured after MAP2 immunolabeling, as a quantitative estimate of neuritic dystrophy. Similar to tubulin-stained cultures, MAP2-labeled neurons showed a dramatic reduction in the length of MAP2-positive dendrites after fA peptide treatment; this effect was abolished by cotreatment with the S3 peptide (Fig. 6). Thus, fA peptide induced a significant 52.3 ± 6.1% reduction in dendritic length compared with vehicle-treated control cultures ($p < 0.0001$; ANOVA and LSD post hoc test); the addition of S3 peptide alone did not alter dendritic length, but it completely inhibited the detrimental effect of fA peptide on dendritic length (Fig. 6A,B). In addition, we observed that other morphological abnormalities induced by fA peptide, such as tortuosity and aberrant branching and sprouting of neurites were also reduced by S3 peptide. Altogether, these experiments indicate that fA peptide-induced neuronal dystrophy requires LIMK1-mediated inactivation of ADF/cofilin.

**LIMK1 activity in AD brain**

We then analyzed the presence of active LIMK1 in brain sections of entorhinal cortex and hippocampal formation in human brain. In normal human brain, sporadic neurons stained positive for P-LIMK1. Immunofluorescence of P-LIMK1 was observed in neuronal cell bodies and processes but was essentially excluded from glial cells (data not shown). Three-dimensional reconstruction images revealed that P-LIMK1 localized as patches at the cell surface of the neuron, suggesting that LIMK1 activation might occur mainly in association with the cell membrane. In sporadic human AD brain, only occasional neurons stained positive for P-LIMK1 in areas free of AD pathology (frequency, 0.68 ± 0.17 neurons/field; see Materials and Methods) (Fig. 8A); in contrast, a dramatic increase in the number of P-LIMK1-positive neurons was clearly observed in AD pathology-rich areas of the same cortex (Fig. 8 B, C). Quantitative analysis confirmed a highly significant increase in the number of neurons that were positive for P-LIMK1 in Aβ pathology-rich areas (frequency, 12.75 ± 2.13 neurons/field) compared with Aβ pathology-free areas ($t = 11.609; df = 18; p < 0.0001$, Student’s $t$ test) (Fig. 8D). Similarly, the number of P-LIMK1-positive neurons significantly increased...
in areas affected by tau pathology (7.87/0.54 neurons/field) compared with tau pathology-free areas (t = 15.79, df = 22; p < 0.0001, Student’s t test) (Fig. 8 D). In addition, P-LIMK1 immunofluorescence was found in neurons showing intracellular accumulation of Ab and diffuse, pretangle phosphorylated tau (Fig. 8 E, F), which are indicators of an early stage of neuronal degeneration (Gouras et al., 2000; Busciglio et al., 2002; Oddo et al., 2003). No P-LIMK1 labeling was associated with late markers of degeneration such as extracellular ghost tangles or Ab-plaque cores (P. Helguera and J. Busciglio, unpublished observation). Thus, LIMK1 activity is increased in AD brain, in neurons displaying markers of AD pathology.

Discussion

Dystrophic neurites are a direct cause of cognitive impairment in AD (McKee et al., 1991; Benzing et al., 1993; Knowles et al., 1999; Brendza et al., 2003; Stern et al., 2004; Tsai et al., 2004), therefore deciphering the molecular mechanism of dystrophy might pave the way for novel therapeutic modalities for AD. The recent findings showing that local applications of antibodies to Ab can rapidly revert neuritic dystrophy in vivo (Lombardo et al., 2003; Brendza et al., 2005) further support this possibility. Here, we demonstrate that Ab dystrophy requires LIMK1-mediated phosphorylation of ADF/cofilin and the remodeling of the actin cytoskeleton. Previous reports showed that Ab deposition was linked to increased accumulation of actin filaments and treatment with actin depolymerizing agents, such as cytochalasin D or lantrunculin B, inhibited Ab degeneration (Furukawa and Mattson, 1995; Himura et al., 2003); our in vitro data are in agreement with these observations and provide a molecular mechanism for Ab-induced actin remodeling. We found that treatment of neuronal cultures with Ab fibrils significantly increased the accumulation of actin filaments and the levels of the active P-LIMK1 and inactive Ser3-phosphorylated ADF/cofilin. Because ADF/cofilin is the only known substrate for LIMK1 in nerve cells (Bamburg and Wiggan, 2002; Meyer and Feldman, 2002), its inactivation by LIMK1 might result in actin filament accumulation. This interpretation is consistent with previously reported effects of LIMK1 (Endo et al., 2003; Rosso et al., 2004). Importantly, the nontoxic soluble-monomeric form of Ab1–40 did not induce dystrophy (Busciglio et al., 1992; Heredia et al., 2004), nor did it promote actin filament accumulation or increase the P-cofilin level, indicating that the conversion of Ab to the fibrillar form is required to activate LIMK1 and to promote actin remodeling and dystrophy. Additionally, we found that other toxic insults that do not induce neuritic dystrophy, such as hydrogen peroxide, serum withdrawal, and NMDA, did not increase the P-LIMK1 level (L. Here-
Figure 7. LIMK1 and fAβ-induced neuronal death. Rat hippocampal cultures were treated with vehicle (control) or 20 μM fAβ1–40 (fAβ1–40), with or without 7.5 μg/ml S3 peptide, and the number of viable neurons was scored. A. In nontransfected cultures, the effect of fAβ1–40 and S3 peptide on neuronal viability was assessed 24 h after treatment by Hoechst staining. B, C. In cultures that were cotransfected with GFP and either wt-LIMK1 (B) or kd-LIMK1 (C), neuronal viability was assessed 18 h after treatment by scoring GFP-expressing neurons. A. Representative experiment showing the effect of fAβ and S3 peptide on neuronal viability. More than 900 neurons were scored, and similar results were found in three independent experiments. Statistical comparisons were made by ANOVA (F(3,24) = 23.32) and LSD post hoc test. *p < 0.001, fAβ versus control, S3 peptide, and fAβ + S3 peptide. B. Effect of fAβ and S3 peptide on neuronal viability in GFP/wt-LIMK1–cotransfected neurons. A representative experiment is shown. >170 neurons were scored, and similar results were found in three independent experiments. Statistical comparisons were made by ANOVA (F(3,12) = 4.1464) and the LSD post hoc test. *p < 0.01, fAβ versus control, S3 peptide, and fAβ + S3 peptide. Note that toxicity of fAβ is blocked by S3 peptide in nontransfected neuronal cultures that express endogenous levels of LIMK1 and in cultures that were transfected with wt-LIMK1. C. Effect of fAβ and S3 peptide on neuronal viability in GFP/kd-LIMK1–cotransfected cultures. A representative experiment is shown. >110 neurons were scored, and similar results were found in three independent experiments. No significant effect of fAβ in neuronal viability was observed; statistical comparisons were made by ANOVA (F(3,12) = 0.6915; p = NS). Note that kd-LIMK transfection inhibits fAβ-induced neuronal death. Error bars indicate SEM.

Aβ fibril-mediated LIMK1 activation might require the participation of cell-surface proteins. We demonstrated that Aβ fibrils are needed for LIMK1 activation in vitro; coincidently, conversion of sAβ to the fibrillar form increases its binding to membrane proteins (Lorenzo et al., 2000). Several membrane proteins have been shown to participate in Aβ-induced apoptosis (Yuan and Yankner, 2000); in contrast, AβPP and/or integrins could be the main cell-surface candidates for Aβ fibril-induced neuronal dystrophy. Both AβPP and integrins participate in mechanisms of neuronal plasticity (Breen et al., 1991; Perez et al., 1997; Benson et al., 2000; Sabo et al., 2001a), bind to Aβ fibrils (Sabo et al., 1995; Lorenzo et al., 2000; Van Nostrand et al., 2002), colocalize in adhesion sites (Storey et al., 1996; Yamazaki et al., 1997), and cluster around Aβ deposits in aberrant focal adhesion structures (Grace and Busciglio, 2003; Heredia et al., 2004). In fact, Aβ dystrophy requires aberrant activation of focal adhesion proteins, including Paxillin (Grace and Busciglio, 2003), activation of which could also depend on AβPP or integrins, through their interaction with different cytoplasmic proteins (Sabo et al., 2001b; Zambrano et al., 2001; Martin et al., 2002). Interestingly, LIMK1 and its activator PAK have been shown to colocalize with Paxillin in focal adhesions (Foletta et al., 2004; Chen et al., 2005). Thus, LIMK1 may be a key downstream effector for Aβ fibril-induced actin remodeling in the aberrant focal adhesion complex, providing the initial forces to remodel neuronal shape around Aβ deposits.

The identity of the upstream pathway involved in fAβ-mediated LIMK1 activation has not been defined. Although noncanonical or aberrant signaling pathways could not be excluded, it is likely that fAβ-mediated LIMK1 phosphorylation may require activation of the Rho family of small GTPases, including role of altered ADF/cofilin activity as an important factor contributing to actin pathology and neuronal degeneration in AD.
pathways of Rho/ROCK or Rac-cdc42/PAK. It was found recently that defects in the PAK pathway may lead to actin pathology and cognitive deficits in AD (Zhao et al., 2006). These authors reported a general reduction in the levels and activity of PAK in AD brain. Interestingly, they also showed that the overall PAK defect is accompanied by abnormal, very intense intraneuronal staining of active PAK in a punctate pattern, and similar abnormalities were also found in neurons surrounding Aβ plaques in 22-month-old APPswe Tg2576 mice and in hippocampal neurons after Aβ treatment (Zhao et al., 2006). These observations suggest that focalized abnormal PAK activation may occur during the progression of AD pathology. Consequently, strategies directed toward abnormal regulation of the pathway of the Rho family of small GTPases in AD neurodegeneration.

The ability of Aβ fibrils to induce the phosphorylation of LIMK1 and to remodel neuritic morphology suggests the activation of a mechanism of neuronal plasticity reminiscent of that triggered by Semaphorin 3A or BMP7, which are soluble, extracellular physiological morphogens that require LIMK1 as a downstream effector for shaping neurites in early stages of neuronal development (Aizawa et al., 2001; Lee-Hoeflich et al., 2004). Physiological morphogens such as Semaphorin 3A or BMP7 are temporally and spatially regulated to model a functional neuronal architecture. In contrast, in AD brain, deposition of Aβ fibrils progresses uncontrolled during adulthood promoting a pathological rearrangement of the extracellular matrix (Thal et al., 2002), suggesting that a continuous alteration in the composition of the extracellular matrix may trigger a pathological mechanism of plasticity. In fact, a pathological mechanism of neuroplasticity has been proposed as a pivotal prime mover in AD (Cotman et al., 1998; Mesulam, 1999; Phinney et al., 1999). In this regard, the results of this work suggest that LIMK1 is a key effector in the mechanism of maladaptive neuroplasticity in AD. Consequently, strategies directed toward suppress abnormal activation of LIMK1 might have therapeutic value to counteract neuronal degeneration and cognitive impairment in AD.

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