Site-specific Phosphorylation of Tau Accompanied by Activation of Mitogen-activated Protein Kinase (MAPK) in Brains of Niemann-Pick Type C Mice*

Naoya Sawamura‡, Jian-Sheng Gong‡, William S. Garver§, Randall A. Heidenreich§, Haruaki Ninomiya†, Kousaku Ohno‡, Katsuhiko Yanagisawa‡, and Makoto Michikawa‡§

From the †Department of Dementia Research, National Institute for Longevity Sciences, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan, ‡Department of Pediatrics, The University of Arizona, Steele Memorial Children’s Research Center, Tucson, Arizona 85724, and §Department of Neurobiology, Tottori University, Faculty of Medicine, Yonago, Tottori 683, Japan

Niemann-Pick type C (NPC) disease is characterized by an accumulation of cholesterol in most tissues and progressive neurodegeneration with the formation of neurofibrillary tangles. Neurofibrillary tangles are composed of paired helical filaments (PHF), a major component of which is the hyperphosphorylated tau. In this study we used NPC heterozygous and NPC homozygous mouse brains to investigate the molecular mechanism responsible for tauopathy in NPC. Immunoblot analysis using anti-tau antibodies (Tau-1, PHF-1, AT-180, and AT-100) revealed site-specific phosphorylation of tau at Ser-396 and Ser-404 in the brains of NPC homozygous mice. Mitogen-activated protein kinase, a potential serine kinase known to phosphorylate tau, was activated, whereas other serine kinases such as glycogen synthase kinase-3β and cyclin-dependent kinase 5 were inactive. Morphological examination demonstrated that a number of neurons, the perikarya of which strongly immunostained with PHF-1, exhibited polymorphous cytoplasmic inclusion bodies and multi-concentric lamellar-like bodies. Importantly, the accumulation of intracellular cholesterol in NPC mouse brains was determined to be a function of age. From these results we conclude that abnormal cholesterol metabolism due to the genetic mutation in NPC1 may be responsible for activation of the mitogen-activated protein kinase-signaling pathway and site-specific phosphorylation of tau in vivo, leading to tauopathy in NPC.

Received for publication, October 25, 2000, and in revised form, December 12, 2000
Published, JBC Papers in Press, January 4, 2001
DOI 10.1074/jbc.M009733200

* This work was supported by Longevity Sciences Research Grant 8A-1, grants from Research on Brain Science from the Ministry of Health and Welfare, Japan, CREST (Core Research for Evolutional Sciences and Technology), Japan, and Ono Medical Research Foundation and Life Science Foundation of Japan, and by National Institutes of Health Grant DK56732. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Dementia Research, National Institute for Longevity Sciences, 36-3 Gengo, Morioka, Obu, Aichi 474-8522, Japan. Tel.: 81-562-46-2311; Fax: 81-562-44-6594; E-mail: michi@nils.go.jp.

‡ The abbreviations used are: NPC, Niemann-Pick type C; AD, Alzheimer’s disease; NPT, neurofibrillary tangle; PHF, paired helical filaments; ECL, enhanced chemiluminescence; MAPK, MAP kinase; GSK-3β, glycogen synthase kinase-3β; PBS, phosphate-buffered saline; RT, room temperature.

feature of NPC is the accumulation of low density lipoprotein-derived cholesterol due to a defect in the sorting/trafficking of cholesterol from lysosomes and late endosomes (1, 2). Neuro-pathologic examination has revealed neuronal distension, swollen axons, and polymorphous cytoplasmic bodies that react with the cholesterol binding reagent, filipin. Since low density lipoprotein-derived cholesterol is inaccessible to the brain and nervous system, the accumulation of cholesterol in neurons must be derived from an additional source. Recent studies using NPC fibroblasts have concluded that endogenously synthesized cholesterol can contribute to cholesterol accumulation as a result of the circulation of cholesterol between the plasma membrane and endosomal/lysosomal compartments (3).

The gene responsible for NPC, referred to as the Niemann-Pick C1 gene (NPC1), was mapped to a region of chromosome 18 in both human and mice and subsequently cloned (4). Although the function of NPC1 remains undefined, studies demonstrate a crucial role for this protein in cholesterol metabolism (5–7). NPC mice share many of the pathophysiological abnormalities observed in patients with NPC (8), including the accumulation of cholesterol in tissues and neurodegeneration marked by decreased Purkinje cell numbers. Use of the murine model for NPC has provided important insights into the role of NPC1 in cholesterol metabolism (4). In a previous study it was reported that NPC mice are asymptomatic at birth, with the earliest definitive symptoms of the disease apparent by 4–6 weeks of age and death ensuing by 10–15 weeks of age (8).

It has been shown that the brains of NPC patients with neurodegeneration have neurofibrillary tangles (NFTs) without amyloid deposits (9–11). Interestingly, the presence of NFTs, which are composed of paired helical filaments (PHF), is one of the diagnostic hallmarks of Alzheimer’s disease (AD) (12). A major component of PHF is tau, which is a microtubule-associated protein (13, 14). It has previously been shown that the phosphorylation of tau prevents it from binding to microtubules (15–19). Although the phosphorylation of tau in AD is the subject of intense investigation, the molecular mechanism responsible for this altered regulation remains to be defined. In this context, it is interesting to note that a perturbation in cholesterol metabolism and NFT formation without amyloid deposits coexist in the brains of NPC patients. This may indicate that a disturbance in cholesterol metabolism is responsible for tauopathy. We have recently demonstrated using cultured neurons that cholesterol deficiency results in axonal degeneration associated with microtubule depolymerization and hyperphosphorylation of tau (20). Therefore, it is important to inves-
Phosphorylation of Tau in NPC Brains

The phosphorylation state of tau in NPC brains may be associated with perturbed cholesterol metabolism in NPC brains.

Studies on the phosphorylation state of tau in the brains of NPC mice have not been performed. Evidence indicates that the defect in NPC1 function together with the perturbation of cholesterol metabolism may be an important tool for elucidating the pathways involved in the modulation of tau phosphorylation, NFT formation, and neurodegeneration. In this work, we have determined the phosphorylation state of tau in brains of BALB/c mice carrying the genetic mutation in NPC1. Our results demonstrate that tau is hyperphosphorylated at Ser-214, Ser-262, and Ser-396 and Ser-404. The elevation of tau phosphorylation in NPC results demonstrate that tau is hyperphosphorylated at Ser-214, Ser-262, and Ser-396 and Ser-404.

**EXPERIMENTAL PROCEDURES**

**NPC Mice**—BALB/c mice carrying the genetic mutation for NPC1 were obtained from The Jackson Laboratory (Bar Harbor, MA). These heterozygous mice were bred to acquire NPC (+/+) and NPC (+/−), and NPC (−/−) mice used for the study. The genotypes of the mice were determined from genomic DNA isolated from tail-snip DNA using a polymerase chain reaction-based method and oligonucleotide primers designed previously (4). Mice used in this study ranged from 6 to 12 weeks of age.

**Antibodies**—The monoclonal antibody Tau-1 was obtained from Chemicon International (Temecula, CA). The monoclonal antibody PHF-1 was kindly provided by Dr. P. Davies (Albert Einstein College of Medicine). The monoclonal antibodies AT-100 and AT-180 were purchased from Immunogenics (Ghent, Belgium). The rabbit polyclonal anti-NPC1 antibody was generated by immunizing rabbits with a MAPK peptide conjugate and purified from serum using peptide-specific affinity chromatography as previously described (21). Rabbit polyclonal anti-phospho-MAPK, rabbit polyclonal anti-phospho-independent-MAPK, and monoclonal anti-phospho-GSK-3β antibody were purchased from New England Biolabs (Beverly, MA). Rabbit anti-p35 antibody, which reacts with the p35 and p25 regulatory subunits of cyclin-dependent kinase 5, was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Fluorescein isothiocyanate-conjugated anti-mouse IgG and biotinylated goat anti-mouse IgG were purchased from Vector (Burlingame, CA).

**Protein Preparation**—Mouse tissues were homogenized in 10 volumes of Tris-saline (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), containing protease inhibitors (Complete®) and phosphatase inhibitors (10 mM NaF, 10 mM NaF, 10 mM sodium orthovanadate) using a motor-driven Teflon homogenizer. The homogenates were centrifuged at 3,000 × g for 10 min at 4 °C, and supernatants were saved for biochemical analyses. Protein concentrations were determined using the bichoncinic acid protein assay kit (Pierce). Aliquots of supernatant containing equal amounts of protein were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis for immunoblot analysis.

**Heat and Alkaline Phosphatase Treatment**—Supernatants containing equal amounts of protein were heat fixed to 95 °C for 10 min and clarified by centrifugation at 20,630 × g for 15 min. To each of these clarified heat-stable supernatants was added the same volume of saturated ammonium sulfate, and the mixtures were kept at 0 °C for 1 h. The 20,630 × g pellets were suspended in 50 mM Tris-HCl buffer, and alkaline phosphatase treatment was performed essentially as described elsewhere (22).

**Immunoblot Analysis**—Proteins separated using SDS-polyacrylamide gel electrophoresis were electrophoretically transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Nonspecific binding was blocked with 5% fat-free milk in phosphate-buffered saline containing 0.1% Tween 20. The blots were then incubated with primary antibodies overnight at 4 °C. For the detection of both monoclonal and polyclonal antibodies, appropriate peroxidase-conjugated secondary antibodies were used in conjunction with SuperSignal chemiluminescence (Pierce) to obtain images saved on film.

**Lipid Analysis**—The concentration of cholesterol and phospholipid in samples was determined using enzymatic methods. Cholesterol was determined using a cholesterol determination kit, PLBC (Wako, Osaka, Japan). Phospholipid determination kit, PLB (Wako, Osaka, Japan).
approximate 10-fold increase in the amount of cholesterol compared with NPC (+/+) mice (24–26) (Fig. 2b). Moreover, the concentration of phospholipids in the liver of 10–12 week old NPC (−/−) mice was 1.8-fold higher than those in NPC (+/−) and NPC (+/+ ) mice of the same age. The concentrations of phospholipids in the cerebrum and cerebellum were similar between the three genotypes.

In direct support of the increased amount of cholesterol measured in 10–12-week-old NPC (−/−) mice, filipin staining demonstrated an accumulation of cholesterol in neurons, particularly in the Purkinje cells of a NPC (−/−) mouse (Fig. 3b, arrows), that was not observed in the neurons from a NPC (+/+) mouse (Fig. 3a, arrowheads). Moreover, an electron micrograph of cerebellum sections from a 12-week-old NPC (−/−) mouse shows polymorphorous cytoplasmic bodies, loosely packed multi-concentric lamellar-like structures in a Purkinje cell, and a smaller type of neuron (Fig. 3, c and d). These features are characteristic of the intracellular accumulations of cholesterol in the brains of patients with NPC (27).

Several well characterized antibodies to tau were used to examine its presence in various tissues of mice from 10 to 12 weeks of age. The immunoblot analysis of tau using Tau-1 antibody, which recognizes dephosphorylated sites of tau at four closely located serine residues, Ser-195, Ser-198, Ser-199, and Ser-202 (28), shows that tau in the cerebrum, cerebellum, and liver was found to have apparent molecular masses between 50 and 70 kDa (Fig. 4a). For samples derived from NPC (−/−) and NPC (+/− ) mice, the main bands immunoreactive to Tau-1 appear to exhibit slower electrophoretic mobility than the bands for samples derived from NPC (+/+) mice (Fig. 4, a and b). These bands of tau exhibiting slower mobility are known to be characteristic of phosphorylated tau. The increased level of tau phosphorylation in NPC (−/−) mice was marked in the cerebellum, which is the most commonly affected region in NPC brains. Thus, we focused on the cerebellum to examine the phosphorylation state of tau. The antibodies used were the site-specific phospho-dependent antibodies PHF1, AT-180, and AT-100, which recognize the phosphorylated tau epitopes Ser-396/Ser-404, Thr-231, and Ser-214/Thr-217, respectively. Only the upper migrating band representing tau was strongly reactive to PHF-1 when samples from the cerebellum of NPC (−/−) mice were analyzed. This was not ob-
served when samples from the cerebella of NPC (+/-) and NPC (+/+ ) mice were analyzed (Fig. 4b), indicating that the Ser-396/Ser-404 sites were highly phosphorylated in NPC (-/- ) mice. In contrast, the intensity of the band reactive to AT-100 and AT-180 was stronger for NPC (+/+ ) mice than for NPC (+/-) and NPC (-/- ) mice. However, the mobility of the AT-100- and AT-180-reactive band for NPC (-/- ) mice was reduced to that of the band for NPC (+/+ ) and NPC (+/- ) mice (Fig. 4b).

Alkaline phosphatase treatment, which induces protein dephosphorylation, revealed a detailed pattern of the tau isoforms with significant shifts in electrophoretic mobility (Fig. 4c). After dephosphorylation, tau from the cerebellum was resolved into three major isoforms with apparent molecular masses of 62, 58, and 52 kDa (Fig. 4c). These three isoforms were detected in samples derived from each genotype. Importantly, the heat-stable supernatant of samples from the cerebellum of NPC (-/-) and NPC (+/-) mice showed similar results. These three isoforms marked the existence of three major subunits of tau in NPC mice.

Immunohistochemical analysis using PHF-1 was performed to determine whether tau phosphorylation was also evident in brain slices. Some of the cerebellum granular neurons (Fig. 5a) and cerebrum cortical neurons (Fig. 5c) in the brain slices of NPC (-/-) mice at 10 weeks of age were shown to be PHF-1 immunopositive, whereas those in the brain slice of NPC (+/+ ) mice were immunonegative (Fig. 5b), lending support to the results shown in Fig. 4b. We also performed electron microscopy to determine whether filaments with PHF exist in the neurons of the cerebrum and cerebellum. However, we could not detect PHF formation in these cells (data not shown).

To determine the molecular basis for increased tau phosphorylation, the expression and the phosphorylation state of several well known tau-directed protein kinases, including MAPK, GSK-3β, and p25, were determined. Immunoblot analysis using the anti-phospho-MAPK antibody, which recognizes only an activated form of MAPK, revealed that brains from 12-week-old NPC (-/-) mice have an 11-fold increase in MAPK activity in comparison with the brains of 12-week-old NPC (+/+ ) mice. Immunoblot analysis using the anti-phospho-GSK-3β antibody showed no alteration in the amount of the active GSK-3β in the brains from the three genotypes (Fig. 6a). The overall level of MAPK was similar for each of the three genotypes (Fig. 6b). Immunoblot analysis using the anti-phospho-GSK-3β antibody showed no alteration in the amount of the active GSK-3β in the brains from the three genotypes (Fig. 6a). Immunoblot analysis using the anti-p35 and p25 antibodies also showed no alteration in the conversion of p35 to p25 from the brains of the three genotypes (Fig. 6a). Together, these results indicate that neither GSK-3β nor p25 are responsible for increased tau phosphorylation. In contrast, markedly increased levels of MAPK activity, in conjunction
with increased phosphorylation of tau, suggest that the MAPK pathway may be responsible for tauopathy in NPC. Additionally, NPC(−/−) mice have increased MAPK activity in the cerebellum (3-fold) compared with NPC(+/+) and NPC(+/-) mice at 6 weeks of age (Fig. 6b); however, cholesterol accumulation was not found (Fig. 2a).

**DISCUSSION**

The mechanism by which NPC1 deficiency, associated with altered cholesterol metabolism, contributes to tauopathy remains undefined. In the present study, we have shown for the first time that (i) the accumulation of intracellular cholesterol in NPC mouse brains was a function of age, (ii) the levels of tau phosphorylation at Ser-396/Ser-404 are markedly increased, (iii) the increase in tau phosphorylation is marked in the cerebellum, which is the most commonly affected region of the brain in NPC, and (iv) MAPK is activated in the brains of NPC (−/−) mice.

Consistent with previous studies (1, 5, 24), our results have demonstrated that the concentration of cholesterol measured in the brains of NPC (−/−) mice at 6 weeks of age were not elevated compared with the brains of NPC (+/+) mice. However, using both biochemical and morphological analysis of NPC(−/−) mice, we demonstrate that the concentration of cholesterol measured in the brains of NPC (−/−) mice 10–12 weeks of age were elevated, similar to other organs (24–26). Cholesterol accumulation has previously been shown to occur as a result of lipoprotein-cholesterol uptake via the coated-pit pathway (25). But a recent study suggests that NPC cells can accumulate cholesterol in the absence of low density lipoprotein, indicating that endogenously synthesized cholesterol and plasma membrane cholesterol can contribute to the accumulation of lysosomal cholesterol (3). In any event, our results indicate that the accumulation of cholesterol is age-dependent. Cholesterol accumulation is detected in 10- to 12-week-old mice but not detected in 6-week-old (this study) or 7-week-old mice, as shown in other studies (24).

There are several possible mechanisms for the increased phosphorylation of tau at the PHF-1 epitope: (i) a decrease in the level of cholesterol at specific cellular compartments due to a defect in cholesterol trafficking, (ii) the accumulation of cholesterol and other lipids in the lysosomal/late endosomal compartment, or (iii) the direct result of a defect in NPC1 function. Based on the results of our study and those presented previously by other investigators, the evidence indicates that a decrease in the level of available cholesterol due to a defect in cholesterol trafficking is responsible for tau phosphorylation in the brains of NPC (−/−) mice. Previous studies show that, despite an accumulation of cellular cholesterol, the rate of cholesterol synthesis and the expression of low density lipoprotein receptors is not down-regulated (24). Moreover, the esterification of excess cholesterol at the endoplasmic reticulum is delayed as a result of cholesterol not being able to gain access to pools responsible for maintaining intracellular cholesterol homeostasis (26, 29). Consistent with this hypothesis, our results suggest that additional cellular compartments may be cholesterol-deficient and, therefore, directly responsible for inducing tau phosphorylation and microtubule depolymerization in the axons of cultured neurons (20).

Cholesterol depletion of plasma membrane caveolae have been shown to cause the activation of MAPK (30). Consistent with these results, we have found that MAPK is activated in cholesterol-deficient neurons and that NPC fibroblasts have significantly decreased levels of caveolae cholesterol (data not shown). In the present study, we demonstrate that MAPK activity is elevated in the brains of NPC (−/−) mice not only at 10–12 weeks of age but also at 6 weeks of age, indicating that the activation of MAPK precedes the accumulation of cholesterol. Since tau is known to be a substrate for MAPK and that Ser-396/Ser-404, but not Ser-214, Thr-217, or Thr-231 sites, are phosphorylated by MAPK (31–33), the assumption that MAPK is responsible for tau phosphorylation in NPC (−/−) brains would well explain our present results that tau was phosho-
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J. Biol. Chem. 2001, 276:10314-10319.
doi: 10.1074/jbc.M009733200 originally published online January 4, 2001

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