Neurobiology of Disease

Induction of Dickkopf-1, a Negative Modulator of the Wnt Pathway, Is Associated with Neuronal Degeneration in Alzheimer’s Brain

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Received Feb. 2, 2004; revised May 19, 2004; accepted May 20, 2004.

We used primary cultures of cortical neurons to examine the relationship between β-amyloid toxicity and hyperphosphorylation of the tau protein, the biochemical substrate for neurofibrillary tangles of Alzheimer’s brain. Exposure of the cultures to β-amyloid peptide (βAP) induced the expression of the secreted glycoprotein Dickkopf-1 (DKK1). DKK1 negatively modulates the canonical Wnt signaling pathway, thus activating the tau-phosphorylating enzyme glycogen synthase kinase-3β. DKK1 was induced at late times after βAP exposure, and its expression was dependent on the tumor suppressing protein p53. The antisense induced knock-down of DKK1 attenuated neuronal apoptosis but nearly abolished the increase in tau phosphorylation in βAP-treated neurons. DKK1 was also expressed by degenerating neurons in the brain from Alzheimer’s patients, where it colocalized with neurofibrillary tangles and distrophic neurites. We conclude that induction of DKK1 contributes to the pathological cascade triggered by β-amyloid and is critically involved in the process of tau phosphorylation.

Key words: Alzheimer’s disease; β-amyloid; Wnt pathway; Dickkopf-1; tau phosphorylation; apoptosis

Introduction

Evidence from human genetics and transgenic mice suggests that an overproduction of β-amyloid peptide (βAP) is a primary event in the pathophysiology of Alzheimer’s disease (AD) (Hardy and Selkoe, 2002). βAP applied to neuronal cultures induces apoptosis (Loo et al., 1993), a phenotype of death that is also observed in the AD brain (Cotman and Anderson, 1995; Smale et al., 1995). How this can be reconciled with the formation of neurofibrillary tangles (NFTs) is uncertain because, at least in vitro, neurons exposed to βAP die too rapidly to allow the formation of NFTs. NFTs might arise in neurons that in vivo escape a fast execution of apoptotic death (Caricasole et al., 2003).

DNA damage associated with p53 expression may be a point of convergence of multiple intracellular pathways related to βAP toxicity (Zhang et al., 2002). p53 induces a number of genes that promote either DNA repair or apoptotic death (Lakin and Jackson, 1999; Shen and White, 2001). We wondered whether a p53 transcription program is involved in the formation of NFTs in βAP-treated neurons. Formation of NFTs results from a hyperphosphorylation of the tau protein, which is potentially driven by glycogen-synthase kinase-3β (GSK3β) (Takahshima et al., 1998; Otth et al., 2002), an enzyme that is under the control of the canonical Wnt signaling pathway (Grimes and Jope, 2001). This pathway is activated by different Wnt secreted glycoproteins that interact with the Frizzled and LRP5/6 (LDL receptor-related protein type 5 and 6) membrane coreceptors (Dale, 1998). Activation of the Wnt pathway leads to inhibition of GSK3β by dissociating the enzyme from a multiprotein complex that involves axin, adenomatous polyposis coli, and β-catenin (Willert and Nusse, 1998) and via phosphorylation of GSK3β on Ser9 (Fukumoto et al., 2001). This results in the stabilization of the underphosphorylated form of β-catenin, which is no longer targeted for degradation by the proteasome and is then made available for its transcriptional and cell adhesion functions (Hinck et al., 1994; Willert and Nusse, 1998). Recent evidence suggests that a loss of Wnt function is implicated in the pathophysiology of neuronal degeneration in AD (De Ferrari and Inestrosa, 2000; Garrido et al., 2002; Inestrosa et al., 2002; De Ferrari et al., 2003). The canonical Wnt pathway is negatively modulated by the extracellular protein Dickkopf-1 (DKK1), which binds to LRP5 preventing their interaction with Wnts (Zorn, 2001). DKK1 is induced by p53 (Wang et al., 2000) and might therefore be a component of the sequence of events leading to neuronal toxicity in response to

DOI:10.1523/JNEUROSCI.1381-04.2004
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The Journal of Neuroscience, June 30, 2004 • 24(26):6021–6027 • 6021
βAP. Induction of DKK1 would prevent the inhibition of GSK3β by βAP, thus facilitating phosphorylation of tau protein and formation of NFTs in neurons that survive a rapid execution of apoptotic death. We now report that DKK1 is expressed in cultured cortical neurons exposed to βAP as well as in neurons from autopic brain samples of AD patients. In addition, we show that βAP-induced DKK1 expression is under the control of p53 and is causally related to hyperphosphorylation of tau in neurons challenged with βAP.

**Materials and Methods**

*Culture preparation, treatments, and assessment of neuronal death.* All experiments were performed in compliance with the European Union use of laboratory animals, the guidelines of the Italian Decreto Legislativo 271/92, number 116, article 7, and according to the institutional approved protocol number 9213. Cultures of pure cortical neurons were obtained from embryonic day (E) 15 rat embryos according to a well-established method that allows the growth of a >99% pure neuronal population (Copani et al., 1999). βAP (25–35) was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). Different lots of the peptide were used. βAP (25–35) was solubilized in sterile, doubly distilled water at an initial concentration of 2.5 mM and stored frozen at −20°C. It was used to a final concentration of 25 μM in the presence of the glutamate receptor antagonists MK-801 ((+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine maleate) (10 μM) and DNQX (30 μM) to avoid the potentiation of endogenous glutamate toxicity (Copani et al., 1999). In some experiments, cultures were treated with the following "end-capped" phosphorothioate antisense (As) oligonucleotides: p53-As, 5'-gacgcaggtgctgtgc-3'; p53-Sense (Sn), 5'-cctgctgacccgtgcgttc-3' (Chen et al., 1999); DKK-As 5'-gtgccggagg gcgcag-3'; DKK-Sn, 5'-gtgcgtgctccctggag-3'. Oligonucleotides (3 μM; MW-Biotec, Firenze, Italy) were applied to cultures 16 hr before the addition of βAP. Assessment of neuronal death was performed by combining MTT assay and cytofluorimetric analysis of hypoploid DNA (Copani et al., 1999; Copani et al., 2002).

**Reverse transcriptase-PCR and real-time PCR analysis.** Total RNA was extracted from the cultures as described previously (Godemann et al., 1999). Total RNA was subjected to Dnal treatment (Roche, Hertfordshire, UK) and 2 μg of total RNA/sample were used for cDNA synthesis using Superscript II (Invitrogen, San Diego, CA) and an oligodT primer. Each reverse transcriptase (RT) product was diluted to 100 using Superscript II (Invitrogen, San Diego, CA) and an oligodT primer.

**Western blot analysis.** Western blot analysis was performed on total cell extracts from the cultures as described previously (Roelen et al., 1994). Primary cortical neuron cultures were transfected with a T-cell factor/lymphoid enhancer factor-based luciferase reporter studies. Transfections and reporter assays were performed essentially as described previously (Caricasole et al., 2002). Transfections of primary cortical neurons were performed in triplcate using Lipofectamine 2000 (Invitrogen). Neurons (~3 × 10^6 per well in 24-well plates) were transfected at 12 d in vitro. A total of 1.02 μg of DNA was transfected into each well, including luciferase reporter plasmid (200 ng), expression construct (250–800 ng for each expression construct, for up to 2 different plasmids), and Renilla luciferase cytomegalovirus-driven internal reporter (20 ng; Promega, Madison, WI), and carrier plasmid DNA (pBluescript; Promega; to 1.02 μg) as appropriate. The luciferase reporter plasmid was the p4TCF, comprising four copies of a T-cell factor (TCF) responsive element upstream of a TATA element-luciferase coding sequence (Caricasole et al., 2002). Transfection medium with culture medium, βAP was added to a final concentration of 25 μM to wells, as appropriate. Luciferase activity was measured using the Promega Dual Luciferase Assay Reagent and read using a Berthold (Bad Wildbad, Germany) LUMAT LB907 tube lumi-meter. Readings were from triplicate transfections and were automatically normalized relative to the internal standard (Renilla luciferase).
and anti-tau (clone AT8; against hyperphosphorylated tau; 1: 2000; Immogenetics, Gent, Belgium) were incubated 30 min at room temperature and at 4 °C overnight. Sections were then washed thoroughly with PBS and incubated at room temperature for 1 hr with the appropriate biotinylated secondary antibody diluted in PBS (rabbit anti-goat Ig, 1:300, Vector Laboratories, Burlingame, CA; 1:200, goat-anti mouse Ig, 1: 200, Dako, Carpinteria, CA). Single-label immunocytochemistry was performed using an avidin-biotin peroxidase method (Vector Elite) with 3,3’-diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin or Congo red and mounted with Depex (BDH Chemicals, Poole, UK). Sections incubated without the primary antibody or with preimmune sera were essentially blank.

For double labeling, sections (after incubation with primary Abs; DKK1 and P53 or AT8) were incubated for 1 d at 4 °C with Alexa Fluor 568 anti-goat IgG and Alexa Fluor 488 anti-mouse IgG (1:200; Molecular Probes, Eugene, OR). To block auto fluorescence caused by the presence of lipofuscin pigment in the tissue, sections were stained with Sudan Black B (Merck, Darmstadt, Germany) for 10 min. Sections were then analyzed by means of a laser scanning confocal microscope (Bio-Rad; MRC1024) equipped with an argon-ion laser.

To analyze the percentage of DKK1-positive structures, sections labeled with DKK1 and PHF-tau (AT8) were digitized using an Olympus Vanox microscope equipped with a DP-10 digital camera (Olympus Optical, Tokyo, Japan). Images (200 × magnification) from representative fields of the lesion in two double-labeled sections of four AD cases were collected with an Apple Macintosh Power PC 82 computer. The total number of neurons stained with PHF-tau, as well as the structures double-labeled for DKK1 and PHF-tau, was counted. We calculated the percentages of structures immunoreactive for PHF-tau that also contained DKK1 immunoreactivity.

**Results**

**Apoptotic death and tau hyperphosphorylation in cultured cortical neurons exposed to βAP**

We used pure cultures of rat cortical neurons devoid of astrocytes and other contaminating cells. These cultures respond to βAP (fragments 1–42, 1–40, or 25–35) with the activation of an unscheduled cell cycle, which leads to an increased p53 expression and apoptotic cell death within 24 hr (Copani et al., 1999, 2002). Treatment with 25 μM βAP enhanced the expression of the pro-apoptotic protein Bax at times that coincide with the increased expression of p53. The antisense-induced knock-down of p53 prevented the induction of Bax and attenuated neuronal death in βAP-treated cultures (Fig. 1). Immunoblots with the PHF-1 antibody (which detects the phosphorylated tau epitopes Ser 396 and 404) showed that a 20 hr exposure to βAP induced an increase in tau protein phosphorylation (Fig. 2). To avoid the fast p53-dependent apoptosis of neurons and allow a full development of tau phosphorylation, βAP-treated cultures were exposed to a caspase-3 inhibitor. The caspase-3 inhibitor Z-Asp-Glu-Val-Asp-CHO (z-DEVD), which prevented βAP-induced apoptosis (percentage of neuronal survival: control, 100 ± 4.3; 24 hr βAP, 42 ± 3.3; 24 hr βAP plus z-DEVD, 20 μM, 78 ± 5.2; 24 hr z-DEVD, 97 ± 2.5; n = 3; *p < 0.05 vs βAP alone), mediated amplification of tau phosphorylation (Fig. 2). Tau phosphoryla-
tion was prevented by lithium ions added to the cultures 7 hr after βAP (Fig. 2). Although lithium has multiple mechanisms of action, this effect might be attributable to the inhibition of GSK3β, an enzyme that phosphorylates tau on the Ser residues recognized by PHF-1 (Godemann et al., 1999). Accordingly, the selective inhibitor of GSK3β, 4-Benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) (50 μM), reproduced the effects of lithium ions when applied to the cultures 7 hr after βAP.

**DKK1 is expressed and required for tau phosphorylation in βAP-treated neurons**

Searching for a mechanism that could link the increase in p53 expression to GSK3β activation, we examined the expression of DKK1, a protein induced by p53 that negatively modulates the Wnt pathway. Cultures constitutively expressed the DKK1/Wnt coreceptors, LRP5 and LRP6, in addition to several Wnt glycoproteins and the other Wnt coreceptor, Frizzled (Fig. 3a). In addition, we could detect a functional response to an overexpressed Wnt member (Wnt7A) in cultures that were cotransfected with a reporter gene (luciferase) under the control of a TCF/lymphoid enhancer factor responsive promoter (Fig. 3b). Thus, the Wnt transduction machinery was functional in our cultures. Interestingly, the response to Wnt7A was reduced in βAP-treated cultures (Fig. 3b), consistent with the reported inhibition of Wnt signaling by βAP (De Ferrari et al., 2003). DKK1, which might account for this reduction, was nearly absent in control cultures but became substantially expressed after 16 hr of exposure to βAP (Fig. 4a). Induction of DKK1 was prevented by p53 antisense oligonucleotides (Fig. 4b). The antisense-induced knock-down of DKK1 had only a small protective effect against neuronal apoptosis (Fig. 5b) but prevented both the activation of GSK3β,
assessed through the reduction of the inactive phospho-Ser9 form of the enzyme, and the increase in tau phosphorylation that were observed after 21 hr of exposure to βAP (Fig. 5c).

Treatment with DKK1 sense oligonucleotides reduced DKK1 mRNA levels induced by βAP but to a much lesser extent than the respective antisenses (Fig. 5a). However, the extent of DKK1 mRNA reduction produced by DKK1 sense oligonucleotides did not appear sufficient to protect against βAP-induced apoptosis (Fig. 5b) or to prevent both GSK3β activation and tau hyperphosphorylation (Fig. 5c).

Expression of DKK1 in the AD brain
We extended the examination of DKK1 to human autopsy brain tissue from five AD patients and five age-matched controls (see Fig. 6 legend). All AD cases displayed a high degree of pathology (Braak score V-VI). Immunohistochemical processing revealed significant DKK1 immunostaining in AD temporal cortex and white matter (Fig. 6b,d). Nondemented age-matched controls showed no evidence of DKK1 expression (Fig. 6a,c). Occasional DKK1 staining was observed in glial cells surrounding Congo red-positive plaques (Fig. 6e). In AD temporal lobe sections that were double immunostained for DKK1 and p53, DKK1 was found in neurons with nuclear p53 expression (Fig. 6f, inset). Neurons with NFT morphology and dystrophic neurites also stained for DKK1 (Fig. 7a,b). In particular, AT8 phosphotau-labeled NFTs, and dystrophic neurites were immunoreactive for DKK1 (Fig. 7c,d; Table 1). DKK1-positive white matter fibers also stained for AT8 (Fig. 7e; Table 1).

Discussion
Recent evidence indicates that abnormalities of Wnt signaling might be involved in human brain diseases, including autism.

Figure 4. p53-dependent induction of DKK1 by βAP in cultured cortical neurons. a, RT-PCR analysis of DKK1 mRNA. Cultures were treated with 25 μM βAP(25–35) for the indicated times. Expression of mRNA in rat cortex (CTX) is shown as a positive control. The 400 bp β-actin band is also shown. The 600 bp β-actin band, assessing genomic DNA contamination, was undetectable. b, Representative immunoblot of DKK1 in protein extracts from cortical neurons treated with 25 μM βAP(25–35) for 20 hr in the absence or presence of p53 As or senses (Sn). Loading controls are included.

Figure 5. DKK1 knock-down attenuates cell death, GSK3β activation, and tau hyperphosphorylation in βAP-treated neurons. a, Real-time PCR analysis of DKK1 mRNA in neurons exposed to 25 μM βAP(25–35) for 16 hr in the presence or absence of DKK1 antisenses (DKK-As) or senses (DKK-Sn). Values are means ± SEM of normalized DKK1 from four determinations. *p < 0.05 versus controls (CTRL), or *# βAP alone (one-way ANOVA followed by Fisher’s LSD test). The effect of DKK-As on neuronal survival (b), GSK3β activation, and tau hyperphosphorylation (c) in cultures exposed to 25 μM βAP(25–35) for 20 hr is shown. In b, values represent the means ± SEM of six determinations. *p < 0.05 versus controls (CTRL), or *# βAP alone (one-way ANOVA followed by Fisher’s LSD test). In c, the immunoblot of PHF-1 was obtained from neuronal cultures treated with βAP in the presence of 20 μM z-DEVD. The β-actin band is shown for comparison. The immunoblot is representative of three independent experiments with similar results. The immunoblot of phospho(ser9)-GSK3β was obtained under the same conditions and repeated twice with similar results.
The AT8 antibody reveals the location of hyperphosphorylated tau (green) in neurons (a). In f, p53-positive cells from temporal cortex of AD are illustrated. A confocal image of DKK1 expression (red) in neurons bearing p53 nuclear expression (green) is shown in the inset. Alz, Alzheimer’s dementia.

**Table 1. DKK1 expression in AT8 phosphotau-labeled NFT and dystrophic neurites**

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<th>Cortex</th>
<th>White matter</th>
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<td>DKK1</td>
<td>85.7 ± 3.3</td>
<td>79.5 ± 5.2</td>
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Data represent percentages of structures immunoreactive for PHF-tau that also contained DKK1 immunoreactivity. Data are expressed as means ± SEM from four AD patients with DKK1-positive structures.

(Wassink et al., 2001), schizophrenia (Cotter et al., 1998), and AD (De Ferrari and Inestrosa, 2000; Garrido et al., 2002; Inestrosa et al., 2002; De Ferrari et al., 2003). In cultured neurons, overexpression of the Wnt antagonist, DKK1, prevents activity-dependent dendritic branching (Yu and Malenka, 2003). Our data demonstrate a role for DKK1 in the mechanisms of βAP toxicity. In tumor cell lines, DKK1 is induced by p53, which interacts with a potential responsive element located in the promoter region of the DKK1 gene (Wang et al., 2000). Consistent with the notion that DKK1 is a p53 target gene (Wang et al., 2000), induction of DKK1 was also p53 dependent in βAP-treated neurons. Thus, distinct p53-activated pathways of neuronal death occur in response to βAP, whereas the expression of Bax might account for the fast execution of apoptosis, the expression of DKK1 might subserve additional roles in the p53-activated program. DKK1 knock-down slightly reduced neuronal death induced by βAP, supporting the hypotheses that the Wnt pathway sustains neuronal survival (De Ferrari and Inestrosa, 2000; Garrido et al., 2002; Inestrosa et al., 2002; De Ferrari et al., 2003), and DKK1 behaves as a proapoptotic factor (Shou et al., 2002). In neurons that were made resistant to apoptosis by inhibition of caspase-3, we could disclose a hyperphosphorylation of tau at times that roughly coincided with the induction of DKK1 (i.e., after 16–21 hr of exposure to βAP). This particular process was inhibited by DKK1 knock-down and by lithium, which mimics the activation of the Wnt pathway by inhibiting GSK3β (De Ferrari et al., 2003). We speculate that DKK1 produced by βAP-treated neurons suppresses the canonical Wnt signaling pathway by interacting with LRP5/6 and therefore facilitates GSK3β activation. This scenario could take place in degenerating neurons of the AD brain, where DKK1 was highly expressed (whereas it was absent in control brains) and colocalized with both NFT and dystrophic neurites labeled by AT8, which recognizes phosphorylated tau in paired helical filaments. Based on recent findings on the effects of DKK1 overexpression on activity-dependent dendritic branching in neuronal cultures (Yu and Malenka, 2003), DKK1 overexpression in the AD brain would result in deficient neuronal plasticity and, together with its capacity to mediate increased tau phosphorylation (this study), likely contribute significantly to neuronal degeneration. Our demonstration that DKK1 is upregulated in the AD brain strengthens the hypothesis that an impairment of the Wnt pathway contributes to the pathophysiology of AD (De Ferrari and Inestrosa, 2000; Garrido et al., 2002; Inestrosa et al., 2002; Caricasole et al., 2003; De Ferrari et al., 2003). It is particularly interesting that the DKK1 receptor LRP5 is also one of the putative receptors for apolipoprotein E (ApoE), and that the genotype 4/4 is an established risk factor for late onset AD (Saunders et al., 2000; Rocchi et al., 2003). Whether ApoE4 binds with high affinity to LRP5 and mimics the action of DKK1 on the Wnt pathway is worthy of investigation. Finally, our findings encourage the search for DKK1 antagonist molecules to be tested as selective neuroprotective agents in experimental models of AD.
References


