Endophilin I Expression Is Increased in the Brains of Alzheimer Disease Patients*

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Alzheimer patients have increased levels of both the 42 amyloid-β-peptide (Aβ) and the amyloid binding alcohol dehydrogenase (ABAD), which is an intracellular binding site for Aβ. The overexpression of Aβ and ABAD in transgenic mice has shown that the binding of Aβ to ABAD results in amplified neuronal stress and impairment of learning and memory. From a proteomic analysis of the brains from these animals, we have identified for the first time that the protein endophilin I increases in Alzheimer diseased brain. The increase in endophilin I levels in neurons is linked to an increase in the activation of the stress kinase c-Jun N-terminal kinase with the subsequent death of the neurons. We also demonstrate in living animals that the expression level of endophilin I is an indicator for the interaction of ABAD and Aβ as its expression levels return to normal if this interaction is perturbed. Therefore this identifies endophilin I as a new indicator of the progression of Alzheimer disease.

Little is known about the early stages of Alzheimer disease before amyloid plaques are formed, although studies have indicated that there is synaptic loss before the formation of plaques (1). The intracellular production of Aβ may be the earliest event occurring in Alzheimer disease, and the identification of early markers for the presence of toxic Aβ would have strong implications for the prevention and treatment of Alzheimer disease (2).

Amyloid-β binding alcohol dehydrogenase (ABAD) is a 27-kDa intracellular binding partner for Aβ at nm concentrations (3). In the absence of Aβ, ABAD can supply energy for the brain by facilitating utilization of ketone bodies, fatty acids, alcohols, and hydroxysteroids (4–7). Alzheimer disease patients have increased expression of ABAD in Alzheimer disease–affected regions, as do transgenic animals that overexpress Aβ (4, 8). Indeed, overexpression of both ABAD and Aβ leads to apoptosis only if the ABAD is catalytically active (3). The crystal structure of ABAD (Protein Data Bank (PDB) code 1 SO8) provided the first three-dimensional structure of an internal binding site for Aβ. This region consists of a 20-amino-acid region close to the active site of the enzyme. A 20-amino-acid peptide of this Aβ binding domain of ABAD, fused to the cell membrane transduction domain of the human immunodeficiency virus Tat protein, was able to block Aβ-induced toxicity (8).

ABAD is predominately localized in mitochondria (3, 5, 8) whose functions are impaired in Alzheimer disease brains (9, 10). Also, transgenic animals expressing both ABAD and a mutated form of the amyloid precursor protein (Tg mAPP/ABAD), resulting in an increase in intracellular Aβ, have an enhanced neuronal cytotoxicity with subsequent changes in spatial learning memory (8). This is via mitochondrial dysfunction because the neurons from these animals show spontaneous generation of reactive oxygen species, a decrease in ATP levels, and the release of cytochrome c from mitochondria (10).

Previously, we identified peroxiredoxin II as a protein whose expression increased within the brains isolated from Tg mAPP/ABAD animals and Alzheimer disease patients. We showed that the physiological role of this increase appears to be the protection of neurons from Aβ-induced toxicity (11). Significantly, we also showed that if the interaction of Aβ with ABAD was perturbed, then these molecular changes are reversed in living animals, thus indicating that ABAD is a suitable target for the treatment of Alzheimer disease (11).

Here we report the identification of a second protein that is increased within the brains isolated from Tg mAPP/ABAD animals and Alzheimer disease patients. This protein is endophilin I, which has not been previously linked to Alzheimer disease but has been shown to influence c-Jun N-terminal kinase (JNK) activity (12). We demonstrate in primary cortical neurons that an increase in endophilin I leads to an increase in JNK activation with the subsequent death of these neurons. Additionally, expression of truncated versions of endophilin I blocked Aβ-induced JNK activation and toxicity. We also show that if the interaction of Aβ with ABAD is perturbed, then these molecular changes are reversed in living animals, thus indicating that endophilin I, like peroxiredoxin II, can act as a marker for the

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interaction of Aβ and ABAD and hence is a suitable target for the treatment of Alzheimer disease.

**EXPERIMENTAL PROCEDURES**

Transgenic Animals and Human Material—Transgenic mice with neuronal targeted expression of ABAD (Tg ABAD) were generated under the control of platelet-derived growth factor-B chain promoter. Transgenic mice expressing a mutant form of human amyloid precursor protein (a minigene encoding hAPP695 and 751 and hAPP70 bearing V717F, K670M/ N671L; Tg mAPP mice, J-20 line) were provided by Dr. Mucke (13). Tg ABAD mice were crossed with Tg mAPP mice to generate double Tg ABAD/mAPP. Tg mice for our studies were in the C57BL/6 background. Characterization of these animals was described in the previous studies (8, 10). Human Alzheimer disease brain tissues were obtained from Columbia Alzheimer’s Disease Research Center brain banks.

Proteomics—Brains from Tg ABAD/mAPP, Tg ABAD, and non-Tg littermates were dissolved in 8 M urea, 2 M thio-urea, 4% CHAPS, 50 mM dithiothreitol, 1% immobilized pH gradient buffer, pH 3–10 (Amersham Biosciences), 40 mM Tris base, protease inhibitor mixture (Sigma). Soluble proteins were purified from this extract by sonication followed by centrifugation at 4 °C at 17,000 × g, 45 mins. From each brain, ~50 mg of soluble proteins was produced. For analysis of protein expression differences, 100 µg of protein was separated in the first dimension by isoelectric focusing on 18-cm, pH 4–7, immobilized pH gradient strips (Amersham Biosciences) before second dimension separation on large 23 × 23 cm 10% polyacrylamide gels (100 V overnight). Proteins were initially visualized by silver staining. To identify proteins, replica gels were run with larger amounts of protein loaded (1 mg). These gels were stained with SyproRuby (Genomic Solutions), and gel images were captured using a fluorescent scanner. The protein spots were excised and subjected to in-gel trypsinization, and masses of the resulting peptides were determined by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry analysis. Proteins were then identified by peptide mass fingerprinting by searching appropriate web-based data base programs (MS-Fit at the ProteinProspector web site).

Western Blot and Immunocytochemistry Analysis—Brain homogenates from Tg animals or Alzheimer disease patients were loaded on a 12% polyacrylamide gel and transferred to nitrocellulose membrane. Immunoblotting was performed by rabbit endophilin I (Zymed Laboratories Inc.) and followed by goat anti-rabbit peroxidase conjugated IgG. The immunoreactive bands were detected using a Lumi-Light kit (Roche Applied Science). Paraffin-embedded sections were stained with rabbit anti-endophilin I (1:50) followed by the secondary antibodies (goat anti-rabbit IgG conjugated with peroxidase, Sigma). Sites of antigens were detected by 3-amino-9-ethylcarbazole chromogen (Sigma).

Preparation of Transfected Embryonic Mouse Cortical Culture—Primary neuronal cultures of embryonic age 14 CD1 wild-type mice were generated following standard protocols. The cerebral cortex was dissected, and 25 mg of trypsin (Worthington Biochemical) was added for 4 min. Following DNase (30 units) addition, the cells were gently triturated to prepare a single cell suspension, and cell number was obtained using a hemacytometer. Neurons were nucleofected with 2 µg of DNA (endophilin I full-length, endophilin I Δ-SH3, endophilin I GFP-SH3, kindly provided by Dr. P McPherson (Montreal Neurological Institute, McGill University)), using the Amaza mouse neuron nucleofector kit (Amaza Biosystems) according to the manufacturer’s instructions. Neurons were seeded onto a 96-well plate (Nunc) precoated with 50 mg/ml poly D-lysine (Sigma) at a density of 200 cells/well or 35-mm Petri dishes precoated with poly D-lysine at a density of 500,000 cells/well. 24 h later, medium was replaced by serum-free medium with B27 supplement (Invitrogen). Following a further 72 h, 50 µM aged Aβ peptide (14) was added to experimental wells.

For cell counting experiments, 24 h after Aβ treatment, cells were fixed and 4′,6-diamidino-2-phenylindole-stained. The total number of live cells (as assessed by 4′,6-diamidino-2-phenylindole staining) per well at the completion of the trial was counted. Each experiment was performed three times. In each experiment, control and Aβ-treated cultures were set up in triplicate.

For immunoblotting, 8 h after Aβ treatment, neurons were harvested in cell lysis buffer (20 mM Tris(pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 µg/ml leupeptin, 1 mM phenylmethysulfonyl fluoride), sonicated for four times for 5 s, and then centrifuged for 15 min at 13,000 rpm, 4 °C. The protein concentration of the supernatant was determined using Bradford’s reagent. Equal amounts of lysate protein were run on a 4–12% precast Bis-Tris gel (Invitrogen) and transferred to nitrocellulose membranes. Nitrocellulose blots were blocked with 3% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with primary antibodies (anti-phospho-specific-JNK, anti-JNK (Cell Signaling)) at a dilution of 1:2000 overnight at 4 °C in Tris-buffered saline containing 0.1% Tween 20 containing 3% bovine serum albumin. After a 1-h incubation with horseradish peroxidase secondary antibodies (1:5000, Bio-Rad), the membranes were subjected to ECL reagents (Pierce) for immunoreactivity detection. Each experiment was repeated three times. All results are expressed as means and standard errors, and the statistical tests were analyzed by analysis of variance using STATVIEW program.

**RESULTS**

Proteomics Analysis of Transgenic Brains—Five-month-old Tg mAPP/ABAD mice show significant behavioral changes and deficits in mitochondrial function as compared with single transgenic animals (8, 15). To maximize the potential changes in protein levels, we performed our initial proteomic analysis on animals of 8 months of age as single Tg mAPP mice also show behavioral changes at this age (16). Whole brains were analyzed from three mice of each genotype (matched for age and sex). The three genotypes analyzed were animals expressing ABAD (Tg ABAD), mAPP, and ABAD (Tg mAPP/ABAD) and non-transgenic litter mates (non-Tg) and have been previously described (8, 15). Proteins were extracted and analyzed as detailed before (11) and under “Experimental Procedures.” All samples were run in duplicate. The two-dimensional gels of the
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Two-dimensional separation of proteins from the total brain extract indicating the identified endophilin I protein isolated from transgenic animals expressing ABAD (Tg ABAD), mAPP, and ABAD (Tg mAPP/ABAD) or non-Tg littermate controls.

Increased Expression of Endophilin I in Tg mAPP/ABAD and Alzheimer Disease Brains—An increase in endophilin I expression has not been reported before for any neurodegenerative disease, although it had been previously predicted (17). Western blot analysis confirmed a consistent up-regulation of endophilin I in the Tg mAPP/ABAD transgenic animals as compared with the Tg ABAD transgenic animals or the non-transgenic littermate controls (Fig. 1). By mass spectrometry, this protein was identified as the Src homology 3 (SH3) domain protein 2A endophilin I (gene: SH3GL2) (Swiss Prot Accession No Q62420). Importantly, the predicted molecular weight and pI value (molecular weight 39,877, pI 5.3, endophilin I) of this protein corresponded favorably to the observed molecular weight (molecular weight 42,000, pI 5.4, endophilin I).

Increased Expression of Endophilin I in Alzheimer Disease Patient and Non-demented Age Matched Control—Endophilin I was predominately present in the neurons of temporal cortex of Alzheimer patients as compared with non-demented age matched controls (ND, n = 6). The right panel indicates quantification of the intensity of immunoreactive bands for endophilin I by NIH image program, n = 5/group. B, immunocytochemistry of endophilin I from temporal cortex of Alzheimer disease patient and non-demented age matched control. The right panel of C shows intensities of immunostaining for endophilin I by quantification with the Universal Image program. Scale bar = 50 μm.

To assess whether the observed increase in endophilin I expression was relevant in Alzheimer disease, we next screened human brain tissue. By both Western blot analysis (Fig. 3A) and immunocytochemistry (Fig. 3B), we observe that there was a consistent increase in endophilin I expression in the temporal cortex of Alzheimer patients as compared with non-demented age matched controls. Immunostaining with specific antibody to endophilin I showed that increased expression of endophilin I was predominately present in the neurons of temporal cortex of Alzheimer disease brain. An altered level of endophilin I suggests its significance in the Alzheimer disease pathogenesis.

Increased Endophilin I Expression Leads to Activation of JNK and a Subsequent Decrease in Neuronal Viability—Endophilin I has been previously linked to JNK activation in the HEK293 cell line where an increase in endophilin I expression led to an increase in JNK activity; additionally, expression of truncated versions of endophilin I (Fig. 4A) led to the blocking of the activation of JNK (12). We confirmed that this also occurs in...
primary cortical neuronal cultures as expression of full-length endophilin I led to an increase in JNK activation as compared with transfected controls (Fig. 4B). The addition of Aβ has been previously shown to activate JNK activity in cortical neurons (14). Notably, in this report, when one of the two differing truncated versions of endophilin I were expressed in these neurons, then JNK activation was blocked even in the presence of toxic levels of Aβ (Fig. 4B).

The activation of JNK has long been associated with a decrease in neuronal viability (18, 19). Therefore as part of the experiments, we additionally counted the number of neurons that survived this process. The results reflect those that were found for the JNK phosphorylation. We saw significant decreases in neuronal cell viability when full-length endophilin I was expressed or with the addition of Aβ in cortical neurons (Fig. 4C). Additionally, the expression of the two truncated versions of endophilin I resulted in an increase in survival of neurons even in the presence of toxic levels of Aβ (Fig. 4C). Thus it would appear that the increased levels of endophilin I may be partly responsible for the increased levels of JNK activation previously observed in the brains of Alzheimer disease sufferers (20).

**Perturbing the Interaction of Aβ with ABAD Results in a Reversal in Expression of Endophilin I in Tg mAPP Animals**—The solving of the crystal structure of Aβ bound to ABAD indicates that residues 94–114 of ABAD interact with Aβ. A peptide spanning this region of ABAD binds to ABAD-Aβ interaction in vitro and protects primary neuronal cultures from Aβ-induced cytotoxicity (8). This peptide has been modified further by the addition of the cell membrane transduction domain of the human immunodeficiency virus-type 1 Tat protein fused with a mitochondrial matrix protein-targeting sequence and the region of ABAD between residues 93 and 116 to produce Tat-mito-ABAD-DP. This chimeric peptide was then successfully used in living animals by intraperitoneal injection to reverse the increased expression of peroxiredoxin II, which is observed in Tg mAPP animals (11). This leads to a subsequent improvement in the learning and memory of the transgenic animals (21). Therefore to determine whether endophilin I expression is also directly due to the interaction of Aβ and ABAD, we performed the same experiments as detailed previously (11), injecting intraperitoneally either the forward (Tat-mito-ABAD-DP) or the reverse peptides (Tat-mito-ABAD-RP) into Tg mAPP/ABAD animals. Western blot analysis of the proteins from hippocampus of Tg mAPP/ABAD animals of 8 months of age showed a significant increase in the expression level of endophilin I as compared with non-transgenic animals using a β-actin expression control. Tg mAPP/ABAD animals treated with the reverse peptide (Tat-mito-ABAD-RP) also show an elevated level of endophilin I (Fig. 5). However, in those animals that were treated with Tat-mito-ABAD-DP, the expression of endophilin I was significantly decreased (Fig. 5). These data confirm that the increased expression of endophilin I is a result of the interaction of Aβ and ABAD and confirms that the Tat-mito-ABAD peptide disrupts this interaction.

**DISCUSSION**

Intracellular Aβ can elicit toxic events by binding to the mitochondrial protein ABAD (8, 15). Alzheimer patients show an increase in the expression levels of both Aβ and ABAD with
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their subsequent binding, leading to mitochondrial dysfunction and the death of neurons (8, 10, 15). Indeed, transgenic animals with elevated levels of both Aβ and ABAD show learning and memory deficits as early as 4 months of age as compared with transgenic animals expressing elevated levels of solely Aβ or ABAD or non-transgenic animals (8). This is thought to be the result of an increase in the oxidative stress by reactive oxygen species within the brains of these animals (8, 15).

Previously, we performed a proteomic analysis of the brains from transgenic animals that were expressing both mAPP and ABAD (Tg mAPP/ABAD) (11). Despite the previously observed cellular dysfunction of Tg mAPP/ABAD animals (15), the overall protein levels were not greatly altered, but we did identify peroxiredoxin II as a protein that was consistently up-regulated in the Tg mAPP/ABAD transgenic animals and the brains of Alzheimer disease patients (11). The apparent function of this increase of this antioxidant protein appeared to be an attempt at protecting neurons from Aβ toxicity (11).

The discovery that within the brains of Tg mAPP/ABAD animals and Alzheimer sufferers, the levels of protein expression for endophilin I are increased is a novel observation. Endophilin I is a cytoplasmic SH3 domain-containing protein, which is present in presynaptic nerve termini (22). Through its SH3 domains, it can bind to other endocytic proteins including synaptojanin and dynamin and hence is known to have a role in synaptic vesicle formation (17). Endophilin I can also complex with signaling molecules including cell surface receptors (23) and the germinal center kinase-like kinase (12). In particular, previous studies have shown that overexpression of endophilin I activates JNK activation in HEK293 cells, whereas expression of truncated parts of endophilin I blocked this activation (12).

Increases in JNK activity have been long associated with Alzheimer disease. It is activated by the addition of Aβ to neuronal cultures (14, 18, 19, 24, 25), it translocates to the nucleus following the course of severity of the disease (26), and it can phosphorylate tau (27, 28), but the mechanisms by which it is activated in vivo remain poorly understood (29, 30). Our studies and those of Ramjaun et al. (12) suggest a potential new signaling pathway contributing to the activation of JNK in Alzheimer disease. Expression of endophilin I in cortical neurons caused a significant increase in JNK activation with a subsequent decrease in neuronal viability. There was also no apparent synergistic increase in JNK phosphorylation when endophilin I and Aβ were added together. This may reflect the maximal activity within this experiment, and this is also suggested from the survival data. Additionally, as was previously reported in HEK293 cells (12), the expression of the truncated versions of endophilin I significantly prevented the activation of JNK with a subsequent increase in neuronal viability, even in the presence of Aβ. Notably, both truncated versions with and without the SH3 domain had the ability to prevent inactivation of JNK. Future studies will determine how this might occur, but it is possible to speculate that prevention of relocation of JNK and/or upstream activators may be responsible, as occurs with the JNK-interacting protein (31). It is also possible to speculate that endophilin I may also have other roles in Alzheimer disease. Indeed, other binding partners for endophilin I include SH3-domain GRB2-like (endophilin) interacting protein 1, which has been identified as a neuronal protein that regulates energy balance (32). A change in the overall energy balance is known to be an important factor in the pathogenesis of Tg mAPP/ABAD animals and also in patients with Alzheimer disease (10). More recently, endophilin I has been shown to increase the production of APP (33). Future studies will explore this new signaling pathway and its involvement in Alzheimer disease and will require the production of further transgenic animals as these events occur over longer periods of time than are achievable from cell culture of embryonic or neonatal neuronal cultures.

Not only is it important to identify factors that underlie the cause of Alzheimer disease, but it would be advantageous if they also reflect its progression. Previously, we identified peroxiredoxin II as one such potential marker. This is because the intraperitoneal injection into Tg mAPP animals of a decoy peptide comprising of ABAD residues 93–116, coupled to a mitochondrial matrix-targeting sequence and a Tat cell-permeating sequence, resulted in the reversal of the increased expression levels of peroxiredoxin II (11). Therefore the reversal of expression to normal levels of endophilin I by the use of the same Tat-mito-ABAD blocking peptide is the identification of another potential factor. It also confirms that the binding of Aβ to ABAD results in increases in endophilin I expression. The actual mechanism of how this occurs and why is unknown. However, it is possible to speculate as it is known that the mitochondria within Tg mAPP/ABAD and Alzheimer patients are under stress due to this interaction, which leads to leakage of reactive oxygen species and enhances CypD translocation, triggering open mitochondrial membrane permeability transition...
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pore (9, 10). The consequence of these events would be to activate signal transduction pathway in the cytosol that leads to increases in endophilin I. Future studies will explore whether this is a general stress response or whether it is specific to Alzheimer disease. As endophilin I is involved in synaptogenesis (17), one possibility is that the increase in endophilin I is an attempt to maintain synaptic connectivity, which is known to be decreased in Alzheimer disease. Our results also confirm previous finding that the prevention of Aβ binding to ABAD is a suitable drug target, as opposed to the production of inhibitors to ABAD, which essentially will have the same effect as the addition of Aβ as this will result in inhibiting the activity of the enzyme (34, 35).

In summary, we have identified proteins that are elevated in Alzheimer disease and remain elevated even after death. Notably, the increases in expression of our identified proteins have differing actions as peroxiredoxin II is an antioxidant, whereas endophilin I will contribute to the toxicity observed in the death of neurons from Alzheimer disease patients (Fig. 6). Our findings suggest that an abnormality for endophilin I, which had previously been predicted (17), has been found, and future studies will investigate the role of this novel signaling pathway further and whether it is involved in other neurodegenerative diseases.

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