primer, 5′-GCGCCTGTTGCTGGTGAT; mouse parkin probe, 5′-AGACAAGGACACGTCGGTAGCTTTGAACCT; mouse Glup forward primer, 5′-GCTTTCTGAAATGACGTTTCCC; mouse Glup priming and reverse primer, 5′-CCGTGTTCCAGCATGTCG; mouse Glup probe, 5′- TGAGTTTTTTGCTCGGCGAGGAATCC. The primers and probe of mouse GAPDH were used from rodent GAPDH control reagent (Applied Biosystems, Warrington, UK). The validity of GAPDH as a housekeeping gene was confirmed by no significant change in mRNA during each stress treatment.

Western blot

Each mouse tissue (C57Black/6, 4 weeks old) was dissected, and homogenized with a Teflon homogenizer in tissue lysis buffer (20 m*M* Tris-HCl, pH 7.5, containing 120 m*M* NaCl, 5 m*M* EDTA, 1% Triton-X 100) with Complete protease inhibitors cocktail (Roche, Mannheim, Germany). Twenty μg of soluble protein from each tissue was subjected to Western blot analysis. Cultured cells were lysed in cell lysis buffer (20 m*M* HEPES, pH 7.4, containing 120 m*M* NaCl, 5 m*M* EDTA, 10% glycerol, 1% Triton X-100) with Complete protease inhibitors cocktail. Soluble cell lysate was analyzed by Western blot analysis with ECL detection reagents (Amersham Bioscience, Buckinghamshire, UK).

RESULTS

Upregulation of parkin is a cell type-specific response to ER stress

To confirm whether induction of parkin is specifically induced by ER stress, we evaluated the effect of a variety of cytotoxic agents on the transcription of parkin or Glup in SH-SY5Y (H) cells. Using primers specific for parkin, Glup, and GAPDH, mRNA levels of parkin in SH-SY5Y (H) cells cultured under various stress conditions for indicated time were measured by quantitative RT-PCR (Fig. 1). The PCR product for each mRNAs formed a single band (Fig. 1A). Consistent with our previous report, treating cells with the reducing agent 2-mercaptoethanol (2-ME; 7.5 m*M*) or the *N*-glycosylation inhibitor tunicamycin (Tunica; 10 μg/ml), both of which are known to cause ER stress, resulted in a significant increase of parkin mRNA levels at 12 and 24 h. In contrast, treatment with the proteasome inhibitor MG-132 (20 μ*M*) or short wavelength ultraviolet light (UV; 40 J/m^2) failed to alter the mRNA level of parkin. Concomitant with upregulation of parkin mRNA, Glup mRNA also increased upon 2-ME or tunicamycin treatment, consistent with the fact that these genes share a common promoter and are co-regulated. However, TCP1α mRNA, which increased upon proteasome inhibition, was unaltered upon treatment with 2-ME or tunicamycin, indicating that parkin or Glup upregulation is specifically increased in response to ER stress in SH-SY5Y (H) cells (Fig. 1B).

In addition to SH-SY5Y (H) cells, 2-ME and tunicamycin also increased parkin and Glup mRNAs in several other neuronal cell lines, including Neuro2a cells (Fig. 1C) and GOTO-P3 cells (Fig. 1D). However, no change was observed in IMR 32 cells (Fig. 1E). Given the different response by parkin alteration upon 2-ME or tunicamycin treatment, our results indicate that upregulation of parkin upon ER stress is cell-type specific.

We then performed Western blot analysis to determine if ER stress also altered parkin expression at the protein level. Consistent with the observation of increased parkin and Glup mRNAs, the parkin and Glup protein expression levels were increased during the ER stress tests in SH-SY5Y (H) (Fig. 2A). The parkin protein was also slightly increased upon MG-132 treatment (Fig. 2A). Given that parkin mRNA level is unaltered and parkin protein is degraded through proteasome, increased parkin protein might be due to decreased degradation of parkin by proteasome inhibition. Heat shock proteins Hsp70 and BiP were also increased upon treatment with ER stress inducers, whereas Hsc70 and HDJ-2 proteins showed no or minor changes (Fig. 2A). Although MG-132 treatment significantly induced TCP1α transcript (Fig. 1B), the protein level remains unaltered (Fig. 2A). Concomitant with ER stress-induced increases in parkin and Glup mRNAs, their protein levels were also increased in Neuro2a cells (Fig. 2B). Although both 2-ME and tunicamycin significantly induced parkin and Glup mRNAs in GOTO-P3 (Fig. 1D), increased parkin and Glup protein levels were only observed in 2-ME treatment (Fig. 2C). Unexpectedly, rather than increases, decreases in parkin and Glup proteins were observed upon tunicamycin treatment (Fig. 2C). Reduction of Glup protein was observed in tunicamycin-treated IMR32 cells, whereas an increase was observed upon 2-ME treatment (Fig. 2D), although both tunicamycin and 2-ME had no effect on Glup mRNA levels (Fig. 1E). An truncated band of parkin was observed in IMR32, and treatment with 2-ME for 24 h caused an increase in this truncated molecule (Fig. 2D), possibly derived from cleavage of full length parkin by activated caspases (14, 15).

Two kinds of SH-SY5Y cell lines demonstrate different characters

Since parkin is differently regulated upon ER stress in a panel of cell lines studied, existence of conflicting data regarding the upregulation of parkin upon tunicamycin treatment in SH-SY5Y cells prompted us to investigate whether this difference is due to different features of SH-SY5Y, possibly derived from different manipulations (such as maintenance or passage conditions) between different laboratories. We therefore compared the effects of a variety of stress inducers on parkin expression in two SH-SY5Y cell lines, which we obtained from two different laboratories. Both 2-ME and tunicamycin treatment increased parkin mRNA level, whereas hydrogen peroxide (H_2O_2; 600 μ*M*), high osmolarity (0.3 *M* sorbitol), the DNA alkylating agent (methyl methanesulfonate (MMS); 100 μg/ml), UV radiation, and heat shock (HS; 42°C, 1 h) had little or no effect in SH-SY5Y(H) cells. However, SH-SY (J) cells responded differently: Dramatic reduction in the parkin mRNA level was observed under UV radiation, whereas 2-ME and tunicamycin had no obvious effect (Fig. 3A). We surmised the different responsiveness might be due to distinct features between these two kinds of SH-SY5Y cells. The effects of

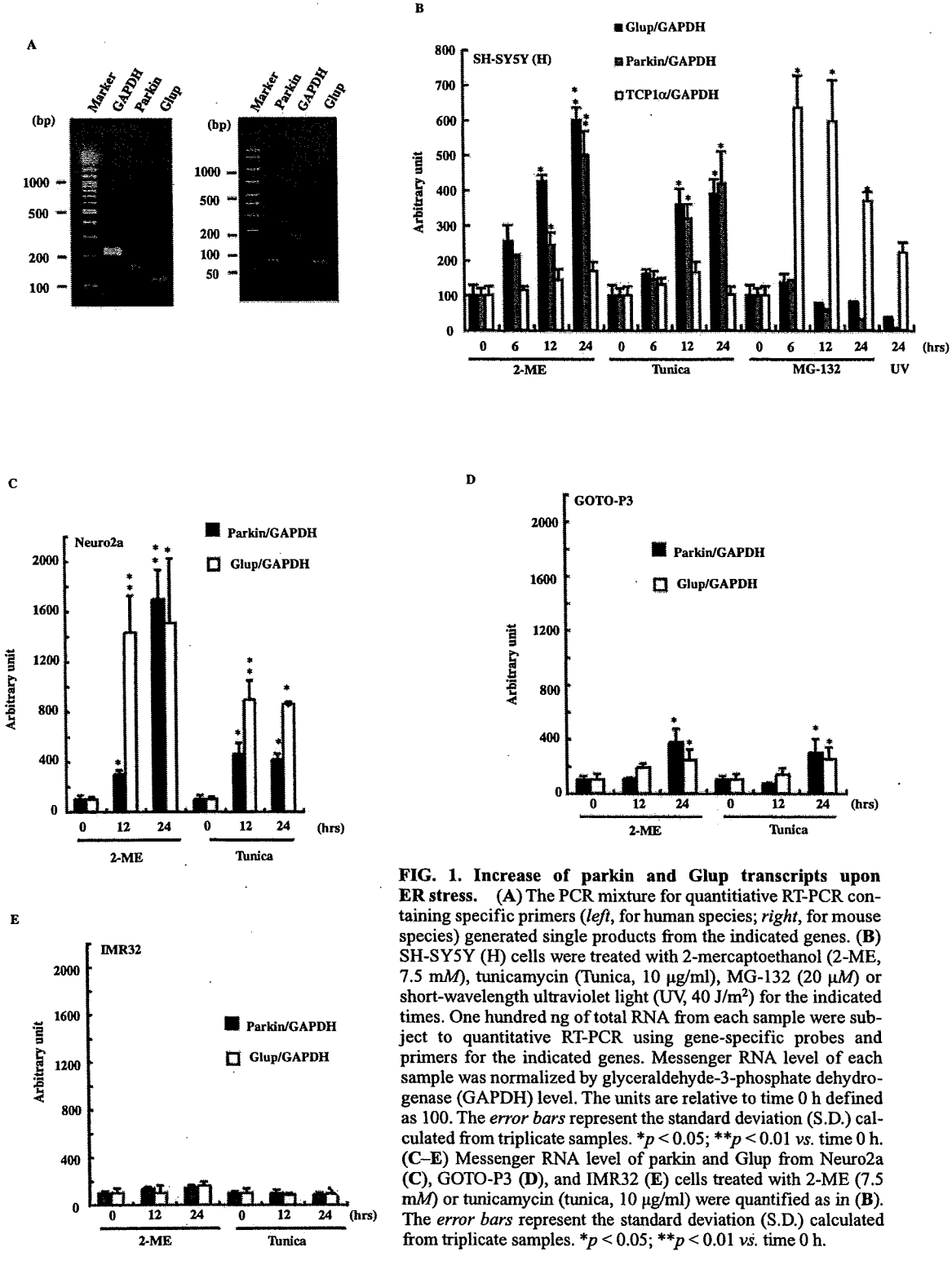

FIG. 1. Increase of parkin and Glup transcripts upon ER stress. (**A**) The PCR mixture for quantitiative RT-PCR containing specific primers (*left*, for human species; *right*, for mouse species) generated single products from the indicated genes. (**B**) SH-SY5Y (H) cells were treated with 2-mercaptoethanol (2-ME, 7.5 m*M*), tunicamycin (Tunica, 10 μg/ml), MG-132 (20 μ*M*) or short-wavelength ultraviolet light (UV, 40 J/m^2) for the indicated times. One hundred ng of total RNA from each sample were subject to quantitative RT-PCR using gene-specific probes and primers for the indicated genes. Messenger RNA level of each sample was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level. The units are relative to time 0 h defined as 100. The *error bars* represent the standard deviation (S.D.) calculated from triplicate samples. $*p < 0.05$; $**p < 0.01$ *vs.* time 0 h. (**C–E**) Messenger RNA level of parkin and Glup from Neuro2a (**C**), GOTO-P3 (**D**), and IMR32 (**E**) cells treated with 2-ME (7.5 m*M*) or tunicamycin (tunica, 10 μg/ml) were quantified as in (**B**). The *error bars* represent the standard deviation (S.D.) calculated from triplicate samples. $*p < 0.05$; $**p < 0.01$ *vs.* time 0 h.

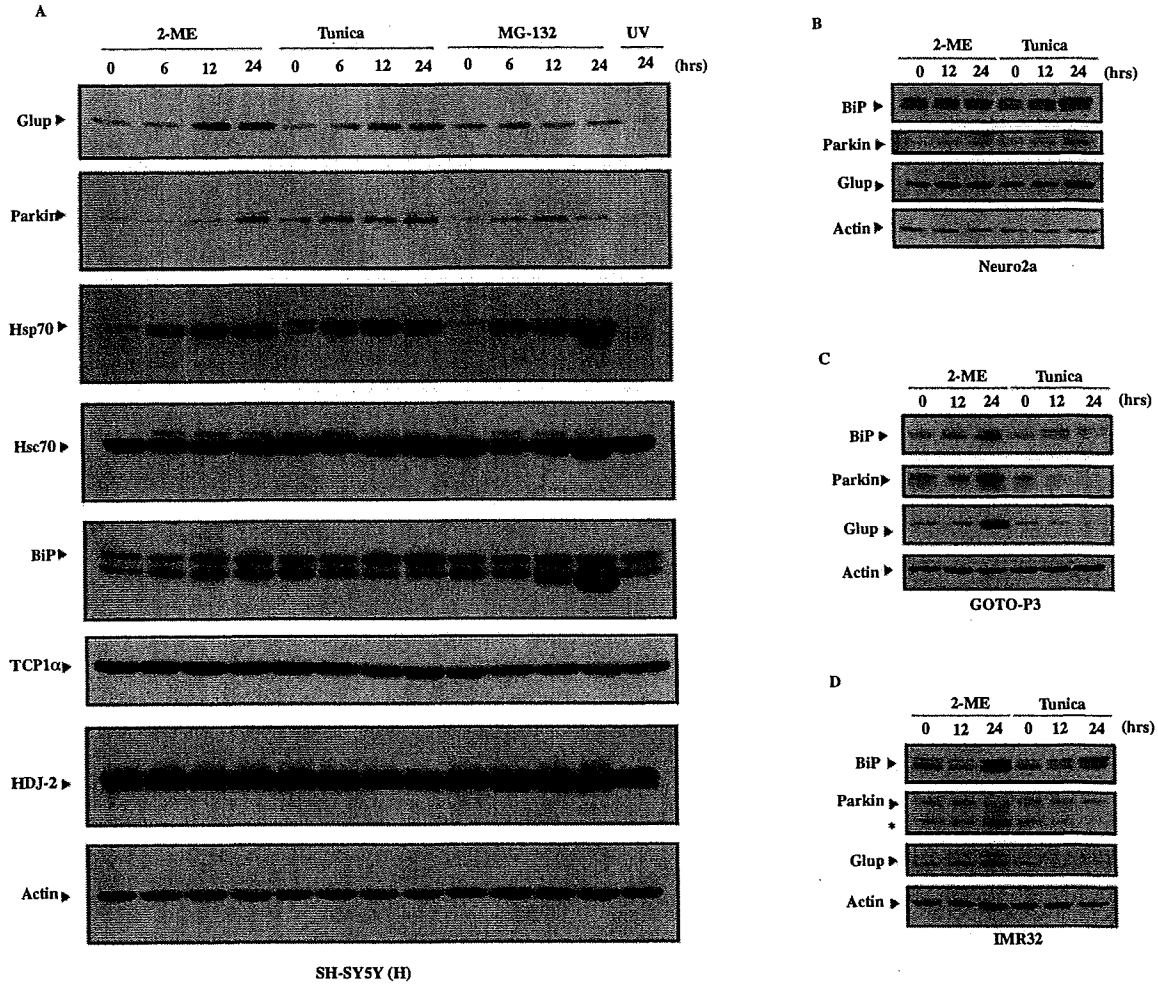

FIG. 2. Protein levels of parkin and Glup upon ER stress. (**A**) Total cell lysates from SH-SY5Y (H) cells in Fig. 1B were analyzed by Western blotting with antibodies against the indicated proteins. ER stress response was assessed by the induction of BiP. Total protein level of each sample was confirmed by the amount of actin. (**B–D**) Total cell lysate from various neural cell lines [(**B**) Neuro2a; (**C**) GOTO-P3; (**D**) IMR32 cells] loaded with ER stress (2-ME, 7.5 m*M*; Tunica, 10 μg/ml) for the indicated times, were analyzed by Western blotting as in (**A**). *An asterisk* in (**D**) indicates the putative processed molecules of parkin.

various differentiation reagents on these cells were therefore investigated. Tyrosine hydroxylase (TH), a dopaminergic neuronal differentiation marker of SH-SY5Y cells, was dramatically induced upon addition of 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 160 n*M*) or N^6, 2′-*O*-dibutyryladenosine-3′:5′-cyclic monophosphate (dcAMP, 1 m*M*) in SH-SY5Y (J) cells. In contrast, SH-SY5Y (H) cells were quite refractory to differentiation into dopaminergic neuronal phenotype, only a slight increase of TH was observed under the same conditions (Fig. 3B).

Increased parkin and Glup mRNAs upon ER stress are not due to direct transcriptional activation

Quantitative RT-PCR demonstrated increased parkin and Glup mRNA levels upon ER stress in SH-SY5Y (H) cells. However, it was not clear whether the increase in parkin mRNA was due to transcriptional or post-transcriptional regulation. We then measured the transcriptional activity of the 500 bp parkin core promoter using luciferase reporter gene assays. The parkin promoter has been found to function as a bidirectional promoter, regulating not only transcription of *parkin*, but also transcription of *Glup*, a gene located 204 bp upstream of and antisense to the *parkin* gene (Fig. 4A). Promoter constructs were transfected into SH-SY5Y (H) cells (Fig. 4B, left) or Neuro2a cells (Fig. 4B, right) and luciferase activity was assayed for basal levels and in response to 2-ME or tunicamycin. Both the parkin promoter vector and the Glup promoter vector failed to show any significant alteration in transcription levels relative to vehicle treated controls (Fig. 4B). This suggested that the increase in parkin and Glup mRNA levels observed in earlier experiments may not be due to direct transcriptional activation of the core promoter region of *parkin* or *Glup*.

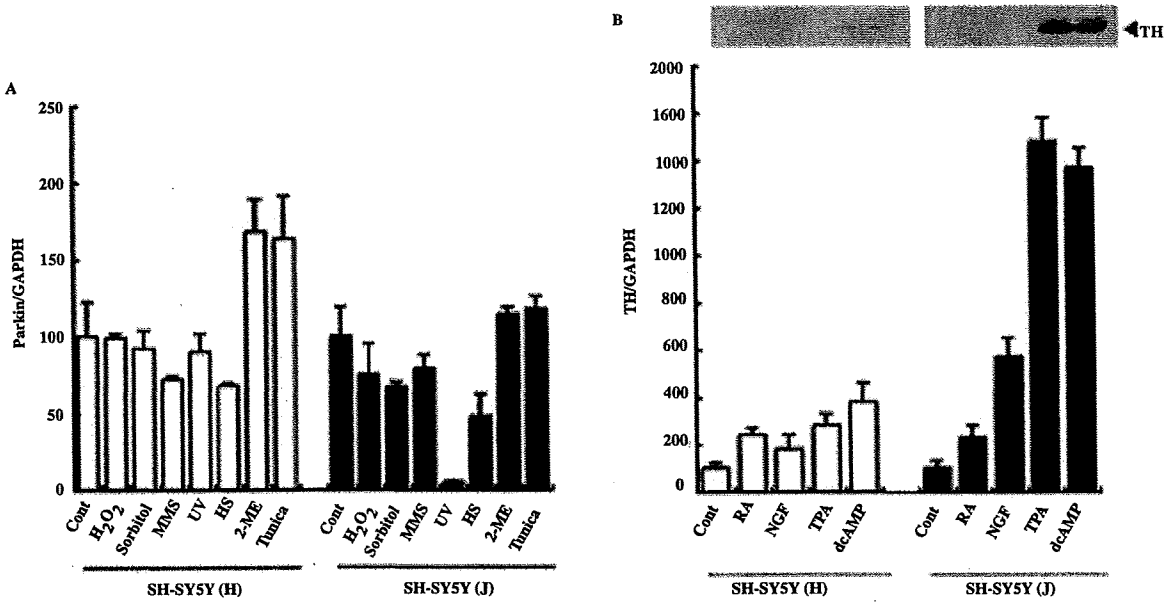

FIG. 3. Characterization of two kinds of SH-SY5Y cell lines. (A) SH-SY5Y (H) and (J) cells were treated with a variety of stresses [hydrogen peroxide (H_2O_2, 600 μ*M*), high osmolarity (sorbitol, 0.3 *M*), a DNA alkylating agent methyl methanesulfonate (MMS, 100 μg/ml), short-wavelength ultraviolet light (UV, 40 J/m^2), and heat shock (42°C for 1 h)]. After 5 h, total RNA was extracted and subject to quantitative RT-PCR as in Figure 1B. The *error bars* represent the standard deviation (S.D.) calculated from triplicate samples. (**B**) SH-SY5Y (H) and (J) cells were cultured with media with 5% horse serum and 5% bovine fetal calf serum containing retinoic acid (RA, 10^{-6} *M*), nerve growth factor-2.5S (NGF, 100 ng/ml), 12-*O*-tetradecanoylphorbol 13-acetate (TPA, 160 n*M*), or N^6, 2′-*O*-dibutyryladenosine-3′:5′-cyclic monophosphate (dcAMP, 1 m*M*). These media were exchanged every 2 days. After 7 days, mRNA level of parkin normalized with GAPDH (Graph) was assessed as in Fig. 1B, and protein level of tyrosine hydroxylase (TH) was analyzed by Western blotting. The *error bars* of the graph represent the standard deviation (S.D.) calculated from triplicate samples.

Tissue distribution of mouse parkin and Glup proteins

Mouse parkin and Glup protein levels were measured in various tissues using Western blot analysis. Mouse Glup protein is mainly expressed in central nervous systems, with almost no protein expression in periphery tissues except for bladder and testis. On the other hand, parkin protein is rather ubiquitously expressed compared with Glup, abundant in central nervous systems, stomach, kidney, thymus, bladder, and testis (Fig. 5). These distinct tissue distribution patterns of parkin and Glup suggest that the protein expression levels of these proteins are regulated differently, although they share the same core promoter.

DISCUSSION

Parkin is generally thought to be neuroprotective due to its role in the ubiquitin proteasome system. Parkin functions as an E3 ligase, targeting specific substrate proteins for degradation by the proteasome. It is conceivable that accumulation of some proteins, which cannot properly degraded because of the absence of parkin, causes ER dysfunction and ER stress. Several studies have focused on the regulation of endogenous parkin in the presence of ER stress. However, there is great disagreement in the literature regarding the expression of parkin upon ER stress. Our previous studies have shown that both parkin mRNA and protein levels were increased upon ER stress in SH-SY5Y cells (11). Higashi *et al.* (5) also demonstrated that parkin protein, as well as ER chaperones BiP and PDI, was upregulated in SH-SY5Y cells upon exposure to manganese (Mn) and overexpression of parkin protected cells from Mn-induced cell death. However, West *et al.* (32) demonstrated a lack of association between parkin and ER stress in SH-SY5Y cells. On the other hand, Mengesdorf *et al.* (21) reported that parkin mRNA was increased upon exposure to thapsigargin in rat cortical neuron cultures, whereas they failed to observe increased parkin protein level. Finally, Ledesma *et al.* (18) showed that when astrocytes and neurons were exposed to conditions associated with ER stress, a selective increase of parkin was observed in rat astrocytes but not hippocampal neurons. These apparent discrepancies prompted us to clarify the association between parkin and ER stress.

In our present studies, we have used 2-ME and tunicamycin to determine if they might result in any alterations in the transcription of parkin in a panel of cell lines. We show here that treatment with ER stress inducers results in upregulation of parkin and Glup at both mRNA and protein levels in a cell

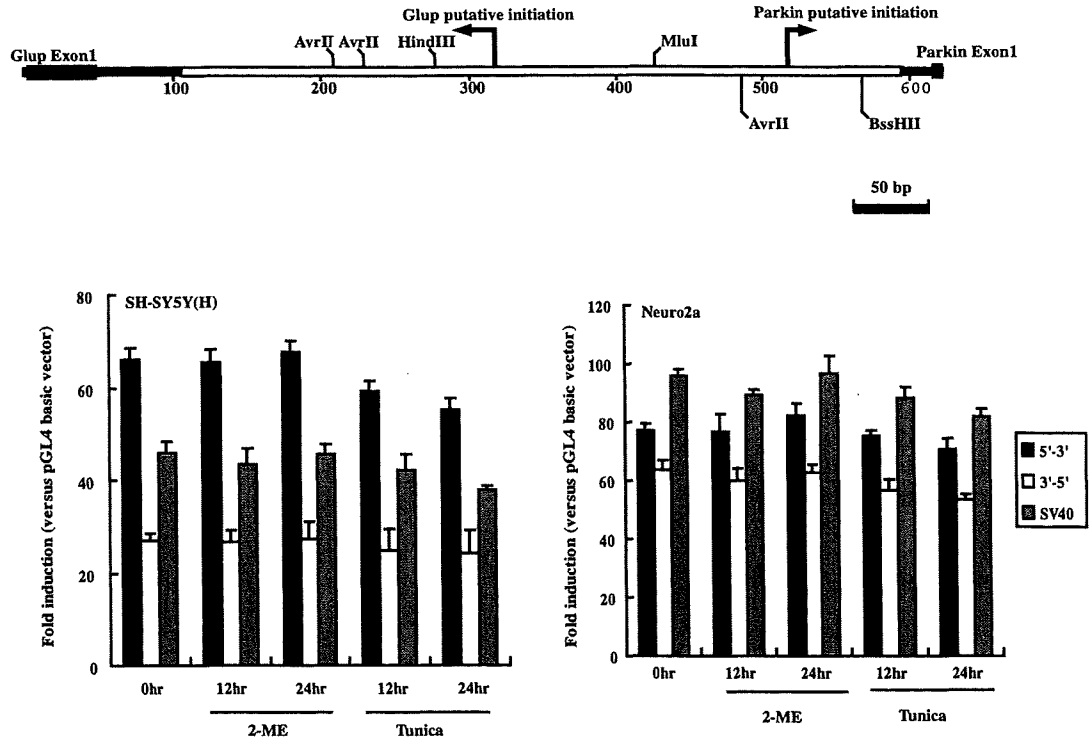

FIG. 4. Parkin and Glup are co-regulated with a bi-derectional promoter in a reporter assay. (**A**) A diagram of the human genomic region between the first exons of Parkin and Glup genes. The sequence used in this reporter assay is shown as an *open box*. (**B**) SH-SY5Y (H) (*left*) and Neuro2a (*right*) cells transfected with reporter plasmids for luciferase (pGL4) driven by the indicated promoters (5′-3′, the region as shown in **A** inserted in the sense direction to parkin gene; 3′-5′, the region as shown in **A** in the sense direction to Glup gene, SV40, SV40 promoter; Basic, without a promoter) and a reporter plasmid for *Renilla* luciferase driven with a TK promoter (phRluc-TK, Promega) were treated with various reagents (2-ME, 7.5 m*M*; Tunica, 10 μg/ml) for the indicated times. The luciferase activity normalized with transfection efficiency by *Renilla* activity was shown by fold induction *vs.* pGL4 basic vector. The *error bars* represent the standard deviation (S.D.) calculated from triplicate samples.

type-specific manner. The function of Glup is not clearly known, but there is some evidence that it may play a role in protection against stress-induced cell death (10, 33). It is noted that some novel components of the UPR that regulates the canonical UPR are expressed in a cell type-specific fashion, suggesting that different cells may have unique responses for adaptation to ER stress (17, 23, 36). The present study therefore suggests that upregulation of parkin and Glup might represent as cell type-specific adaptation to ER stress. Moreover, the parkin and Glup protein levels do not always reflect their mRNA levels, suggesting a complicated mechanism by which regulates their transcription, translation and/or degradation in different types of cells upon ER stress.

Upregulation of parkin or Glup transcripts upon ER stress is not ascribable to direct transcriptional activation since the 500 bp promoter region shared by parkin and Glup fails to confer transcriptional activation upon ER stress as assessed by luciferase assays. A previous report showed that the parkin promoter region containing 4,500 bp sequence 5′ to parkin exon 1 was unresponsive to ER stress, consistent with our results. The increased parkin or Glup mRNA levels might be derived from the result of post-transcriptional events, such as increased mRNA stability and/or decreased mRNA turnover. Alternatively, given the bulk of introns in the *parkin* gene, some regulatory elements outside our luciferase constructs might affect parkin transcription upon ER stress, which should be clarified in the future study.

The cell lines used in this study are all derived from neuroblastomas with neuronal properties. IMR 32 is a neuroblastoma cell line derived from human. The culture of IMR 32 is a mixture of two morphologically distinct cell types: the pre-

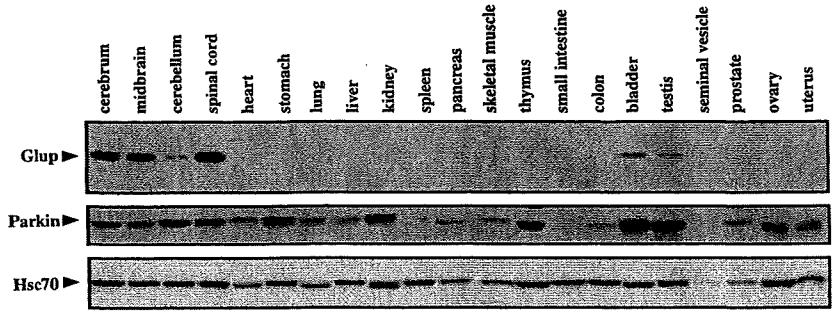

FIG. 5. Tissue distribution of parkin and Glup protein. Mouse various tissue samples were analyzed with Western blot for parkin, Glup, and Hsc70.

dominant one is a small neuroblast-like cell and the other is a large hyaline fibroblast-like cell (31). GOTO-P3 has been established in tissue culture from a human neuroblastoma arising from the adrenal grand. The cells of GOTO-P3 are small and fibroblast-like (26). SH-SY5Y is a thrice-cloned subline of bone marrow biopsy-derived line SK-N-SH, both epithelial-like and neuroblast-like morphologies have been reported (2, 3, 13). In our hands, SH-SY5Y (H) cells are epithelial-like, whereas SH-SY5Y (J) cells demonstrate small neuroblast-like. Neuro2a is a mouse neuroblastoma line established from the spontaneous tumor of *albino* mouse strain. The cells are neuronal and ameboid-like (24).

The presence of conflicting data regarding different parkin alteration upon ER stress in the "same" SH-SY5Y cells prompted us to further characterize two SH-SY5Y cell lines, which we obtained from two different laboratories. Actually, these two cell lines demonstrated different properties among manipulation. SH-SY5Y (H) but not SH-SY5Y (J) responded to ER stress by upregulation of parkin. On the other hand, SH-SY5Y (H) was more refractory to induced differentiation compared with SH-SY5Y (J), strongly suggesting that SH-SY5Y (H) and SH-SY5Y (J) are different in their properties. It is thus conceivable that conflicting data observed previously in SH-SY5Y cells might be due to different properties of SH-SY5Y cells, possibly acquired in the process of maintenance in individual laboratories.

It remains unclear how ER stress causes upregulation of parkin in a subset of these neuroblastomas with neuronal properties. It is noted that upregulation of parkin was not observed in IMR32 as well as in SH-SY5Y (J) cells. Like SH-SY5Y (J) cells, IMR 32 has been demonstrated readily to differentiate into dopaminergic neuronal phenotype, since TH proteins were already detectable upon addition of TPA for only 3 days (19). Morphologically, parkin appeared to be upregulated in predominantly fibroblast-like SH-SY5Y (H), GOTO-P3, and Neuro2a cells, but not in predominantly neuroblast-like SH-SY5Y (J) and IMR32 cells. Consistent with the data from Ledesma *et al.* (18), our current experiments suggest that upregulation of parkin may not occur in response to ER stress in neurons.

It is likely that certain cell-type specific ER stress related molecules affect parkin expression. Recently it has been reported that OASIS (old astrocyte specifically induced substance) is a novel ER stress transducer that specifically regulates UPR signaling in astrocytes (7, 17). Although we examined whether OASIS is responsible for the cell type-specific upregulation of parkin upon ER stress, only negative results were obtained (data not shown). Although parkin and Glup share the same promoter, the tissue distribution patterns of these proteins are different, suggesting the presence of cell type- or tissue-specific regulators for parkin and Glup protein expression (Fig. 5). Further work is necessary to examine the mechanisms whereby the upregulation of parkin occurs specifically in certain types of cells upon ER stress.

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ABBREVIATIONS

AR-JP, autosomal recessive juvenile parkinsonism; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; ERAD, ER associated degradation; FCS, fetal calf serum; Glup, gene adjacent to parkin; 2-ME, 2-mercaptoethanol; Mn, manganese; OASIS, old astrocyte specifically induced substance; PACRG, parkin coregulated gene; PCR, polymerase chain reaction; PD, Parkinson's disease; RT, reverse transcript; TH, tyrosine hydroxylase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; UPR, unfolded protein response.

REFERENCES

1. Abbas N, Lucking CB, Ricard S, Durr A, Bonifati V, De Michele G, Bouley S, Vaughan JR, Gasser T, Marconi R, Broussolle E, Brefel-Courbon C, Harhangi BS, Oostra BA, Fabrizio E, Bohme GA, Pradier L, Wood NW, Filla A, Meco G, Denefle P, Agid Y, and Brice A. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe. French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. *Hum Mol Genet* 8: 567–574, 1999.

2. Biedler JL, Helson L, and Spengler BA. Morphology and growth, tumorigenicity, and cytogenetics of human neuroblastoma cells in continuous culture. *Cancer Res* 33: 2643–2652, 1973.
3. Biedler JL, Roffler-Tarlov S, Schachner M, and Freedman LS. Multiple neurotransmitter synthesis by human neuroblastoma cell lines and clones. *Cancer Res* 38: 3751–3757, 1978.
4. Cookson MR. The biochemistry of Parkinson's disease. *Annu Rev Biochem* 74: 29–52, 2005.
5. Higashi Y, Asanuma M, Miyazaki I, Hattori N, Mizuno Y, and Ogawa N. Parkin attenuates manganese-induced dopaminergic cell death. *J Neurochem* 89: 1490–1497, 2004.
6. Holtz WA and O'Malley KL. Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. *J Biol Chem* 278: 19367–19377, 2003.
7. Honma Y, Kanazawa K, Mori T, Tanno Y, Tojo M, Kiyosawa H, Takeda J, Nikaido T, Tsukamoto T, Yokoya S, and Wanaka A. Identification of a novel gene, OASIS, which encodes for a putative CREB/ATF family transcription factor in the long-term cultured astrocytes and gliotic tissue. *Brain Res Mol Brain Res* 69: 93–103, 1999.
8. Imai Y, Soda M, Hatakeyama S, Akagi T, Hashikawa T, Nakayama KI, and Takahashi R. CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol Cell* 10: 55–67, 2002.
9. Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, and Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* 105: 891–902, 2001.
10. Imai Y, Soda M, Murakami T, Shoji M, Abe K, and Takahashi R. A product of the human gene adjacent to parkin is a component of Lewy bodies and suppresses Pael receptor-induced cell death. *J Biol Chem* 278: 51901–51910, 2003.
11. Imai Y, Soda M, and Takahashi R. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J Biol Chem* 275: 35661–35664, 2000.
12. Imai Y and Takahashi R. How do Parkin mutations result in neurodegeneration? *Curr Opin Neurobiol* 14: 384–389, 2004.
13. Jalava AM, Heikkila J, Akerlind G, Pettit GR, and Akerman KE. Effects of bryostatins 1 and 2 on morphological and functional differentiation of SH-SY5Y human neuroblastoma cells. *Cancer Res* 50: 3422–3428, 1990.
14. Kahns S, Kalai M, Jakobsen LD, Clark BF, Vandenabeele P, and Jensen PH. Caspase-1 and caspase-8 cleave and inactivate cellular parkin. *J Biol Chem* 278: 23376–23380, 2003.
15. Kahns S, Lykkebo S, Jakobsen LD, Nielsen MS, and Jensen PH. Caspase-mediated parkin cleavage in apoptotic cell death. *J Biol Chem* 277: 15303–15308, 2002.
16. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, and Shimizu N. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392: 605–608, 1998.
17. Kondo S, Murakami T, Tatsumi K, Ogata M, Kanemoto S, Otori K, Iseki K, Wanaka A, and Imaizumi K. OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes. *Nat Cell Biol* 7: 186–194, 2005.
18. Ledesma MD, Galvan C, Hellias B, Dotti C, and Jensen PH. Astrocytic but not neuronal increased expression and redistribution of parkin during unfolded protein stress. *J Neurochem* 83: 1431–1440, 2002.
19. Mangoura D, Theofilopoulos S, Karouzaki S, and Tsirimonaki E. 12-O-tetradecanoyl-phorbol-13-acetate-dependent up-regulation of dopaminergic gene expression requires Ras and neurofibromin in human IMR-32 neuroblastoma. *J Neurochem* 97 Suppl 1: 97–103, 2006.
20. Mata IF, Lockhart PJ, and Farrer MJ. Parkin genetics: one model for Parkinson's disease. *Hum Mol Genet* 13: R127–133, 2004.
21. Mengesdorf T, Jensen PH, Mies G, Aufenberg C, and Paschen W. Down-regulation of parkin protein in transient focal cerebral ischemia: A link between stroke and degenerative disease? *Proc Natl Acad Sci USA* 99: 15042–15047, 2002.
22. Moore DJ, West AB, Dawson VL, and Dawson TM. Molecular pathophysiology of Parkinson's disease. *Annu Rev Neurosci* 28: 57–87, 2005.
23. Nagamori I, Yabuta N, Fujii T, Tanaka H, Yomogida K, Nishimune Y, and Nojima H. Tisp40, a spermatid specific bZip transcription factor, functions by binding to the unfolded protein response element via the Rip pathway. *Genes Cells* 10: 575–594, 2005.
24. Olmsted JB, Carlson K, Klebe R, Ruddle F, and Rosenbaum J. Isolation of microtubule protein from cultured mouse neuroblastoma cells. *Proc Natl Acad Sci USA* 65: 129–136, 1970.
25. Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, and Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. *J Neurosci* 22: 10690–10698, 2002.
26. Sekiguchi M, Oota T, Sakakibara K, Inui N, and Fujii G. Establishment and characterization of a human neuroblastoma cell line in tissue culture. *Jpn J Exp Med* 49: 67–83, 1979.
27. Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K, and Suzuki T. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat Genet* 25: 302–305, 2000.
28. Smith WW, Jiang H, Pei Z, Tanaka Y, Morita H, Sawa A, Dawson VL, Dawson TM, and Ross CA. Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant alpha-synuclein-induced toxicity. *Hum Mol Genet* 14: 3801–3811, 2005.
29. Takahashi R and Imai Y. Pael receptor, endoplasmic reticulum stress, and Parkinson's disease. *J Neurol* 250 Suppl 3: III25–29, 2003.
30. Takahashi R, Imai Y, Hattori N, and Mizuno Y. Parkin and endoplasmic reticulum stress. *Ann NY Acad Sci* 991: 101–106, 2003.
31. Tumilowicz JJ, Nichols WW, Cholon JJ, and Greene AE. Definition of a continuous human cell line derived from neuroblastoma. *Cancer Res* 30: 2110–2118, 1970.
32. West AB, Gonzalez-de-Chavez F, Wilkes K, O'Farrell C, and Farrer MJ. Parkin is not regulated by the unfolded protein response in human neuroblastoma cells. *Neurosci Lett* 341: 139–142, 2003.
33. West AB, Lockhart PJ, O'Farell C, and Farrer MJ. Identification of a novel gene linked to parkin via a bi-directional promoter. *J Mol Biol* 326: 11–19, 2003.
34. Yamamuro A, Yoshioka Y, Ogita K, and Maeda S. Involvement of endoplasmic reticulum stress on the cell death induced by 6-hydroxydopamine in human neuroblastoma SH-SY5Y cells. *Neurochem Res* 31: 657–664, 2006.
35. Yang Y, Nishimura I, Imai Y, Takahashi R, and Lu B. Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in *Drosophila*. *Neuron* 37: 911–924, 2003.
36. Zhang K, Shen X, Wu J, Sakaki K, Saunders T, Rutkowski DT, Back SH, and Kaufman RJ. Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. *Cell* 124: 587–599, 2006.

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Forum Review

Expanding Insights on the Involvement of Endoplasmic Reticulum Stress in Parkinson's Disease

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ABSTRACT

Parkinson's disease (PD) is the second most common neurodegenerative disease characterized by selective loss of dopaminergic neurons and the presence of Lewy bodies. The pathogenesis of PD remains incompletely understood. Environmental factors, oxidative damage, misfolded protein aggregates, ubiquitin–proteasome system impairment, and mitochondrial dysfunction might all be involved. Recent studies point to activation of endoplasmic reticulum (ER) stress-mediated cell death linked to PD. Accumulation of unfolded and/or misfolded proteins in the ER lumen induces ER stress. To withstand such potentially lethal conditions, intracellular signaling pathways collectively termed the unfolded protein responses (UPR) are activated. The UPR include translational attenuation, induction of ER resident chaperones, and degradation of misfolded proteins through the ER-associated degradation. In case of severe and/or prolonged ER stress, cellular signals leading to cell death are activated. Accumulating evidence suggests that ER stress induced by aberrant protein degradation is implicated in PD. Here the authors review the emerging role of ER stress in PD and related disorders, and highlight current knowledge in this field that may reveal novel insight into disease mechanisms and help to provide novel avenues to potential therapies. *Antioxid. Redox Signal.* 9, 553–561.

INTRODUCTION

PARKINSON'S DISEASE (PD) is the most common neurodegenerative movement disorder among elderly people. The classical symptoms of the disease include rigidity, resting tremor, bradykinesia, and postural instability. The pathological hallmarks underlying the clinical phenotypes are characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc), together with the presence of intraneuronal inclusions termed Lewy bodies (7). Although the molecular mechanisms underlying neurodegeneration remain elusive, its pathogenesis begins to be considered as a multifactorial cascade of deleterious factors. Mitochondrial dysfunction, protein aggregation, impairment of the ubiquitin–proteasome system (UPS), and activation of the stress kinase signaling pathways have been supposed to be involved in the pathogenesis of PD. Recently, emerging lines of evidence from familial forms of PD, coupled with those findings from toxin-induced PD models, raise the possibility of widespread involvement of unfolded protein responses [UPR, also known as endoplasmic reticulum (ER) stress responses], the term given to an imbalance between the cellular demand for ER function and ER capacity (2, 43, 44), in the pathogenesis of this disease.

Neuronal loss in both familial and sporadic forms of neurodegenerative disorders is often accompanied by formation of inclusion bodies and aggregation of misfolded proteins (45). Upregulation of ER stress markers has been observed in postmortem brain tissues and cell culture models of many neurodegenerative diseases including PD, Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS), and expanded polyglutamine diseases such as Huntington's disease and spinocerebellar ataxias (4, 26). Several chaperones ameliorate the accumulation of misfolded proteins triggered by oxidative or nitrosative stress, or of mutated gene products (26, 40, 58). The hypothesis that ER dysfunction plays an important role in the development of dopaminergic neuronal

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loss in PD has recently been put forward by observations that parkin has been associated with ER stress-induced cell death. Mutations in the *PARK2* gene coding for parkin cause autosomal recessive juvenile Parkinsonism (AR-JP), the most common form of familial PD. This review summarizes new observations implying that impairment of ER functioning is a common denominator of neuronal death in PD.

ER STRESS AND ER STRESS RESPONSES

Besides calcium storage and signaling, a central function of the ER is quality control for membrane or secretory proteins, which comprise nearly one-third of all cellular proteins (29). The importance of the ER for normal cell function is highlighted by the observation that blocking of the protein folding or processing reactions can be lethal for cells. Indeed, in various cases such as depletion of ER calcium stores, blocking the proteasome that is required for degradation of unfolded proteins, or genetic mutations resulting in proteins that cannot be properly folded, the ER functions are impaired and unfolded proteins accumulate in the ER. Accumulation of unfolded proteins in the ER is a severe form of stress that will induce apoptosis if ER function cannot be restored. To cope with conditions associated with impairment of ER function, cells activate highly conserved stress response, the UPR (2, 43, 44). The main purpose of UPR is to remove aberrant substrates and restore the ER to an efficiently operating maturation compartment. The UPR pathway functions as a tripartite signal that comprises (i) inhibition of general translation to attenuate the load of proteins to the ER, (ii) transcriptional activation of ER chaperones to increase protein folding and processing capacity; (iii) activation of ER-associated degradation (ERAD) to promote degradation of terminally misfolded proteins. However, when the ER stress is severe or prolonged, the cells eventually activate apoptotic signals, leading to cell death (5, 29) (Fig. 1).

Cells have developed two pathways for removing unfolded proteins from the lumen of the ER, increasing folding capacity through upregulation of ER chaperones (Fig. 2A) and promoting degradation of terminally misfolded proteins through activation of ERAD (Fig. 2B). The ERAD pathway is

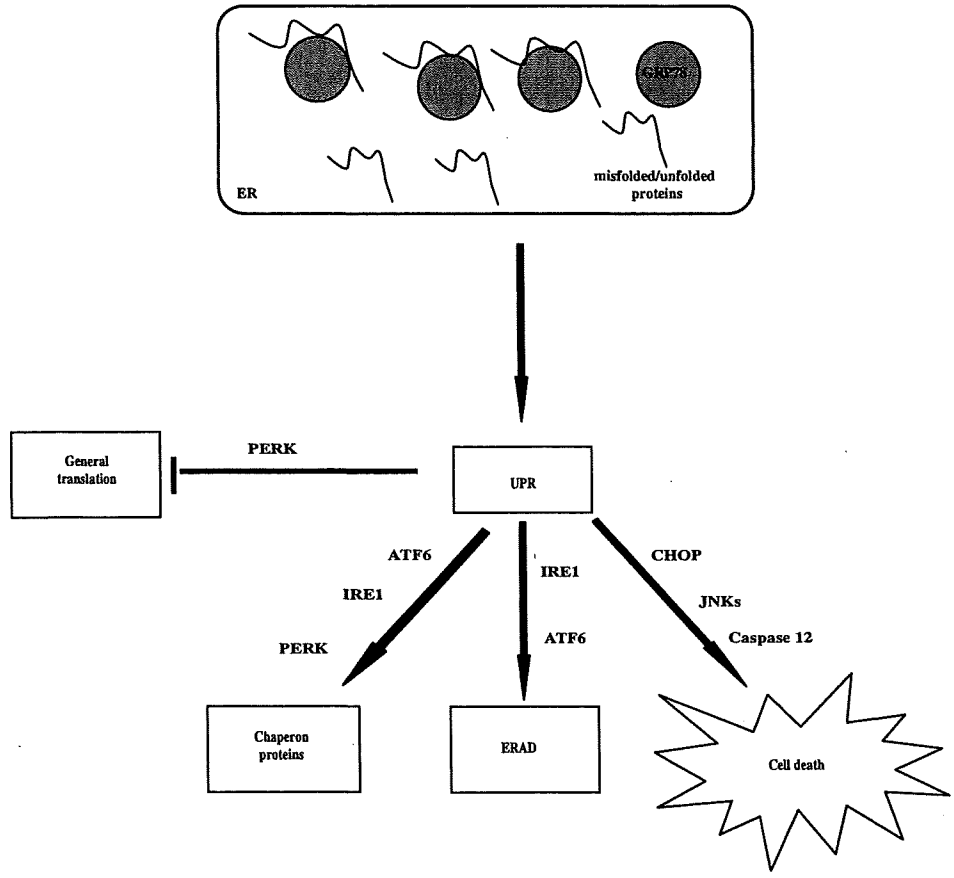

FIG. 1. The tripartite unfolded protein response. Three primary transducers of the unfolded protein response (UPR) signal, known as ATF6, IRE1, and PERK, seek to relieve ER stress through suppression of translational initiation, increased folding capacity of ER, and degradation of terminally misfolded proteins until the aberrations have been alleviated. However, severe or prolonged ER stress eventually activates apoptotic pathway.

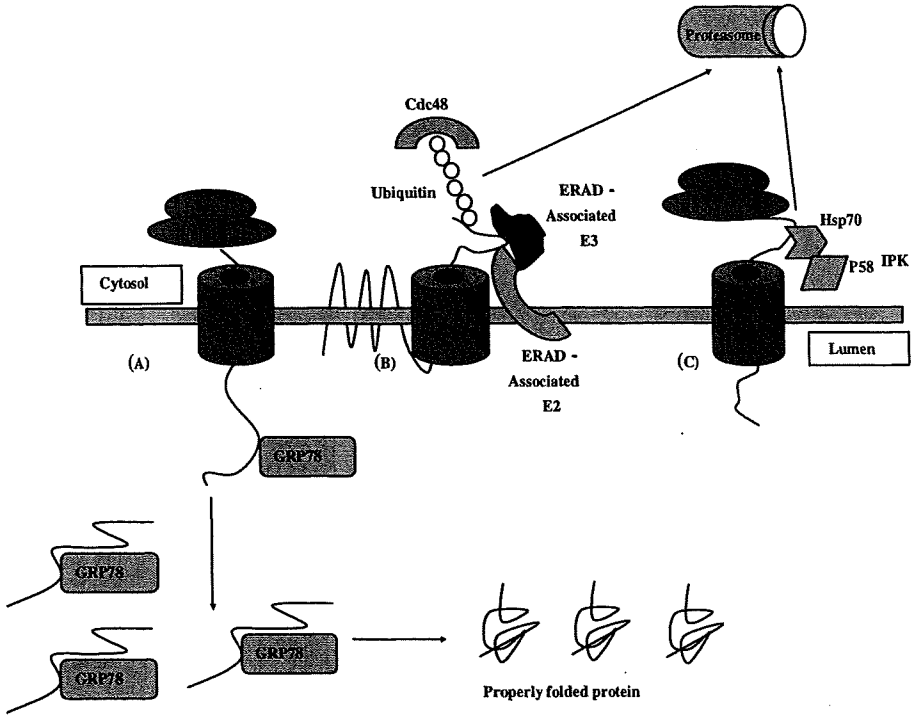

FIG. 2. The pathways related to clearance of unfolded proteins upon ER stress. Under ER stress conditions, cells develop three pathways to clear unfolded proteins in the ER. **(A)** ER chaperones such as GRP78 are upregulated to facilitate proper substrate folding. **(B)** Activation of ER associated degradation (ERAD)-mediated degradation of terminally unfolded proteins. **(C)** Cotranslational degradation of newly synthesized proteins trapped in the Sec61 translocon to decrease load burden to the ER.

characterized by the polyubiquitination and subsequent degradation of misfolded proteins (36–38). With the aid of the cytosolic AAA-ATPase p97/Cdc48, the misfolded ER protein is extruded through the ER membrane conduit Sec61, where it is then polyubiquitinated and delivered to the proteasome for degradation in the cytosol (Fig. 2B).

A recent study has revealed a new layer in the UPR pathway that permits the cotranslocational degradation of secretory proteins involving $P58^{IPK}$/DNAJC3, which collaborates with cytosolic chaperone networks and appears to assist in the co-translational/translocational degradation of nascent polypeptide chains that are stalled in ER translocons (Fig. 2C). This function diminishes the biosynthetic burden on the ER by degrading proteins at a stage earlier than previously envisioned. This protective effect might reflect a reduction in protein flux into the stressed ER's lumen. Alternatively, intervention early in the protein biogenesis by $P58^{IPK}$/DNAJC3 might allow the maturation and quality control machinery to focus its attenuation on the pre-existing improperly folded proteins that triggered the initial UPR signal (34).

ER STRESS IN FAMILIAL FORMS OF PD

As in most cases of PD, the degeneration is idiopathic, the etiology of the disease remains unknown. The recent identification of genetic mutations in familial cases of PD has advanced our understanding of the molecular mechanisms that cause the neurodegeneration. So far, six PD-associated genes have been identified.

Autosomal dominant forms of PD

Three rare missense mutations in the α-*synuclein* gene (A30P, E46K, and A53T) cause autosomal dominant familial PD (23, 39). Although the function of α-synuclein is still unclear, the discoveries that α-synuclein is a main component of Lewy bodies (48) and that its overexpression and gene triplication can cause neurodegeneration (1, 46) suggest that abnormalities of α-synuclein might be crucial for the pathogenesis of both familial and sporadic forms of PD. α-Synuclein transgenic mouse or *Drosophila* at least partially recapitulated PD phenotype including α-synuclein positive aggregate formation, although no obvious dopaminergic neuronal loss was observed in transgenic mice (12, 49). Lentivirus-mediated overproduction of α-synuclein in rat substantia led to significant cell death (27). Leucine-rich repeat kinase 2 (LRRK2) has recently been added to the list of genes that are implicated in autosomal dominant PD (35, 59). LRRK2 is a GTP/GDP-regulated protein kinase, and increased kinase activity appears to be implicated in neurodegeneration (47). Another gene, ubiquitin carboxyl-terminal esterase L1 (UCHL1), has been associated with the dominantly inherited disease, but the genetic evidence for its pathogenicity is not established since only a single mutation with low penetrance has been identified in one family (39).

Autosomal recessive forms of PD

Three recessive forms of parkinsonism have been identified, including mutations in the genes that encode parkin, DJ1, and PTEN-induced kinase 1 (PINK1).

Mutations in the *parkin* gene were originally discovered from the linkage study of Japanese AR-JP families, the most frequent type of familial PD (20). Thereafter its mutations have been found worldwide. Parkin is a 465 amino acid protein characterized by a ubiquitin-like domain at its NH2-terminus, as well as two RING (really interesting new gene) finger domains flanking a domain known as the IBR (in-between RING) at its COOH-terminus (RING–IBR–RING). Like many other proteins containing a RING domain, parkin has been found to function as an ubiquitin ligase (E3) (Fig. 3). E3s are part of the cellular machinery that tags proteins with ubiquitin, thereby targeting them for degradation by the proteasome. The UPS plays a major role in many vital cellular processes, and its dysfunction has been implicated in the pathogenesis of neurodegenerative disorders including sporadic PD. Parkin mutants associated with AR-JP reduce or abolish its E3 activity. Therefore, the most straightforward mechanism by which the dysfunction of parkin would cause neurodegeneration is accumulation of some neurotoxic substrate protein(s), which leads to dysfunction and eventually the death of susceptible neurons.

Mutations in *PINK1* were initially identified in three large consanguineous families with autosomal recessive forms of PD (52). Mutations in *PINK1* have differential effects on protein stability, localization, and kinase activity (3). As the kinase domain is the hot spot of mutations, disruption of the kinase activity is the most probable disease mechanism. Although functional data are limited, wild-type PINK1 protected neurons from mitochondrial dysfunction and apoptosis induced by oxidative stress (11), supporting an involvement of mitochondria in the pathogenesis.

A third gene linked to recessively inherited albeit rare PD is *DJ-1* (6). DJ1 has been assigned various functions, but perhaps the most relevant function in terms of the pathogenesis of PD is its potential role in oxidative stress response, either as a redox sensor or antioxidant protein (8).

In this review, rather than attempting to overview the entire picture, we focus on potential involvement of ER stress in this disease according to published data.

ER stress in α-synuclein-associated PD

In a *Drosophila* model of PD engineered to express wild-type and mutant α-synuclein, expression of molecular chaperone heat shock protein 70 (Hsp70) prevented dopaminergic cell loss mediated by accumulation of α-synuclein (1). However, Hsp70 is not directly activated in the UPR. A recent study provided direct evidence indicating the implication of ER stress in α-synuclein-mediated cell death (47). In a mammalian cell culture model, induction of the expression of A53T α-synuclein induced ER stress, as evidenced by the elevation in expression of CHOP and GRP78, increased phosphorylation of eIF2α, and activation of caspase-12. Furthermore, decrease of eukaryotic initiation factor 2α (eIF2α) phosphorylation by inhibitor, or knockdown of caspase-12 levels by RNA interference partially protected against cell death (47), indicating that ER stress at least partially contribute to A53T α-synuclein-induced cell death. Overexpression of mutant forms of α-synuclein in cultured neuronal cells leads to decrease in proteasome activity (51). The mechanism underlying mutant α-synuclein-induced impairment of proteasome activity remains to be identified. α-Synuclein is reported to be degraded through several different pathways including macroautophagy, chaperone-mediated autophagy, and proteasome (10, 50, 53). Since α-synuclein interacts with a subunit of proteasome regulatory complexes (15), it is possible that mutant α-synuclein directly affects the proteasome complex. It is of interest that an important means of removing misfolded proteins from the ER is their degradation by proteasomes. In addition, it has been reported that partial inhibition of the proteasome activity by poly-Q was sufficient to cause ER stress in primary neurons (33). Therefore, ER stress observed in overexpression of mutant forms

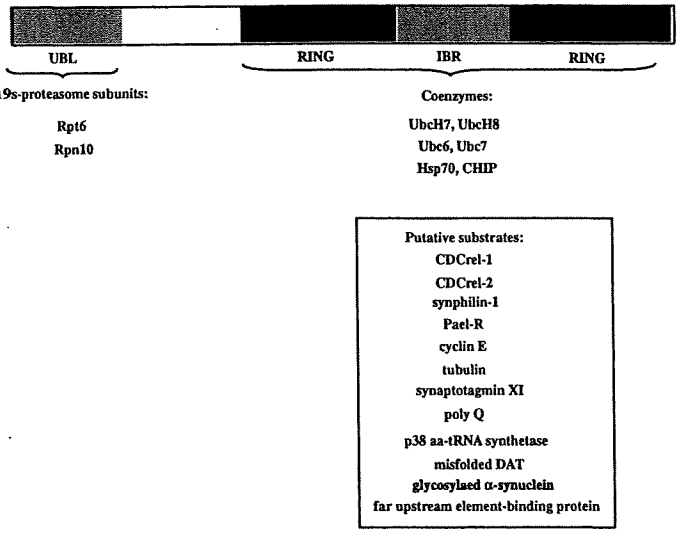

FIG. 3. Modular structure of parkin. Parkin has a modular structure, containing a ubiquitin-like (UBL) domain at the amino-terminus and two real interesting new gene (RING) fingers at its carboxy-terminus. In addition, an in-between RING (IBR) domain is inserted in the middle portion between two RING finger motifs. The two RINGs and IBR are named as a RING box. Furthermore, the linker region is located between UBL and RING box. The UBL binds to 19S proteasome subunits, and the RING–IBR–RING domain binds to specific co-enzymes and substrates (except for glycosylated α-synuclein, which binds to the UBL domain).

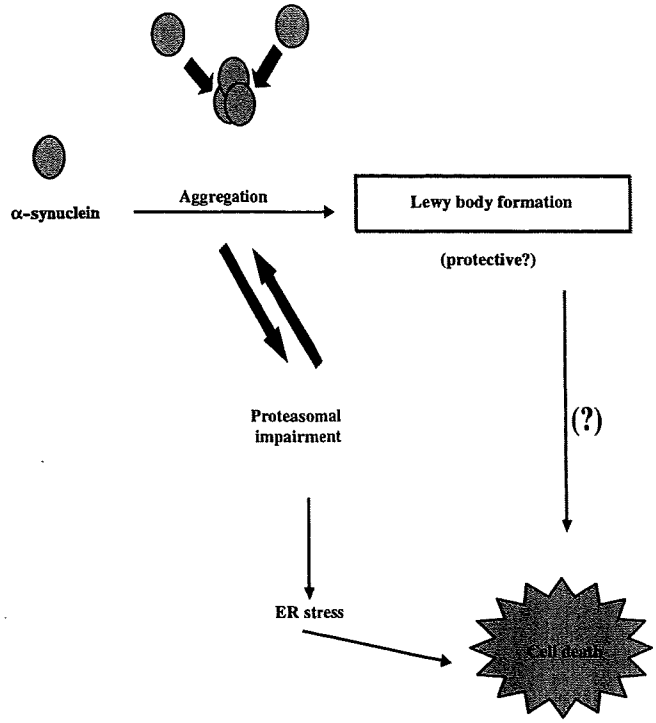

FIG. 4. Hypothetical mechanism of ER stress-mediated cell death induced by α-synuclein. The insidious loop feedback between α-synuclein aggregation and proteasomal impairment induces ER stress, which at least in part contributes to α-synuclein-mediated cell death.

of α-synuclein in cultured neuronal cells possibly derive from disruption of proteasome activity (Fig. 4).

ER stress in parkin-associated PD

Evidence supporting the involvement of ER stress in parkin mutations-induced cell death is more potent. Parkin is an E3 enzyme that interacts with Hsp70 and CHIP and plays a general role in protein degradation during ER stress (17–19). In line with this notion, cognate E2 (ubiquitin conjugating enzyme) partners of parkin include Ubc6 and Ubc7, which are ER-associated E2s involved in ERAD, indicating parkin is a component of ERAD machinery. It is easily conceivable that disruption of parkin function directly leads to ER stress, since ERAD and ER stress are coordinately regulated and deletion of ERAD components results in ER stress (13).

Given that accumulation of the substrate of parkin might play a key role in the neurodegenerative process, identification of parkin substrates has therefore been a major focus of many laboratories working on parkin. Typically, one expects an E3 to be highly specific for one or possibly a small number of substrates. Unexpectedly, a large number of putative parkin substrates have been reported (Fig. 3). Interestingly, several parkin substrates are misfolded or aggregation-prone proteins and are components of Lewy bodies. Considering that misfolded proteins, associated molecular chaperones, and proteasomal subunits are accumulated in Lewy bodies, the substrates of parkin may represent a subset of misfolded proteins. The C-terminus of Hsc70 interaction protein (CHIP), a U-box containing E3, has been shown to recognize misfolded protein through the heat-shock protein Hsp70 and is proposed to be a "quality control E3" that is contributed to the clearance of misfolded proteins (31). Given that parkin also binds to Hsp70, parkin may have a similar function to CHIP in dealing with misfolded proteins.

Among these substrates of parkin, one of the best-characterized parkin substrates, Pael-R underscores the ER stress-mediated cell death in the pathogenesis of AR-JP (18). Pael-R is a multipass G protein-coupled transmembrane protein with homology to the endothelin receptor type B, the function of which is unknown. Folding of Pael-R is a formidable challenge to cells. When overexpressed in cultured cells, Pael-R tends to become unfolded and insoluble; at the early stage of Pael-R accumulation, ER chaperones showed transcriptional upregulation, indicating that accumulation of Pael-R actually induced ER stress. Interestingly, CHIP serves as a cofactor of parkin. When Pael-R misfolding exceeds the cellular chaperone capacity, CHIP is upregulated, which sequesters Hsp70 and facilitates parkin-mediated ubiquitination of Pael-R (17). Under these conditions, parkin apparently acts as part of the ERAD machinery, utilizing the ER associated E2 enzymes Ubc6 and Ubc7 as the collaborating partners.

The UPR induces upregulation of parkin mRNA per se, and cells overexpressing parkin, but not mutant parkins found in AR-JP patients, are particularly resistant to unfolded protein-induced cell death (19). Furthermore, when astrocytes and neurons were exposed to conditions associated with ER stress, parkin protein levels were upregulated in astrocytes

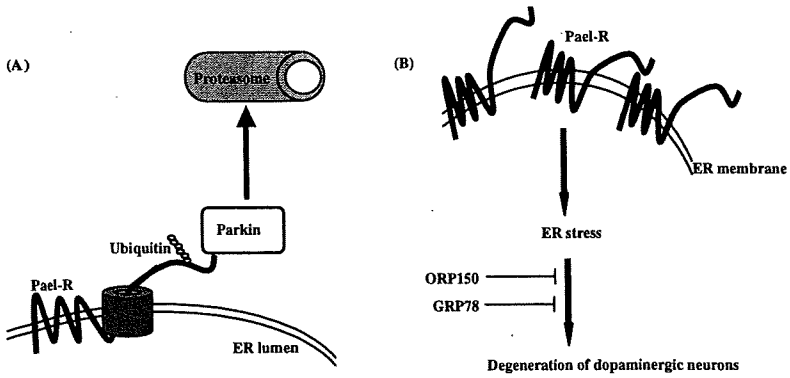

FIG. 5. Implication of ER stress in Pael-R-induced neuronal degeneration. (A) Pael-R is a difficult protein to be folded, and parkin ubiquitinates misfolded Pael-R and facilitates its degradation by UPS. (**B**) When dysfunction of parkin or overexpression of Pael-R, unfolded Pael-R accumulates in the ER and causes ER stress. ER chaperones, GRP78 and ORP150, suppress Pael-R-induced neuronal degeneration, possibly through enhancing the folding capacity of the ER.

but not in neurons (25). In the brain, Pael-R is primarily expressed in oligodendrocytes and shows little expression in neurons, except for a few distinct subpopulations of neurons, including hippocampal neurons and dopaminergic neurons in the SNpc. This implies that dopaminergic neurons of patients suffering from AR-JP are less well protected from neurotoxicity arising under conditions of Pael-R accumulation-induced ER stress. Thus, the inability of neurons to respond to ER stress by activating the expression of parkin and distributional pattern of Pael-R may contribute to the high vulnerability of dopaminergic neuronal cells. Using a transgenic *Drosophila* expressing human Pael-R, Yang *et al.* (55) found that this fly model revealed the age-dependent selective degeneration of dopaminergic neurons in spite of pan-neuronal expression of Pael-R. This Pael-R mediated dopaminergic neuronal loss was suppressed by the coexpression of human parkin and exacerbated by knockdown of endogenous parkin in the *Drosophila* by RNA interference. Recent *in vivo* observations in mice further highlights the important role of ER stress in Pael-R-mediated toxicity (21). Adenovirus-mediated overexpression of Pael-R in dopaminergic neurons induced ER stress and degeneration. This Pael-R-mediated neuronal death was suppressed by increased GRP78 or oxygen regulated protein 150 (ORP150), whereas cell death was exacerbated by downregulation of parkin or ORP150 (Fig. 5). Furthermore, a complicated interplay between ER stress and dopamine toxicity might present a mechanism underlying Pael-R-induced selective dopaminergic neuronal death, as evidenced by a neuroprotective effect of a tyrosine hydroxylase (TH) inhibitor (21).

ER STRESS IN TOXIN-INDUCED PD MODELS

Mitochondria toxins, 6-OHDA, rotenone, and MPP^+, are believed to contribute to dopaminergic neuronal death. These reagents can promote the generation of reactive oxygen species (ROS) via the inhibition of mitochondrial complex I or their oxidative function (14). Using functional genomics approaches to identify transcriptional alterations, numerous changes in genes associated with UPR were identified (42). Notably, a major target of the UPR pathway, the transcription factor CHOP, was dramatically upregulated by these reagents, as well as numerous markers of UPR including GRP78, splicing of XBP1, PERK, and the JNKs pathway. The assumption that ER dysfunction may play a role in the pathological process resulting in PD is corroborated by the observation that exposing cells to 6-OHDA, rotenone, or MPP^+, which are cellular models mimicking pathological disturbances associated with PD, induces a striking increase in transcripts associated with UPR (16, 42, 54). A number of reports have shown that both proteasome inhibition and ROS can trigger ER stress-mediated cell death pathways. One possible mechanism of ER stress induced by these mitochondria toxins is that accumulation of damaged oxidized proteins by the effects of these reagents on mitochondrial respiration causes ER stress (13). Alternatively, oxidative stress can directly compromise proteasomal components (41). However, the oxidative stress caused by the effects of these agents on mitochondrial respiration may not be totally attributable since a nonselective oxidant does not trigger ER stress. In addition, neurons lacking expression of PERK are defective in ER response and are significantly more sensitive to the death-promoting effects of PD mimetics (42). Thus, not only do mitochondria toxins provoke ER stress, but neurons lacking the capacity to deal with this by inducing an appropriate UPR are at greater risk of death, suggesting that ER stress is likely to play a causative role in neuronal cell death induced by these mitochondria toxins. Coupled with evidence from familial forms of PD, the induction of UPR and ER stress in these generally used neurotoxin models raise the possibility of widespread involvement of ER stress-mediated cell death in the pathogenesis of PD and other related disorders (Fig 6).

CROSSTALK OF ER STRESS WITH OXIDATIVE STRESS IN PD

PD has been closely associated with oxidative stress and mitochondrial dysfunction. In addition, dopaminergic neurons are particularly subjected to increased oxidative stress due to production of free radicals during dopamine autooxidation and dopamine metabolism (24). ER stress is intricately connected to oxidative stress. As described above, oxidative stress can directly or indirectly induce ER stress (13, 41). Evidence is also accumulating for a converse

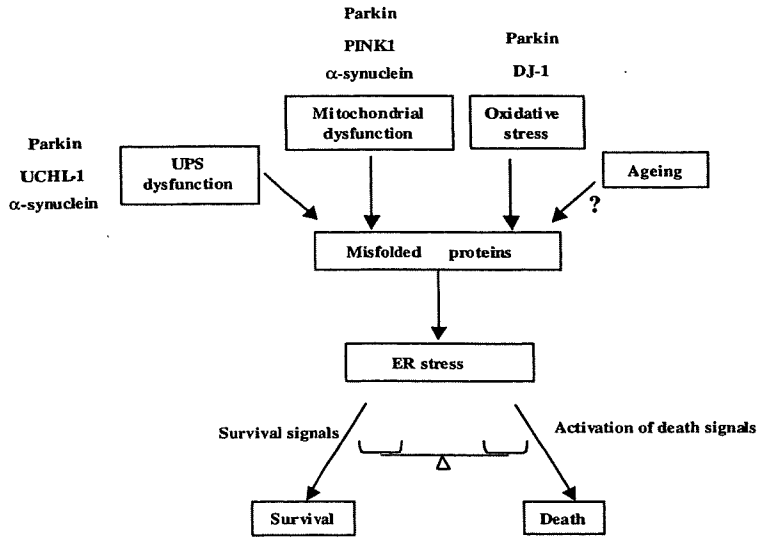

FIG. 6. Widespread involvement of ER stress in the pathogenesis of Parkinson's disease. In Parkinson's disease, proteasome dysfunction, oxidative stress, mitochondrial dysfunction, and possibly aging could directly or indirectly cause accumulation of misfolded proteins in the ER, thus induce ER stress. Cells counteract ER stress by activation of unfolded protein responses (UPR), which activates protective signals to eliminate misfolded proteins. However, when UPR fails to eliminate misfolded proteins, cells undergo apoptosis.

mechanism, whereby ER stress can result in secondary oxidative damage. One target of CHOP is ER oxidoreductase ERO1α, which participates in protein disulfide bond formation during protein refolding in the ER to help relieve ER stress, but in doing so also promotes production of ROS (28). The interplay between ER stress and oxidative stress might be mediated in part by parkin. Parkin is itself sensitive to oxidative stress, and is inactivated by nitric oxide-mediated nitrosylation or dopamine, which could lead to a simultaneous ER stress and oxidative damage (9, 24, 56). Inactivation of parkin is likely to create a feed-forward amplification loop, rendering dopaminergic cells more susceptible to oxidative and ER stress.

CONCLUSIONS

Evidence has been presented in various experimental studies that impairment of ER function may be involved in the neuronal cell death in PD. Environmental toxins, oxidative damage by dopamine itself, and mitochondrial abnormalities are all believed to play a role in sporadic PD (30). All these affect protein folding in the cytoplasm and lead to ER stress by compromising the process of ERAD (4). Alternatively, a genetic defect such as parkin mutation could impair the ability of cells to adapt to ER stress through impairment of its E3 activity. Since some novel components of the canonical UPR are expressed in a cell type-specific fashion, different types of cells may have unique responses for adaptation to ER stress (22, 32, 57). It is noted that parkin is upregulated in astrocytes, but not in neurons upon ER stress (25), suggesting parkin may represent as another unique response for adaptation to ER stress. Further investigation of parkin regulators will improve our chances of identifying novel targets for designing effective therapeutic strategies to impede the pathological processes.

ABBREVIATIONS

AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; AR-JP, autosomal recessive juvenile Parkinsonism; bZIP, basic leucine zipper; CHIP, C-terminus of Hsc70 interaction protein; CHOP, C/EBP homologous protein; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; eIF2α, eukaryotic initiation factor 2α; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERO1α, ER oxidoreductase 1α; GRP78, glucose regulated protein 78; Hsp70, heat shock protein 70; IBR, in-between RING; JNK, c-Jun NH2-terminal kinase; Pael-R, parkin associated endothelin like-receptor; ORP150, oxygen regulated protein 150; PINK1, PTEN-induced kinase 1; PD, Parkinson's disease; RING, really interesting new gene; ROS, reactive oxygen species; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; UPR, unfolded protein response; UPS, ubiquitin–proteasome system.

REFERENCES

1. Auluck PK, Chan HY, Trojanowski JQ, Lee VM, and Bonini NM. Chaperone suppression of alpha-synuclein toxicity in a *Drosophila* model for Parkinson's disease. *Science* 295: 865–868, 2002.
2. Back SH, Schroder M, Lee K, Zhang K, and Kaufman RJ. ER stress signaling by regulated splicing: IRE1/HAC1/XBP1. *Methods* 35: 395–416, 2005.
3. Beilina A, Van Der Brug M, Ahmad R, Kesavapany S, Miller DW, Petsko GA, and Cookson MR. Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. *Proc Natl Acad Sci USA* 102: 5703–5708, 2005.
4. Bence NF, Sampat RM, and Kopito RR. Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* 292: 1552–1555, 2001.
5. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, and Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol* 2: 326–332, 2000.

6. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, van Dongen JW, Vanacore N, van Swieten JC, Brice A, Meco G, van Duijn CM, Oostra BA, and Heutink P. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. *Science* 299: 256–259, 2003.
7. Braak H, Del Tredici K, Rub U, de Vos RA, Jansen Steur EN, and Braak E. Staging of brain pathology related to sporadic Parkinson's disease. *Neurobiol Aging* 24: 197–211, 2003.
8. Canet–Aviles RM, Wilson MA, Miller DW, Ahmad R, McLendon C, Bandyopadhyay S, Baptista MJ, Ringe D, Petsko GA, and Cookson MR. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. *Proc Natl Acad Sci USA* 101: 9103–9108, 2004.
9. Chung KK, Thomas B, Li X, Pletnikova O, Troncoso JC, Marsh L, Dawson VL, and Dawson TM. S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. *Science* 304: 1328–1331, 2004.
10. Cuervo AM, Stefanis L, Fredenburg R, Lansbury PT, and Sulzer D. Impaired degradation of mutant alpha-synuclein by chaperone-mediated autophagy. *Science* 305: 1292–1295, 2004.
11. Deng H, Jankovic J, Guo Y, Xie W, and Le W. Small interfering RNA targeting the PINK1 induces apoptosis in dopaminergic cells SH-SY5Y. *Biochem Biophys Res Commun* 337: 1133–1138, 2005.
12. Fernagut PO and Chesselet MF. Alpha-synuclein and transgenic mouse models. *Neurobiol Dis* 17: 123–130, 2004.
13. Friedlander R, Jarosch E, Urban J, Volkwein C, and Sommer T. A regulatory link between ER-associated protein degradation and the unfolded-protein response. *Nat Cell Biol* 2: 379–384, 2000.
14. Gee P and Davison AJ. Intermediates in the aerobic autoxidation of 6-hydroxydopamine: relative importance under different reaction conditions. *Free Radic Biol Med* 6: 271–284, 1989.
15. Ghee M, Fournier A, and Mallet J. Rat alpha-synuclein interacts with Tat binding protein 1, a component of the 26S proteasomal complex. *J Neurochem* 75: 2221–2224, 2000.
16. Holtz WA and O'Malley KL. Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. *J Biol Chem* 278: 19367–19377, 2003.
17. Imai Y, Soda M, Hatakeyama S, Akagi T, Hashikawa T, Nakayama KI, and Takahashi R. CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. *Mol Cell* 10: 55–67, 2002.
18. Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, and Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* 105: 891–902, 2001.
19. Imai Y, Soda M, and Takahashi R. Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin–protein ligase activity. *J Biol Chem* 275: 35661–35664, 2000.
20. Kitada T, Asakawa S, Hattori N, Matsumine H, Yamamura Y, Minoshima S, Yokochi M, Mizuno Y, and Shimizu N. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* 392: 605–608, 1998.
21. Kitao Y, Imai Y, Ozawa K, Kataoka A, Ikeda T, Soda M, Namekawa K, Kiyama H, Stern DM, Hori O, Wakamatsu K, Ito S, Itohara S, Takahashi R, and Ogawa S. Pael receptor induces death of dopaminergic neurons in the substantia nigra via endoplasmic reticulum stress and dopamine toxicity, which is enhanced under condition of parkin inactivation. *Hum Mol Genet* 16: 50–60, 2007.
22. Kondo S, Murakami T, Tatsumi K, Ogata M, Kanemoto S, Otori K, Iseki K, Wanaka A, and Imaizumi K. OASIS, a CREB/ATF-family member, modulates UPR signalling in astrocytes. *Nat Cell Biol* 7: 186–194, 2005.
23. Kruger R, Kuhn W, Muller T, Woitalla D, Graeber M, Kosel S, Przuntek H, Epplen JT, Schols L, and Riess O. Ala30Pro mutation in the gene encoding alpha-synuclein in Parkinson's disease. *Nat Genet* 18: 106–108, 1998.
24. LaVoie MJ, Ostaszewski BL, Weihofen A, Schlossmacher MG, and Selkoe DJ. Dopamine covalently modifies and functionally inactivates parkin. *Nat Med* 11: 1214–1221, 2005.
25. Ledesma MD, Galvan C, Hellias B, Dotti C, and Jensen PH. Astrocytic but not neuronal increased expression and redistribution of parkin during unfolded protein stress. *J Neurochem* 83: 1431–1440, 2002.
26. Lindholm D, Wootz H, and Korhonen L. ER stress and neurodegenerative diseases. *Cell Death Differ* 13: 385–392, 2006.
27. Lo Bianco C, Schneider BL, Bauer M, Sajadi A, Brice A, Iwatsubo T, and Aebischer P. Lentiviral vector delivery of parkin prevents dopaminergic degeneration in an alpha-synuclein rat model of Parkinson's disease. *Proc Natl Acad Sci USA* 101: 17510–17515, 2004.
28. Marciniak SJ, Yun CY, Oyadomari S, Novoa I, Zhang Y, Jungreis R, Nagata K, Harding HP, and Ron D. CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev* 18: 3066–3077, 2004.
29. Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. *Cell* 101: 451–454, 2000.
30. Mouradian MM. Recent advances in the genetics and pathogenesis of Parkinson disease. *Neurology* 58: 179–185, 2002.
31. Murata S, Minami Y, Minami M, Chiba T, and Tanaka K. CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein. *EMBO Rep* 2: 1133–1138, 2001.
32. Nagamori I, Yabuta N, Fujii T, Tanaka H, Yomogida K, Nishimune Y, and Nojima H. Tisp40, a spermatid specific bZip transcription factor, functions by binding to the unfolded protein response element via the Rip pathway. *Genes Cells* 10: 575–594, 2005.
33. Nishitoh H, Matsuzawa A, Tobiume K, Saegusa K, Takeda K, Inoue K, Hori S, Kakizuka A, and Ichijo H. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev* 16: 1345–1355, 2002.
34. Oyadomari S, Yun C, Fisher EA, Kreglinger N, Kreibich G, Oyadomari M, Harding HP, Goodman AG, Harant H, Garrison JL, Taunton J, Katze MG, and Ron D. Cotranslocational degradation protects the stressed endoplasmic reticulum from protein overload. *Cell* 126: 727–739, 2006.
35. Paisan–Ruiz C, Jain S, Evans EW, Gilks WP, Simon J, van der Brug M, Lopez de Munain A, Aparicio S, Gil AM, Khan N, Johnson J, Martinez JR, Nicholl D, Carrera IM, Pena AS, de Silva R, Lees A, Marti–Masso JF, Perez–Tur J, Wood NW, and Singleton AB. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron* 44: 595–600, 2004.
36. Plemper RK, Deak PM, Otto RT, and Wolf DH. Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase yscY species. *FEBS Lett* 443: 241–245, 1999.
37. Plemper RK and Wolf DH. Endoplasmic reticulum degradation. Reverse protein transport and its end in the proteasome. *Mol Biol Rep* 26: 125–130, 1999.
38. Plemper RK and Wolf DH. Retrograde protein translocation: ERADication of secretory proteins in health and disease. *Trends Biochem Sci* 24: 266–270, 1999.
39. Polymeropoulos MH, Lavedan C, Leroy E, Ide SE, Dehejia A, Dutra A, Pike B, Root H, Rubenstein J, Boyer R, Stenroos ES, Chandrasekharappa S, Athanassiadou A, Papapetropoulos T, Johnson WG, Lazzarini AM, Duvoisin RC, Di Iorio G, Golbe LI, and Nussbaum RL. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045–2047, 1997.
40. Rao RV and Bredesen DE. Misfolded proteins, endoplasmic reticulum stress and neurodegeneration. *Curr Opin Cell Biol* 16: 653–662, 2004.
41. Reinheckel T, Ullrich O, Sitte N, and Grune T. Differential impairment of 20S and 26S proteasome activities in human hematopoietic K562 cells during oxidative stress. *Arch Biochem Biophys* 377: 65–68, 2000.
42. Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, and Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. *J Neurosci* 22: 10690–10698, 2002.
43. Schroder M and Kaufman RJ. ER stress and the unfolded protein response. *Mutat Res* 569: 29–63, 2005.
44. Schroder M and Kaufman RJ. The mammalian unfolded protein response. *Annu Rev Biochem* 74: 739–789, 2005.
45. Selkoe DJ. Folding proteins in fatal ways. *Nature* 426: 900–904, 2003.

46. Singleton AB, Farrer M, Johnson J, Singleton A, Hague S, Kachergus J, Hulihan M, Peuralinna T, Dutra A, Nussbaum R, Lincoln S, Crawley A, Hanson M, Maraganore D, Adler C, Cookson MR, Muenter M, Baptista M, Miller D, Blancato J, Hardy J, and Gwinn–Hardy K. alpha-Synuclein locus triplication causes Parkinson's disease. *Science* 302: 841, 2003.
47. Smith WW, Jiang H, Pei Z, Tanaka Y, Morita H, Sawa A, Dawson VL, Dawson TM, and Ross CA. Endoplasmic reticulum stress and mitochondrial cell death pathways mediate A53T mutant alpha-synuclein-induced toxicity. *Hum Mol Genet* 14: 3801–3811, 2005.
48. Spillantini MG, Schmidt ML, Lee VM, Trojanowski JQ, Jakes R, and Goedert M. Alpha-synuclein in Lewy bodies. *Nature* 388: 839–840, 1997.
49. Springer W and Kahle PJ. Mechanisms and models of alpha-synuclein-related neurodegeneration. *Curr Neurol Neurosci Rep* 6: 432–436, 2006.
50. Stefanis L, Larsen KE, Rideout HJ, Sulzer D, and Greene LA. Expression of A53T mutant but not wild-type alpha-synuclein in PC12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. *J Neurosci* 21: 9549–9560, 2001.
51. Tanaka Y, Engelender S, Igarashi S, Rao RK, Wanner T, Tanzi RE, Sawa A, V LD, Dawson TM, and Ross CA. Inducible expression of mutant alpha-synuclein decreases proteasome activity and increases sensitivity to mitochondria-dependent apoptosis. *Hum Mol Genet* 10: 919–926, 2001.
52. Valente EM, Abou–Sleiman PM, Caputo V, Muqit MM, Harvey K, Gispert S, Ali Z, Del Turco D, Bentivoglio AR, Healy DG, Albanese A, Nussbaum R, Gonzalez–Maldonado R, Deller T, Salvi S, Cortelli P, Gilks WP, Latchman DS, Harvey RJ, Dallapiccola B, Auburger G, and Wood NW. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. *Science* 304: 1158–1160, 2004.
53. Webb JL, Ravikumar B, Atkins J, Skepper JN, and Rubinsztein DC. Alpha-Synuclein is degraded by both autophagy and the proteasome. *J Biol Chem* 278: 25009–25013, 2003.
54. Yamamuro A, Yoshioka Y, Ogita K, and Maeda S. Involvement of endoplasmic reticulum stress on the cell death induced by 6-hydroxydopamine in human neuroblastoma SH-SY5Y cells. *Neurochem Res* 31: 657–664, 2006.
55. Yang Y, Nishimura I, Imai Y, Takahashi R, and Lu B. Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in Drosophila. *Neuron* 37: 911–924, 2003.
56. Yao D, Gu Z, Nakamura T, Shi ZQ, Ma Y, Gaston B, Palmer LA, Rockenstein EM, Zhang Z, Masliah E, Uehara T, and Lipton SA. Nitrosative stress linked to sporadic Parkinson's disease: *S*-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. *Proc Natl Acad Sci USA* 101: 10810–10814, 2004.
57. Zhang K, Shen X, Wu J, Sakaki K, Saunders T, Rutkowski DT, Back SH, and Kaufman RJ. Endoplasmic reticulum stress activates cleavage of CREBH to induce a systemic inflammatory response. *Cell* 124: 587–599, 2006.
58. Zhao L and Ackerman SL. Endoplasmic reticulum stress in health and disease. *Curr Opin Cell Biol* 18: 444–452, 2006.
59. Zimprich A, Biskup S, Leitner P, Lichtner P, Farrer M, Lincoln S, Kachergus J, Hulihan M, Uitti RJ, Calne DB, Stoessl AJ, Pfeiffer RF, Patenge N, Carbajal IC, Vieregge P, Asmus F, Muller–Myhsok B, Dickson DW, Meitinger T, Strom TM, Wszolek ZK, and Gasser T. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron* 44: 601–607, 2004.

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Capillary beds are decreased in Alzheimer's disease, but not in Binswanger's disease

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Abstract

Morphological abnormalities of the cortical microvessels have been reported in Alzheimer's disease (AD), but not in Binswanger's disease (BD), a form of vascular dementia. Therefore, we compared the capillary beds in AD and BD brains, using a modified Gallyas silver impregnation method and immunohistochemistry for β amyloid. Eight autopsied brains with AD and seven with BD were compared with six control brains. The cortical microvessels in AD were frequently narrowed, and torn off, especially in close proximity to the senile plaques. The capillary densities in AD were significantly decreased as compared with the control brains. In contrast, there were no significant changes in the capillary densities and their morphologies in BD brains. Immunohistochemistry for β amyloid revealed numerous deposits in the vascular wall and perivascular neuropil exclusively in AD brains. Cortical microvascular changes in AD and their absence in BD may indicate a role of β amyloid for the microvessel pathology in AD.

Keywords: Microvessel; Alzheimer's disease; Vascular dementia; Binswanger's disease; Silver impregnation; Amyloid protein

Alzheimer's disease (AD) and vascular dementia are major causes of dementia and disabilities in the elderly. These two conditions have been believed to have an independent pathoetiology. However, in recent studies, co-morbid factors have been revealed in AD and vascular dementia [10,12]. These factors include hypertension, diabetes mellitus, hyperlipidemia, apo E4 ε genotype, cholinergic deficits, and white matter lesions. In addition, patients with vascular lesions reportedly develop dementia more frequently than those without vascular lesions among those subjects with senile changes [18]. Taken together, this evidence has shed light on the interrelationship between AD and vascular dementia, and raised the hypothesis that vascular factors may have a role in the pathogenesis of AD. In concordance with this hypothesis, previous electron microscopic studies have reported thickening of the basement membrane, denervation of the perivascular nerves, and bulging or narrowing of the cortical microvessels in AD brains [14,17].

Binswanger's disease (BD) is a form of vascular dementia, featured by diffuse white matter lesions, lacunar infarcts and fibrohyaline thickening of the microvessel [13]. Fibrohyaline thickening of the microvessels is marked in BD, and significant but less severe in AD in the cerebral white matter [19]. However, with respect to the cortical microvessels, there are no studies in BD. Therefore, we aimed to compare the alterations of the cortical microvessels in AD and BD using a modified Gallyas silver impregnation method and immunohistochemistry for β amyloid, which enable us to examine the network of the brain capillaries, and senile plaques.

We examined 21 brains, including 8 from patients with AD (3 males), 7 from patients with BD (4 males), and 6 from patients who did not have any neuropsychiatric symptoms or brain lesions (3 males). The age was 79 ± 12 years (mean ± S.D.) in the AD, 74 ± 13 years in the BD and 73 ± 4 in the control groups, respectively, among which no significant differences were observed ($p < 0.05$). The brain weight was 1020 ± 111 g in the AD, 1093 ± 112 g in the BD, and 1244 ± 57 g in the control groups, respectively. The brain weight in the AD group was significantly lower than in the control and BD groups ($p < 0.05$). The patients with AD and BD, but not the control patients, met

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the diagnostic criteria for dementia (diagnostic and statistical manual of mental disorders; DSM-IV) [1] in the occasion of diagnosis and, most of these patients suffered in a bed-ridden condition in their terminal stages.

The diagnosis of AD was made based on the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) diagnostic neuropathologic criteria [15] and excluded the brains with large cerebral infarctions. The diagnosis of BD was made clinico-pathologically, and retrospectively met the pathological inclusion criteria including (1) presence of diffuse white matter lesions, (2) lacunar infarctions in the perforator territory, (3) arteriolosclerosis such as fibrohyalinosis and fibrinoid necrosis and (4) absence of cortical infarctions, as well as the clinical criteria by Bennett et al. [3], and excluded the brains with significant pathologic hallmarks of AD. The control group included two patients with pneumonia, one patient each with renal cancer, lung cancer, chronic renal failure and pulmonary emphysema.

The tissue blocks were sectioned in a cryostat (30 or 100 μm-thick) and kept in 0.1 M phosphate buffer (pH 7.4). The tissue sections were processed according to the silver impregnation method described by Gallyas [8]. Briefly, the sections were incubated for 30 min in 5% periodic acid (HIO4) solution. The sections were then immersed in 4% sodium hydroxide solution for 30 min, and washed in 0.5% acetic acid solution in double-distilled water for 5 min. They were further incubated in a mixture (pH 13.0) of 90 ml of an ammoniated silver nitrate solution (AgNO3, 0.5 g and ammonium nitrate, 2.5 g in 900 ml double-distilled water), and 10 ml of 4% sodium dihydroxide in double-distilled water for 30 min at 20 °C. These sections were left in a physical developer solution, which was composed of a mixture of 10 ml of solution A, 5 ml of solution B and 5 ml of solution C at 25 °C. The composition of each solution was as follows; sodium carbonate, 50 g in 1000 ml of distilled water (solution A); ammonium nitrate 1.9 g, silver nitrate, 2.0 g; tungsto-silicic acid ($SiO_2 \cdot 2WO_3$), 10 g in 1000 ml of distilled water (solution B); and ammonium nitrate, 1.9 g; silver nitrate, 2.0 g; tungsto-silicic acid ($SiO_2 \cdot 2WO_3$), 10.0 g; and 6.1 ml of 40% formalin solution in 1000 ml of distilled water (solution C). The reaction was terminated in 0.5% acetic acid solution, and the extent of silver impregnation was monitored intermittently under light microscopy.

For immunohistochemistry, autoclaved paraffin sections were incubated with a mouse anti-amyloid β protein antibody (Dakopatts, diluted 1:200), biotinylated anti mouse IgG (Vector laboratories, diluted 1:200) and an avidin biotinylated peroxidase complex (Vector Laboratories, diluted 1:200). They were finally visualized with 0.01% diaminobenzidine tetrahydrochloride and 0.005% H_2O_2 in 0.05 M Tris–HCl (pH 7.6). To test for the specificity of the immunohistochemical reaction, control sections were incubated with normal mouse IgG instead of the primary antibody.

The density of the capillary beds was determined by the test grid method [7], in which the number of vascular intersections were counted against 6 × 6 square test grids each with a 50 μm width. The average counts from five representative fields in the layers II–IV of the frontal and parietal cortices, respectively, were used as the capillary densities in each patient. The data were expressed as means ± S.D. and the Mann–Whitney *U*-test was used to compare between the groups.

Using the modified Gallyas stains, the microvessels in the cerebral cortices appeared smooth and regular in diameter in the non-neurological control and BD brains (Fig. 1A and C, respectively). There were no senile plaques nor neurofibrillary tangles. In contrast, the brains with AD had numerous senile plaques and neurofibrillary tangles, which were intermingled by irregularly-shaped microvessels in both frontal and parietal cortices (Fig. 1B). The microvessels were frequently narrowed and irregular in diameter for a variable length of the vessel (Fig. 1D–F). These vessels often showed bulging of their walls. In close proximity to the senile plaques, the microvessels were blunted and torn off in the sections with a thickness of 100 μm (Fig. 1E). These microscopic changes were not observed in the non-neurological control and BD groups.

With immunohistochemistry, β amyloid-immunoreactivity was localized in senile plaques which accumulated numerously in the superficial layer, as well as perivascular deposits in the vascular wall itself and perivascular neuropil in the AD group (Fig. 1G–I). Beta amyloid-immunopositive fine texture fibrils were distributed in the neuropil with or without contact to the microvessels. In contrast, there was almost no deposit of β amyloid in the cerebral cortices of the non-neurological control and BD groups. In the semi-quantitative measures of the microvessels, the microvascular densities were significantly lower in the AD group as compared to the other two groups in both frontal and parietal cortices (Fig. 2).

The capillaries in AD have been shown to exhibit thickening of their basement membrane, atrophy, perivascular fibrosis and degeneration of the pericytes [5,6,16], which may correspond to the bulging of the microvessels observed here. In semi-quantitative measures, some authors have not observed any decrease in the capillary densities [2], while others showed a decrease in selected or non-selected regions of AD brains [4,7]. The present study underscored the morphological abnormalities of the capillaries, and further revealed their numerical decrease in AD and absence of capillary damages in BD. The actual reduction rate in the capillary density of AD brains may be more severe, because significant atrophy in this group should have ameliorated the reduction ratio. The fact that there were no β amyloid-deposits nor damages in the cortical microvessels in BD brains was not contradictory to the major site of the pathologic process, which involve subcortical white matter and perforator territory in BD. However, in previous studies, slight but significant neuronal dysfunction has been noted in the cerebral cortex, such as a decrease in the synaptic densities and neuronal viabilities [11,22].

The reduction in the vascular densities and the spatial proximity of β amyloid deposits to the microvascular changes may suggest some vascular toxicity due to β amyloid. Indeed, preamyloid deposits were found in the extracellular space and extended directly into the capillaries [16]. Vinters and Farag [20] raised a neurovascular hypothesis, in which β amyolid accumulates on the outer side of the basement membrane and

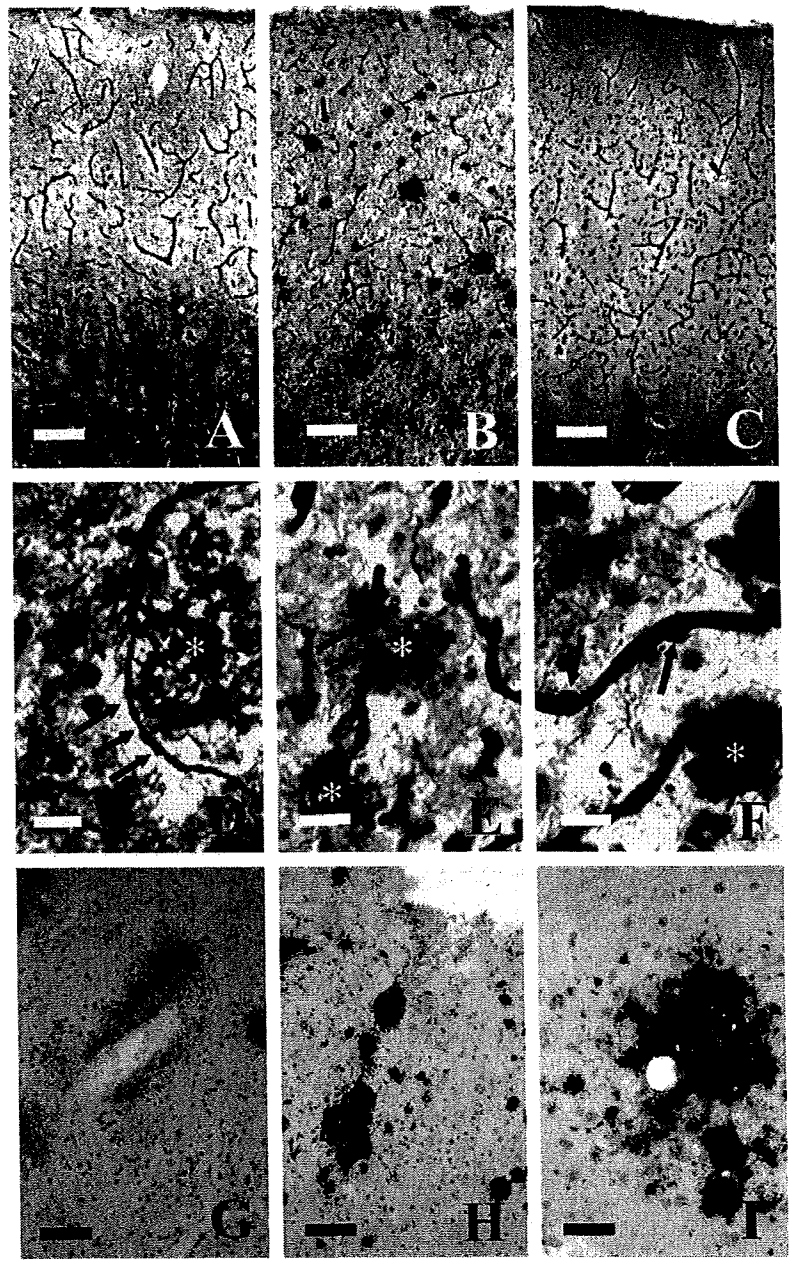

Fig. 1. Photomicrograph of Gallyas stains (A)–(F) and immunohistochemistry for β amyloid (G)–(I). The photographs were taken in the cerebral cortex of non-neurological (A); AD (B), (D)–(I); and BD patients (C). Numerous senile plaques (asterisks) were observed exclusively in AD brains. The capillary density appears less dense in AD as compared with the non-neurological and BD brains. The small vessels showed narrowing (arrows in D), tearing off (arrows in E) and scattered bulging (arrows in F). There were perivascular deposit in the vascular wall which extended into the neuropil diffusely (G). A heavy accumulation of perivascular β amyloid was also seen in the tangential (H) and axial planes (I), in which the perivascular deposits continued into the neuroglial deposits. Bars indicate 100 μm for (A)–(C), (I), 30 μm for (D)–(F) and 200 μm in (G), (H).

may promote local neurovascular inflammation. In support for this hypothesis, GAX, a gene encoding a homeodomain-transcription factor box gene related to vascular differentiation [9], is downregulated in AD brains. The downregulation of GAX activates a proapoptotic pathway, and may result in a decrease in the number of cerebral microvessels and cerebral blood flow (CBF), by way of activating the forkhead transcription factor, AFX-1. This activation may also downregulate low density lipoprotein receptor-related prtoein-1 (LRP) which enhances efflux of β amyloid from the brain [21]. This impaired clearance of β amyloid may further increase soluble β amyloid and fibrillary β amyloid levels [23]. Finally, we hypothesized a vicious cycle in which β amyloid may cause microvascular regression, brain hypoperfusion and neurovascular inflammation, although this will be addressed in future studies.

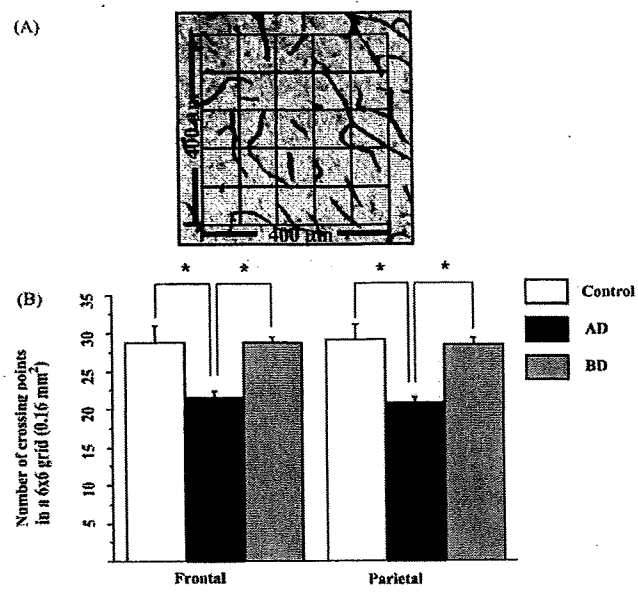

Fig. 2. Quantitative evaluation of capillary densities in the cerebral cortex. (A) indicates the grid applied for a non-neurological control brain; (B) indicates the capillary densities in the non-neurological, AD and BD brains. $*p < 0.05$ by Mann–Whitney U-test.

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References

[1] American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders, fourth ed., Washington, DC, American Psychiatric Association, 1994.
[2] M.A. Bell, M.J. Ball, Morphometric comparison of hippocampal microvasculature in ageing and demented people: diameters and densities, Acta Neuropathol. 53 (1981) 299–318.
[3] D.A. Bennett, R.S. Wilson, D.W. Gilley, J.H. Fox, Clinical diagnosis of Binswanger's disease, J. Neurol. Neurosurg. Psychiatry 53 (1990) 961–965.
[4] L. Buee, P.R. Hof, C. Bouras, A. Delacourte, D.P. Perl, J.H. Morrison, H.M. Fillit, Pathological alterations of the cerebral microvasculature in Alzheimer's disease and related dementing disorders, Acta Neuropathol. 87 (1994) 469–480.
[5] J.C. de la Torre, Hemodynamic consequences of deformed microvessels in the brain in Alzheimer's disease, Ann. N Y Acad. Sci. 826 (1997) 75–91.
[6] E. Farkas, P.G. Luiten, Cerebral microvascular pathology in aging and Alzheimer's disease, Prog. Neurobiol. 64 (2001) 575–611.
[7] V.W. Fischer, A. Siddiqi, Y. Yusufaly, Altered angioarchitecture in selected areas of brains with Alzheimer's disease, Acta Neuropathol. 79 (1990) 672–679.
[8] F. Gallyas, Silver staining of collagen and reticulin fibers and cerebral capillaries by mean of physical development, J. Microscopy 91 (1970) 119–124.
[9] D.H. Gorski, K. Walsh, Control of vascular cell differentiation by homeobox transcription factors, Trends Cardiovasc. Med. 13 (2003) 213–220.
[10] V. Hachinski, D.G. Munoz, Cerebrovascular pathology in Alzheimer's disease: cause, effect or epiphenomenon? Ann. N Y Acad. Sci. 826 (1997) 1–6.
[11] M. Ihara, H. Tomimoto, K. Ishizu, T. Mukai, H. Yoshida, N. Sawamoto, M. Inoue, T. Doi, K. Hashikawa, J. Konishi, H. Shibasaki, H. Fukuyama, Decrease in cortical benzodiazepine receptors in symptomatic patients with leukoaraiosis: a positron emission tomography study, Stroke 35 (2004) 942–947.
[12] R.N. Kalaria, Similarities between Alzheimer's disease and vascular dementia, J. Neurol. Sci. 203–204 (2002) 29–34.
[13] J.-Xi. Lin, H. Tomimoto, I. Akiguchi, M. Matsuo, H. Wakita, H. Shibasaki, H. Budka, Vascular cell components of the medullary arteries in Binswanger's disease brains; a morphometric and immunoelectron microscopic study, Stroke 31 (2000) 1838–1842.
[14] G.L. Mancardi, F. Perdelli, C. Rivano, A. Leonardi, O. Bugiani, Thickening of the basement membrane of cortical capillaries in Alzheimer's disease, Acta Neuropathol. 49 (1980) 79–83.
[15] S.S. Mirra, A. Heyman, D. McKeel, S.M. Sumi, B.J. Crain, L.M. Brownlee, F.S. Vogel, J.P. Hughes, G. van Belle, L. Berg, The Consortium to Establish a Registry for Alzheimer's Disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease, Neurology 41 (1991) 479–486.
[16] T. Miyakawa, S. Katsuragi, K. Yamashita, K. Ohuchi, Morphological study of amyloid fibrils and preamyloid deposits in the brain with Alzheimer's disease, Acta Neuropathol. 83 (1992) 340–346.
[17] A.B. Scheibel, Alterations of the cerebral capillary bed in the senile dementia of Alzheimer, Ital. J. Neurol. Sci. 8 (1987) 457–463.
[18] D.A. Snowdon, L.H. Greiner, J.A. Mortimer, K.P. Riley, P.A. Greiner, W.R. Markesbery, Brain infarction and the clinical expression of Alzheimer disease, JAMA 277 (1997) 813–817.
[19] H. Tomimoto, I. Akiguchi, H. Akiyama, K. Ikeda, H. Wakita, J.-Xi. Lin, I. Akiguchi, M. Kinoshita, H. Shibasaki, Vascular changes in white matter lesions of Alzheimer's disease, Acta Neuropathol. 97 (1999) 629–634.
[20] H.V. Vinters, E.S. Farag, Amyloidosis of cerebral arteries, Adv. Neurol. 92 (2003) 105–112.
[21] Z. Wu, H. Guo, N. Chow, J. Sallstrom, R.D. Bell, R. Deane, A.I. Brooks, S. Kanagala, A. Rubio, A. Sagare, D. Liu, F. Li, D. Armstrong, T. Gasiewicz, R. Zidovetzki, X. Song, F. Hofman, B.V. Zlokovic, Role of the MEOX2 homeobox gene in neurovascular dysfunction in Alzheimer disease, Nat. Med. 11 (2005) 959–965.
[22] S.S. Zhan, K. Beyreuther, H.P. Schmitt, Synaptophysin immunoreactivity of the cortical neuropil in vascular dementia of Binswanger type compared with the dementia of Alzheimer type and nondemented controls, Dementia 5 (1994) 79–87.
[23] B.V. Zlokovic, Neurovascular mechanisms of Alzheimer's neurodegeneration, Trends Neurosci. 28 (2005) 202–208.