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ORIGINAL ARTICLE

Differential Expression of Oxidative Phosphorylation Genes in Patients With Alzheimer's Disease

Implications for Early Mitochondrial Dysfunction and Oxidative Damage

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Abstract

In Alzheimer's disease (AD) pathogenesis, increasing evidence implicates mitochondrial dysfunction resulting from molecular defects in oxidative phosphorylation (OXPHOS). The objective of the present study was to determine the role of mRNA expression of mitochondrial genes responsible for OXPHOS in brain specimens from early AD and definite AD patients. In the present article, using quantitative real-time polymerase chain reaction (PCR) techniques, we studied mRNA expression of 11 mitochondrial-encoded genes in early AD patients ($n = 6$), definite AD patients ($n = 6$), and control subjects ($n = 6$). Using immunofluorescence techniques, we determined differentially expressed mitochondrial genes—NADH 15-kDa subunit (complex I), cytochrome oxidase subunit 1 (complex IV), and ATPase δ-subunit (complex V)—in the brain sections of AD patients and control subjects. Our quantitative reverse transcription (RT)-PCR analysis revealed a downregulation of mitochondrial genes in complex I of OXPHOS in both early and definite AD brain specimens. Further, the decrease of mRNA fold changes was higher for subunit 1 compared to all other subunits studied, suggesting that subunit 1 is critical for OXPHOS. Contrary to the downregulation of genes in complex I, complexes III and IV showed increased mRNA expressions in the brain specimens of both early and definite AD patients, suggesting a great demand on energy production. Further, mitochondrial gene expression varied greatly across AD patients, suggesting that mitochondrial DNA defects may be responsible for the heterogeneity of the phenotype in AD patients. Our immunofluorescence

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analyses of cytochrome oxidase and of the ATPase δ-subunit suggest that only subpopulations of neurons are differentially expressed in AD brains. Our double-labeling immunofluorescence analyses of 8-hydroxyguanosine and of cytochrome oxidase suggest that only selective, overexpressed neurons with cytochrome oxidase undergo oxidative damage in AD brains. Based on these results, we propose that an increase in cytochrome oxidase gene expression might be the result of functional compensation by the surviving neurons or an early mitochondrial alteration related to increased oxidative damage.

Index Entries: Oxidative phosphorylation; mitochondrial genes; Alzheimer's disease; postmortem brains; GAPDH; mitochondrial abnormalities; oxidative damage; 8-OHG.

Introduction

Mitochondrial defects are known to occur in aging, cancer, heart disease, and a wide variety of degenerative diseases, such as Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease (Beal, 1998; Schapira et al., 1999; Wallace, 1999). More specifically, mitochondrial abnormalities of oxidative phosphorylation (OXPHOS) of electron transport have been reported to be involved in AD pathogenesis (Beal, 1998; Blass et al., 1997; Blass, 2001; Bonilla et al., 2001; Castellani et al., 2002; Chandrasekaran et al., 1996, 1997; de la Monte et al., 2000; Egensperger et al., 1997; Hutchin et al., 1997; Lin et al., 1992; Lin et al., 2002; Ojaimi and Byrne, 2001; Orth and Schapira, 2001; Shoffner et al., 1993). The precise relationship between OXPHOS defects and AD pathogenesis is still unknown. However, it has been proposed that OXPHOS defects cause mitochondrial dysfunction by the escape of electrons from the electron transport chain, thus leading to the formation of hydroxyl radicals and hydrogen peroxide from superoxides (de la Monte et al., 2000; Schoffner, 2000). The resulting, increased oxidative stress could cause further free-radical damage to mitochondria, mitochondrial DNA, and calcium dysregulation in the AD brain (Blass, 2001; Mattson et al., 1999).

In OXPHOS, five complexes (I, II, III, IV, and V) contain flavins, coenzyme Q10, iron–sulfur clusters, hemes, and protein-bound copper (Shoffner, 1997). To assemble functional OXPHOS complexes, nuclear DNA (nDNA) genes and mitochondrial DNA (mtDNA) genes are coordinately expressed (Wallace, 1999). OXPHOS polypeptides encoded by nDNA genes are transported to the mitochondria, where they join OXPHOS polypeptides encoded by the mtDNA (Shoffner, 1997). Thirteen polypeptide genes are involved in the production of components of the OXPHOS complexes. Mitochondrial DNA encodes 7 out of 43 subunits of complex I, 1 of 11 subunits of complex III, 3 of 13 subunits of complex IV, and 2 of 17 subunits of complex V (Wallace, 1999). It is important to determine the role of mRNA expression in mitochondrial genes in the OXPHOS of electron transport in AD patients.

To understand the role of mitochondrial abnormalities of OXPHOS in AD pathogenesis, several studies have investigated mRNA expression of mitochondrial-encoded genes in complexes I and IV (Aksenov et al., 1999; Chandrasekaran et al., 1994, 1997; Simonian and Hyman, 1994) and of nuclear-encoded mitochondrial genes in complexes IV and V (Aksenov et al., 1999; Chandrasekaran et al., 1994, 1997). These studies suggested that decreased mRNA expression might be the result of mitochondrial DNA mutations in AD patients. In contrast, Hirai et al. (2001) recently found increased mDNA and cytochrome oxidase gene expressions in AD brain specimens compared to those of control subjects. Further, Strazielle et al. (2003) found increased cytochrome oxidase activity in the dentate gyrus and CA2–CA3 region of the hippocampus, the anterior cingulate and primary visual cortex, and the neostriatum of APP23 transgenic mice.

To determine the connection between mitochondrial gene expression and AD pathogenesis, several issues need to be addressed: whether changes in mitochondrial gene expression are the early cellular events in AD progression; within the components of the OXPHOS complexes, which complex is involved in AD pathogenesis; and whether a particular population of neurons is affected by changes in mitochondrial gene expression in AD patients.

To address these issues, in the present study, we measured the mRNA expression of 11 mitochondrial-encoded genes in the brain specimens of early AD, definite AD, and control subjects, using previously established real-time polymerase chain reaction (PCR) techniques in our laboratory (Gutala and Reddy, 2004). Using immunofluorescence techniques, we also studied NADH-ubiquinone, cytochrome-*c* oxidase, ATPase, and 8-hydroxyguanosine (8-OHG; a marker for oxidative damage) proteins in these brain specimens. In addition, using double-labeling immunofluorescence analysis of 8-OHG and cytochrome oxidase, we studied whether overexpressed neurons with cytochrome oxidase are vulnerable to oxidative damage in AD brains compared to the brains of control subjects.

Materials and Methods

Postmortem Brains

Eighteen postmortem brain specimens from AD patients and age-matched control subjects were obtained from the Harvard Tissue Resource Center. Table 1 presents the characteristics of the brain specimens used in this study. Twelve specimens were from patients diagnosed with AD according to Braak criteria (Braak and Braak, 1991) and six were from age-matched control subjects. Based on quantitative pathological features, including senile plaques, neurofibrillary tangles, and neuronal density, the AD brain specimens were classified as specimens from early AD, definite AD patients, or control subjects.

RNA Extraction

Total RNA was isolated from the frontal cortices (broadman area 9) of all brains using a RNAeasy kit from Qiagen (Valencia, CA), according to the manufacturer's recommendations. The concentration of RNA samples was ascertained by measuring optical density at 260 nm. The quality of RNA was confirmed by the detection of 18S and 28S bands after agarose-formaldehyde gel electrophoresis. To remove residual DNA contamination, the RNA samples were incubated with RNAse-free DNAse I at 37°C for 20 min. Then, the DNAse I was inactivated at 65°C for 10 min, and RNA samples were purified with a RNAeasy kit.

cDNA Synthesis

Total RNA from each sample was used to generate cDNA with a Superscript II first-strand cDNA synthesis kit (Invitrogen Inc., Carlsbad, CA) with oligo (dT) primers, according to the manufacturer's protocol. Briefly, 1 µg of Dnase-treated total RNA was used as starting material, to which we added 1 µL of oligo (dT), 1 µL of 10 m*M* dNTP, 4 µL of 5X first-strand buffer, 2 µL of 0.1 *M* dithiothreitol (DTT), and 1 µL of RNase Out. The reagents RNA, oligo (dT), and dNTPs were mixed and then heated at 65°C for 5 min. They were chilled on ice until the other components were added. The samples were incubated at 42°C for 2 min. Then 1 µL of Superscript II (40 units/µL) was added, and the samples were incubated at 42°C for 50 min. The reaction was inactivated at 70°C for 15 min.

Oligonucleotide Primers

Using Primer Express software (Applied Biosystems Inc., Foster City, CA), we designed the primers used for the housekeeping gene GAPDH and the mitochondrial-encoded genes NADH-SU 1, NADH-SU 2, NADH-SU 3, NADH-SU 4, NADH-SU 6, Cyt. b, COX 1, COX 2, COX 3, ATPase 6, and ATPase 8. The primer sequences are listed in Table 2. (*Note:* Oligonucleotide primers for NADH-SU 5 and NADH-4DL polypeptide genes of complex I of OXPHOS did not work for real-time PCR.)

Quantitative Real-Time PCR in AD Brains Using Sybr-Green Chemistry

The main features of real-time quantitative reverse transcription (RT)-PCR technology are the direct measurement of the PCR product accumulation during the log-linear phase of the PCR reaction and the simultaneous combination of amplification, detection, and quantification of PCR product (Bustin, 2000). To identify the unregulated endogenous reference gene in AD patients, our laboratory measured mRNA expressions of several commonly used reference genes, including β-actin, 18S rRNA, and GAPDH. It has been observed that β-actin and 18S rRNA are differentially expressed in the brain specimens of both AD and control subjects, whereas GAPDH is similarly expressed in AD and control brain specimens,

Table 1
Demographic Details of Control and AD Postmortem Brain Specimens Obtained From the Harvard Tissue Resource Center

No.	Case type	Age/sex	Postmortem interval (in h)	Braak stage of AD brains[a]
1	Control	68/M	21	None
2	Control	90/F	12	None
3	Control	82/M	17	None
4	Control	74/F	12	None
5	Control	74/F	23	None
6	Control	74/M	15	None
7	AD	76/F	7	I/II
8	AD	96/F	13	I/II
9	AD	67/F	10	I/II
10	AD	68/M	21	I/II
11	AD	92/F	22	I/II
12	AD	76/F	24	I/II
13	AD	77/M	18	III/IV
14	AD	72/F	12	III/IV
15	AD	69/M	18	III/IV
16	AD	75/M	24	III/IV
17	AD	82/M	19	III/IV
18	AD	80/M	19	III/IV
Average		78	16.7	

[a]Data from Braak and Braak, 1991.

suggesting that GAPDH can be used as an endogenous reference gene in the study of AD brains (Gutala and Reddy, 2004).

To determine the mRNAs of mitochondrial genes (listed in Table 2) in AD patients, the cycle threshold (CT) values (Bustin, 2000) of GAPDH and mitochondrial genes were studied. The CT value is the cycle number at which the fluorescence is generated within a reaction that crossed the threshold within the linear phase of the amplification profile. The CT value is an important quantitative parameter in real-time PCR analysis (Bustin, 2000). In our study, real-time quantitative PCR-amplification reactions were carried out in an ABI Prism 7700 sequence detection system (Applied Biosystems) in a 25-µL volume. The reaction mixture consisted of 1X PCR buffer containing Sybr-Green, 3 m*M* $MgCl_2$, 100 nm of each primer, 200 n*M* each of dATP, dGTP, and dCTP, 400 nm of dUTP, 0.01 U/µL of AmpErase UNG, and 0.05 U/µL of AmpliTaq Gold. Fifty nanograms of cDNA template was added to each reaction.

All real-time PCR reactions were carried out in duplicate with no template control. The PCR conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and then 60°C for 1 min. The fluorescent spectra were recorded during the elongation phase of each PCR cycle. To distinguish specific amplicons from nonspecific amplifications, a dissociation curve was generated. The CT values were calculated with sequence-detection system (SDS) software v 1.7 (Applied Biosystems) and an automatic setting of baseline, which was the average value of PCR, cycles 3–15, plus CT generated 10 times its standard deviation. The amplification plots and CT values were exported from the exponential phase of PCR directly into a Microsoft Excel worksheet for further analysis.

The mRNA transcript level was normalized against GAPDH for each mitochondrial gene. To compare GAPDH and mitochondrial genes, relative quantification was performed as outlined in the Relative Quantification of Gene Expression: ABI Prism 7700 SDS (Applied Biosystems). A

Table 2
Oligonucleotide Primers Used in Real-Time PCR Analysis of Mitochondrial-Encoded Genes of Oxidative Phosphorylation in AD Brain Specimens

	OXPHOS complex	Real-time PCR forward primer	Real-time PCR reverse primer
Mitochondrial-encoded genes			
NADH SU 1	I	5′ CCCTAAAACCCGCCACATCT 3′	5′ CGATGGTGAGAGCTAAGGTC 3′
NADH SU 2	I	5′ CACCCTTAATTCCATCCACCC 3′	5′ TGGGCAAAAAGCCGGTTAG 3′
NADH SU 3	I	5′ AGAAAAATCCACCCCTTACGAGT 3′	5′ TGGAGAAAGGGACGCGG 3′
NADH SU 4	I	5′ ACCTTGGCTATCATCACCCG 3′	5′ TAGGAAGTATGTGCCTGCGTTC 3′
NADH SU 6	I	5′ TGGTTGTCTTTGGATATACTACAGCG 3′	5′ CCAAGACCTCAACCCCTGAC 3′
Cyto. b	III	5′ CCCCACCCCATCCAACAT 3′	5′ TCAGGCAGGCGCCAAG 3′
COX 1	IV	5′ TCCGCTACCATAATCATCGCT 3′	5′ CCGTGGAGTGTGGCGAGT 3′
COX 2	IV	5′ TGCCCGCCATCATCCTA 3′	5′ TCGTCTGTTATGTAAAGGATGCGT 3′
COX 3	IV	5′ CCAATGATGGCGCGATG 3′	5′ CTTTTTGGACAGGTGGTGTGTG 3′
ATPase 6	V	5′ CCAATAGCCCTGGCCGTAC 3′	5′ CGCTTCCAATTAGGTGCATGA 3′
ATPase 8	V	5′ CAACTAAAAATATTAAACACAAACTACCACC 3′	5′ CGTTCATTTTGGTTCTCAGGG 3′
GAPDH			
Reference gene		5′ GAAGGTGAAGGTCGGAGTC 3′	5′ GAAGATGGTGATGGGATTTC 3′

Note: Oligonucleotide real-time PCR primers did not work for NADH SU 5 and NADH 4DL polypeptide genes in OXPHOS.

comparative CT method was used as outlined in User Bulletin no. 2 (Applied Biosystems, 1997) and according to Aarskog and Vedeler (2000), Sieber and colleagues (2002), and Gutala and Reddy (2004). Briefly, this comparative CT method involved averaging duplicate samples taken as the CT values for each mitochondrial gene and GAPDH. The ΔCT value was obtained by subtracting the average GAPDH CT value from the average CT value of mitochondrial genes. The present study used the average ΔCT of six control subjects as the calibrator. The fold change was calculated according to the formula $2^{-(\Delta\Delta CT)}$, where ΔΔCT was the difference between ΔCT and the ΔCT calibrator value.

Immunofluorescence Analysis of Mitochondrial and Oxidative Damage-Related Genes in AD Brains and Control Subjects

To determine the localization of mitochondrial-encoded NADH in complex I, cytochrome oxidase in complex IV, ATPase in complex V, and 8-OHG using immunofluorescence techniques, we studied NADH 15-kDa subunit, cytochrome oxidase subunit 1, ATPase δ-subunit, and 8-OHG proteins in the frontal cortex brain (broadman area 9) sections from early AD patients ($n = 6$) and control subjects ($n = 6$) (Table 1). Brain specimens were paraffin embedded, and sections were cut at 15 μm. We deparaffinized the brain sections by washing them with xylene for 10 min and then for 5 min each in a serial dilution of alcohol: 95%, 70%, and 50%. The sections were washed for 10 min with double-distilled H_2O (ddH_2O) and six more times for 5 min each with phosphate-buffered saline (PBS) at pH 7.4.

Further, to reduce the autofluorescence of brain sections, we treated deparaffinized sections with sodium borohydrate. The sections were washed two times each for 30 min in a freshly prepared 0.1% sodium borohydrate solution dissolved in PBS at pH 8.0. Then, the sections were washed three times each for 5 min with PBS at pH 7.4. To block the endogenous peroxidase, sections were treated 30 min with H_2O_2 and then with 0.5% Triton dissolved in PBS, pH 7.4. The sections were blocked with a solution (0.5% Triton in PBS + 10% goat serum + 1% bovine serum albumin [BSA]).

The sections were incubated overnight at room temperature with the anti-cytochrome oxidase (1 : 100, dilution), anti-ATPase δ-subunit (1 : 200, dilution), anti-NADH ubiquinone subunit 15 kDa (1 : 75, dilution) (Molecular Probes, Eugene, OR), and anti-8-OHG, a marker for oxidative damage (1 : 50, dilution) (Alpha Diagnostics, San Antonio, TX) monoclonal antibodies. On the day after primary antibody incubation, the sections were washed with a washing buffer (0.5% Triton in PBS). Then, they were incubated with a secondary biotinylated anti-mouse antibody, 1 : 2000 dilution (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections were washed three times for 10 min each with PBS. Then the sections were blocked for 1 h with blocking solution (Molecular Probes) and then incubated with the labeled streptavidin horseradish peroxidase (HRP) solution for 1 h (Molecular Probes). The sections were washed three times each for 10 min with PBS at pH 7.4, then treated with the fluorescent dye Alexa 594 (red color) (Molecular Probes) for 10 min at room temperature, and counterstained with Hoechst (1 : 1000) (blue color) for nuclear labeling. They were cover-slipped with Cytoseal and photographed using a confocal microscope.

Double-Labeling Immunofluorescence Analysis of Cytochrome Oxidase and 8-Hydroxyguanosine in AD Brains and Control Subjects

To determine whether neurons showing increased cytochrome oxidase are more vulnerable to oxidative damage, we conducted double-labeling immunofluorescence analysis using an anti-mouse cytochrome oxidase monoclonal antibody (Molecular Probes) and an anti-goat 8-OHG antibody (Alpha Diagnostics, San Antonio, TX). As described earlier, the sections from AD patients and control subjects were deparaffinized and also treated with sodium borohydrate to reduce autofluorescence.

For the first labeling, the sections were incubated overnight at room temperature with the goat anti-8-OHG polyclonal antibody (1 : 50, dilution) (Alpha Diagnostics, San Antonio, TX). On the day after primary antibody incubation, the sections were washed with a washing buffer (0.5% Triton in PBS). Then, they were incubated with a secondary biotinylated anti-goat secondary antibody at a 1 : 500 dilution (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Then, the sections were blocked

for 1 h with blocking solution (Molecular Probes) and incubated with the labeled streptavidin HRP solution for 1 h (Molecular Probes). The sections were washed three times each for 10 min with PBS at pH 7.4 and treated with the fluorescent dye Alexa 488 (green color) (Molecular Probes) for 10 min at room temperature.

For the second labeling, the sections were blocked for 1 h with a blocking solution containing 0.5% Triton in PBS + 10% donkey serum + 1% BSA. Then, the sections were incubated overnight at room temperature with the mouse anti-cytochrome oxidase (1 : 100 dilution; Molecular Probes). Next, the sections were incubated with the donkey anti-mouse secondary antibody labeled with Alexa 594 (red color) for 1 h at room temperature. They were coverslipped with Cytoseal and photographed using a confocal microscope.

Statistical Considerations

The software STATISTICA (Tulsa, OK) was used for statistical analysis. The mean mRNA fold change, standard deviation, and standard error were calculated for each mitochondrial gene investigated for real-time PCR (*see* Tables 3 and 4 for details). To determine the statistical significance of mRNA fold changes between early AD and definite AD groups, we used a two-sample *t*-test.

Results

mRNA Expression of Mitochondrial-Encoded Genes

To determine the role of mitochondrial-encoded genes in AD pathogenesis, using quantitative real-time PCR analysis with Sybr-Green fluorescent dye, we calculated the mRNA fold change for 11 (out of the total 13) mitochondrial-encoded genes in complexes I, III, IV, and V of oxidative phosphorylation in both early AD and definite AD brain specimens (Table 3).

Complex I

The mRNA fold changes were decreased for all subunits in the frontal cortex brain specimens studied in five out of the six early AD patients and in three out of the six definite AD brain specimens, suggesting that mRNA expressions are altered in early AD patients (Table 3). Further, the decrease of mRNA fold change was higher for subunit 1 compared to all other subunits studied, suggesting that subunit 1 is critical for oxidative phosphorylation (Tables 3 and 4). In addition, mRNA levels were increased in brain specimens from three out of the six definite AD patients, suggesting a tendency for mRNA expression to increase during AD progression.

To determine statistical significance, if any, between early AD patients and definite AD patients for mitochondrial gene expressions, we calculated *t* values for all subunits of OXPHOS genes. We found no statistically significant difference between early AD and definite AD patients for all mitochondrial genes studied (Table 4). However, the mean mRNA fold change difference was large for subunit 1 of complex I. There was also a large patient–patient mitochondrial gene expression variation for both early and definite AD patients (Tables 3 and 4).

Complex III

As shown in Table 3, cytochrome-*b* mRNA levels increased for both early AD (with a range of 1–33 fold) and definite AD brain specimens (with a range of 1–100 fold), and the mean mRNA fold change was 9.5 for early AD brain patients and 20.5 for definite AD patients, suggesting a progressive increase in mRNA levels from early to definite AD progression (Table 4).

Complex IV

As shown in Table 3, mRNA levels increased in all three subunits of cytochrome oxidase for both early and definite AD brain specimens. The mean mRNA fold change was 22.1 for early AD brain specimens, 27.9 for definite AD brain specimens in subunit 1, 10.4 for early AD brain specimens, 21.4 for definite AD brain specimens in subunit 2, 2.1 for early AD, and 1.1 for definite AD brain specimens in subunit 3.

Complex V

The mRNA levels increased in two of six early AD brain specimens, four of six definite AD brain specimens for ATPase 6, and three of six early AD and four of six definite AD cases for the ATPase 8 subunit of complex V (Tables 3 and 4). Although the mean mRNA fold change of early AD cases was not different from that of definite AD cases, there was a trend for mRNA to increase in definite AD brain

Table 3
mRNA Fold Changes of Mitochondrial Genes in the Frontal Cortex Brain Specimens From Early AD and Definite AD Patients

		NADH SU 1	NADH SU 2	NADH SU 3	NADH SU 4	NADH SU 6	Cyt. b	COX 1	COX 2	COX 3	ATPase 6	ATPase 8
Early AD	7	–2.0	–2.6	–1.2	–1.3	–1.1	9.1	7.1	1.4	1.4	2.7	1.9
	8	15.4	12.1	11.6	9.1	25.0	33.0	100.0	50.0	11.1	5.3	2.1
	9	dna	dna	dna	–10.4	–1.6	1.0	1.3	–1.4	–2.5	–4.2	–1.4
	10	–130.5	–9.9	–5.9	–7.9	–1.1	1.4	1.2	1.8	–1.9	–1.8	–3.6
	11	–16.7	–14.1	–13.3	1.3	9.1	9.1	16.6	6.6	2.9	1.1	5.0
	12	–6.3	–1.4	1.5	–1.2	2.0	3.1	6.6	4.1	1.9	2.8	1.8
Definite AD	13	–4.1	–2.5	–1.6	–18.2	1.1	1.9	4.1	1.0	–9.0	dna	–3.5
	14	–120.5	–12.1	–4.0	–9.5	–4.9	–2.7	–3.2	–3.0	–9.1	–3.7	–3.5
	16	–1.5	–21.5	–2.8	dna	9.1	1.0	2.0	11.1	–2.0	–6.9	–1.4
	17	1.1	3.6	2.6	2	7.1	14	50.0	14.3	7.7	3.3	12.5
	18	8.6	2.2	dna	4.3	4.0	6.6	14.3	4.8	16.6	2.7	25.0
	19	–112.4	14.9	40.6	1.4	10.0	100.0	100.0	100.0	2.6	4.1	1.8

Note: dna = data not available.

Table 4
Statistical Analysis of mRNA Fold Changes in the Frontal Cortex Brain Specimens From Early AD and Definite AD Patients

Gene	Mean definite AD	Mean early AD	Mean difference	Standard error	*t* Value	*p*-Value (parametric)	*p*-Value (nonparametric)
NADH 1	–38.1	–28.0	–10.1	36.2	–0.28	0.79	0.72
NADH 2	–2.6	–3.2	0.6	7.0	0.09	0.94	0.72
NADH 3	7.0	–1.5	8.41	9.4	0.89	0.40	0.61
NADH 4	–4	–1.7	–2.3	5.0	–0.45	0.67	0.80
NADH 6	4.4	5.4	–1.0	4.8	–0.20	0.84	0.68
Cyt. b	20.5	9.5	10.7	16.9	0.64	0.55	0.81
COX 1	27.9	22.1	5.7	22.7	0.25	0.81	0.94
COX 2	21.4	10.4	11.0	17.8	0.61	0.55	0.64
COX 3	1.1	2.1	–1.0	4.6	–0.22	0.83	0.75
ATPase 6	0.98	–0.11	–1.09	2.52	–0.44	0.67	0.93
ATPase 8	5.15	0.97	4.18	24.34	–0.87	0.42	1.00

specimens, suggesting that while AD progresses, there is an increasing demand for mitochondrial gene expression.

Immunofluorescence Analysis of Mitochondrial-Encoded Genes in Brain Specimens From AD Patients and Control Subjects

To determine the localization of mitochondrial gene expressions and to understand differentially expressed neurons, using immunofluorescence techniques, we studied the NADH 15-kDa subunit in complex I, cytochrome oxidase subunit 1 in complex IV, and ATPase δ-subunit in complex V from the frontal cortex brain sections from AD patients and control subjects. Our immunofluorescence analysis revealed that cytochrome oxidase (Fig. 1A,B) and ATPase protein levels (Fig. 2A,B) were high in the brains sections of AD patients compared to those of control subjects. In real-time PCR analysis of mitochondrial genes in complex I, we found decreased subunit 2 expressions in the brain specimens of AD patients (Fig. 3A) compared to those of control subjects (Fig. 3B). We also noted that these altered gene expressions were selective in cortical layers II and III and in deep layers IV and VI (data not shown).

Immunofluorescence of 8-OHG in Brain Specimens From AD Patients and Control Subjects

To determine oxidative damage, using anti-8-OHG antibody, we studied the expression of 8-OHG in the brain specimens of AD patients and control subjects. Our immunofluorescence analysis revealed an increased expression of 8-OHG in the cytoplasm of cortical neurons in the brain sections of AD patients (Fig. 4A) compared to control subjects (Fig. 4B), suggesting oxidative damage in AD patients. We also noted 8-OHG-positive neurons in the brain sections from control subjects, suggesting that oxidative damage is related to the aging process (Fig. 4B).

Double-Labeling Immunofluorescence Analysis of Cytochrome Oxidase and 8-OHG in Brain Specimens From AD Patients and Control Subjects

To determine whether neurons showing increased cytochrome oxidase are vulnerable to oxidative damage, we conducted double-labeling immunofluorescence analysis. This analysis of cytochrome oxidase (green in Fig. 5A) and 8-OHG (red in Fig. 5B) revealed that overexpressed neurons

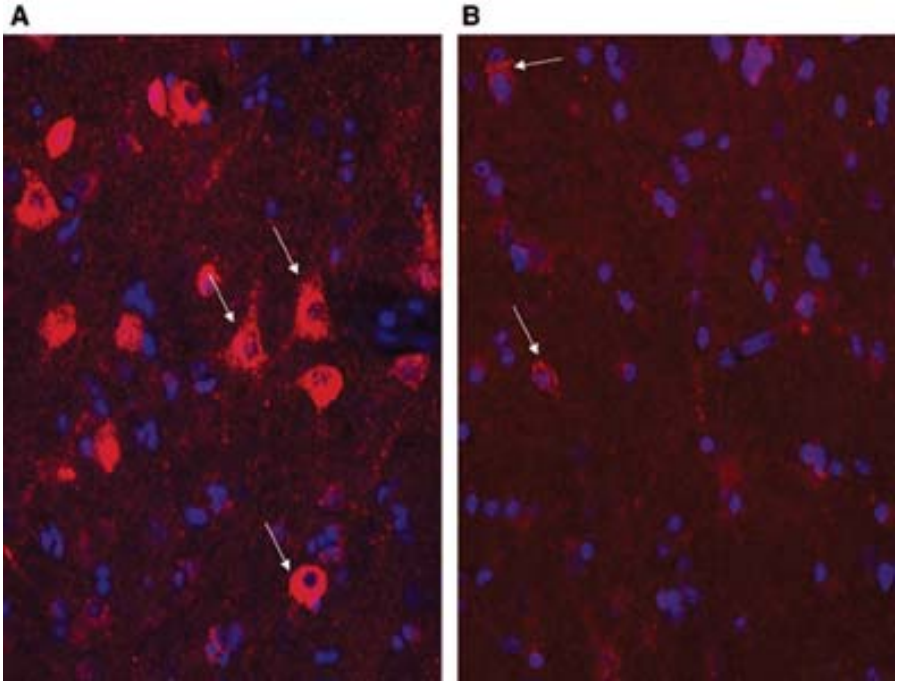

Fig. 1. Immunoreactivity with anti-cytochrome oxidase. Increased cytochrome oxidase immunofluorescence in red is shown in the cytoplasm of cortical pyramidal neurons in the brain sections of AD patients **(A)** compared to control subjects **(B)**. Blue nuclear counterstaining (with Hoechst) in nucleated cells suggests that selective neurons are overexpressed for cytochrome oxidase in AD patients. Arrows indicate overexpressions of cytochrome oxidase. Images were photographed at ×400 the original magnification of sections.

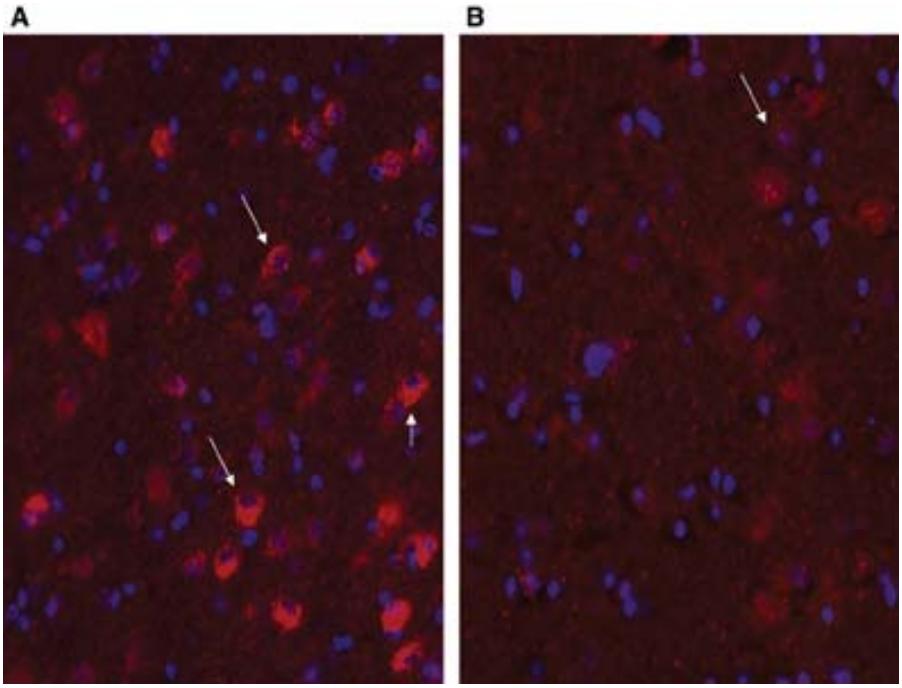

Fig. 2. Immunoreactivity with anti-ATPase. Increased ATPase immunofluorescence in red is shown in the cytoplasm of cortical pyramidal neurons in the brain sections of AD patients **(A)** compared to control subjects **(B)**. Blue nuclear counterstaining (with Hoechst) in nucleated cells suggests that selective neurons are overexpressed for ATPase in AD patients. Arrows indicate the overexpressions of ATPase. Images were photographed at ×400 the original magnification of sections.

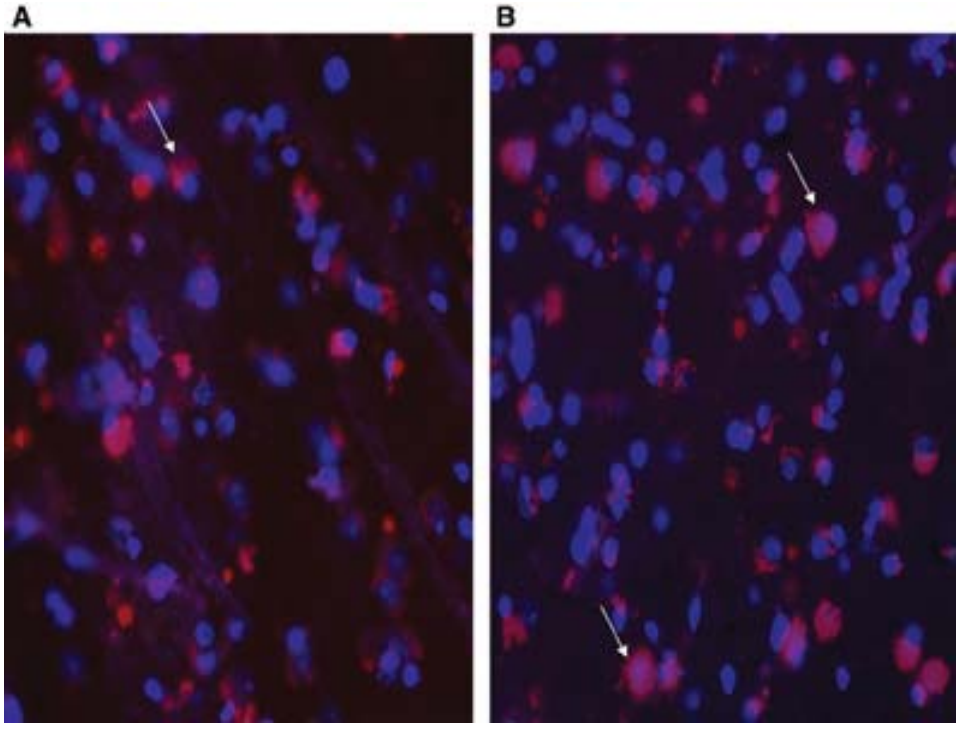

Fig. 3. Immunoreactivity with the anti-NADH 15-kDa subunit. Decreased NADH-ubiquinone is shown in the cytoplasm of neurons in the brain sections of AD patients **(A)** compared to control subjects **(B)**. Blue nuclear counterstaining (with Hoechst) in nucleated cells from AD brain specimens suggests that selective neurons exhibit decreased expression of NADH-ubiquinone. Arrows indicate expressions of NADH-ubiquinone. Images were photographed at ×400 the original magnification of sections.

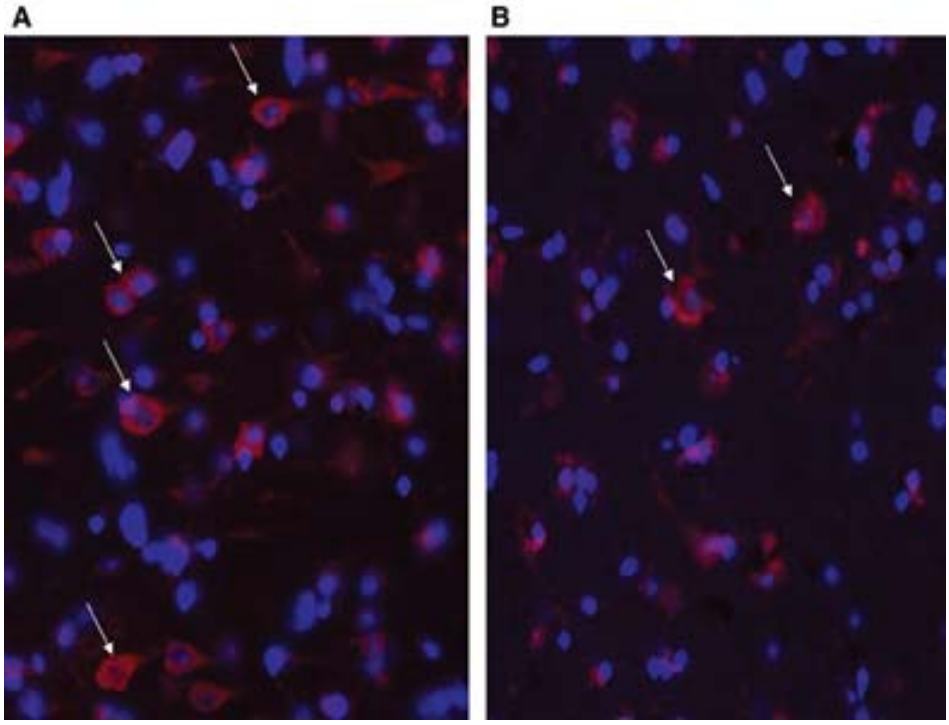

Fig. 4. Immunoreactivity with anti-8-OHG. Increased 8-OHG is shown in the cytoplasm of neurons in the brain sections of AD patients **(A)** compared to control subjects **(B)**. Blue nuclear counterstaining (with Hoechst) in nucleated cells from AD brain specimens suggests that selective neurons show a decreased expression of 8-OHG in AD. Arrows indicate expressions of 8-OHG. Images were photographed at ×400 the original magnification of sections.

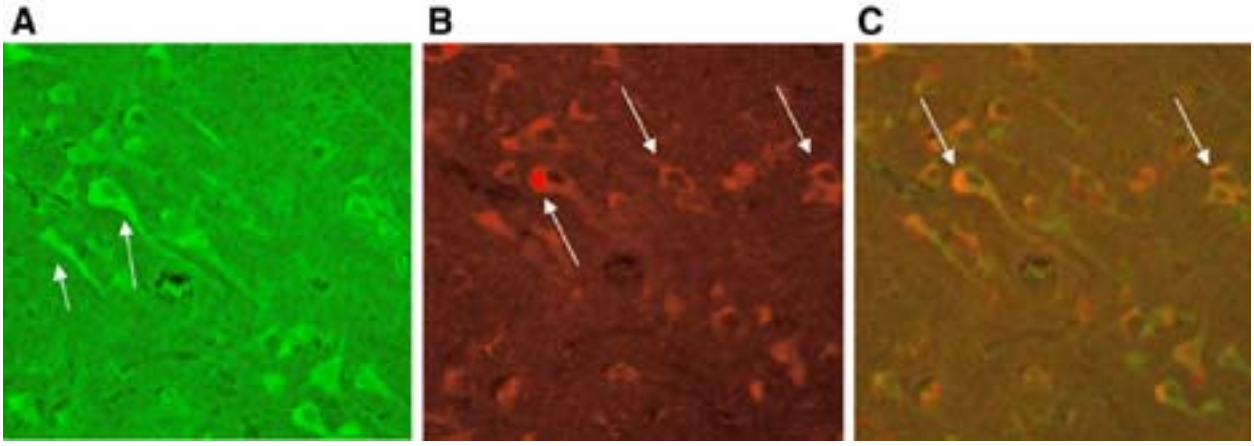

Fig. 5. Double-labeling immunofluorescence analysis of cytochrome oxidase and 8-OHG: **(A)** cytochrome oxidase immunoreactivity in the frontal cortex brain section of an AD patient; **(B)** 8-OHG immunoreactivity of the same section labeled with cytochrome oxidase; **(C)** the overlay of both cytochrome oxidase and 8-OHG immunoreactivities from the same section. Arrows indicate expressions of cytochrome oxidase and 8-OHG.

with cytochrome oxidase in the brains sections of AD patients showed positive immunoreactivity for 8-OHG (overlaid with both cytochrome oxidase (green) and 8-OHG (red); Fig. 5C).

Discussion

To understand the role of mitochondrial abnormalities in AD pathogenesis, it is critical to determine the connection, if any, between altered mitochondrial gene expression and the pathogenesis of AD, for all mitochondrial polypeptides in oxidative phosphorylation. Using highly sensitive, quantitative real-time PCR techniques (Gutala and Reddy, 2004), in the present study we calculated mRNA fold changes for 11 mitochondrial-encoded polypeptide genes in 6 brain specimens from early AD and 6 definite AD patients. Further, using immunofluorescence techniques, we studied differentially expressed mitochondrial genes in these brain specimens.

The development of real-time PCR techniques has revolutionized gene expression studies (Bustin, 2000; Aldea et al., 2002; Olney et al., 2002), particularly in detecting and quantifying mRNA levels in postmortem intervals up to 24 h (Gutala and Reddy, 2004). Unlike Northern blot analysis, which requires 5–10 µg of total RNA, real-time PCR techniques require only 1 µg total RNA for RT-PCR reactions (Gutala and Reddy, 2004). Further, in combination with Sybr-Green chemistry, real-time PCR enhances the sensitivity of mRNA expression, allows for high throughput, and reduces post-PCR manipulations (Bustin, 2000; Aldea et al., 2002; Olney et al., 2000).

In the present study, although the number of fold changes in the cytochrome-*b* and cytochrome oxidase subunits were high in some brain specimens, we found all subunits of complex I consistently downregulated. We also found cytochrome-*b* in complex III and all three subunits of cytochrome oxidase in complex IV consistently upregulated in the brain specimens of AD patients compared to those of control subjects.

Downregulation of Mitochondrial-Encoded Polypeptide Genes in Complex I of Oxidative Phosphorylation

The present study revealed decreased mitochondrial gene expression for all subunits in the frontal cortex brain specimens of both early AD and definite AD patients, suggesting that mRNA expressions are altered early in AD pathogenesis (Table 3). Further, we found that the decrease of mRNA fold changes was higher for subunit 1 compared to all other subunits studied, suggesting that subunit 1 is critical for complex I of OXPHOS. Our findings of the downregulation of

complex I subunits agree with earlier studies (Aksenov et al., 1999; Fukuyama et al., 1996; Chandrasekaran et al., 1997). Our study is the first gene expression study investigating 11 subunits of OXPHOS complexes.

A possible reason for the decreased expression of complex I subunits in both early and definite AD patients might be the presence of DNA mutations in complex I subunits (Cavelier et al., 2001; Lin et al., 1992). A recent investigation of mitochondrial mutations in OXPHOS polypeptide genes in 17 AD patients, 10 elderly control subjects, and 14 younger control subjects by Lin et al. (2002) revealed a high mutation rate in complex I polypeptide genes in AD patients and elderly control subjects compared to the mutation rate of younger control subjects, suggesting that DNA mutations in complex I subunits might be responsible for abnormalities of OXPHOS electron transport in AD patients and elderly control subjects. The presence of a high mutation rate in complex I subunits in AD patients and elderly control subjects observed by Lin et al. (2002) might explain partly the decreased mRNA expression of complex I subunits that we observed in AD patients in this study. A thorough investigation of both gene expressions and DNA defects in the complex I subunits in the brain specimens of AD patients and elderly control subjects might provide a precise connection between gene expressions and complex I DNA defects.

We also found a gradual decreased downregulation of gene expression in complex I subunits from subunits 1 to 6 in AD specimens. Further, we found a gradual decreased downregulation of mRNA expression in early AD brain specimens compared to definite AD brain specimens. Although we found a statistically insignificant difference in mitochondrial gene expression between early AD and definite AD brain specimens, mitochondrial gene expression tended to increase in early through definite AD brain specimens, suggesting that mitochondrial abnormalities might be early events in the disease process and might be responsible for the progression of AD.

*Upregulation of Cytochrome-*b*, Cytochrome Oxidase, and ATPase Subunits*

In the brain specimens of both early and definite AD patients, the present study revealed an increased expression of cytochrome-*b* in complex III and in subunits 1 and 2 of cytochrome oxidase of OXPHOS. This finding suggests that, in both early AD and definite AD patients, there is a great demand for mRNA expression in cytochrome-*b* of complex III and subunits 1 and 2 in complex IV of OXPHOS in the brain specimens. The increased gene expressions in complex III and IV genes might compensate for the decreased ATP production in the brains of AD patients (Hatanpaa et al., 1998; Strazielle et al., 2003). However, in the same brain specimens that showed increased expressions of cytochrome-*b* and COX 1 and 2 (Table 3), we also found decreased downregulation of subunit 3 in complex III and of ATPase 6 and 8 in complex V. These decreases could be the result of unexplained compensatory mechanisms acting simultaneously in OXPHOS of the electron transport in AD patients.

Our study provides evidence that altered mitochondrial gene expressions occur in early AD patients, suggesting that mitochondrial defects might lead to oxidative damage early in the progression of AD. Oxidative stress could cause an imbalance between the generation of reactive oxygen species and an antioxidant defense mechanism. Important factors that may favor oxidative stress in the brains of AD patients include the brain's high oxygen-consumption rate, its abundant polyunsaturated fatty acid content, the short lifespan of mitochondrial DNA, the high rate of mitochondrial DNA defects, and the relative lack of antioxidant defenses in the brain compared to other tissues (Bonilla, 1999).

Another interesting finding from our study is the large patient-to-patient variation of mitochondrial mRNA expressions in a small portion of both early and definite AD brain specimens (Tables 3 and 4). This patient–patient mitochondrial gene expression variation cannot be explained by the presence of pathological features, such as number of senile plaques or neurofibrillary tangles, or neuronal density.

It is possible that long postmortem intervals might contribute to the degradation of high-molecular-weight mRNA. However, in the brain specimens of the present study, we recently found that factors such as postmortem intervals up to 24 h and age at death did not influence mRNA expression (Gutala and Reddy 2004). Further, in most of the brain specimens from both early and definite AD patients, we found

the downregulation of complex I subunits and the upregulation of cytochrome-*b* in complex III and of cytochrome oxidase subunits 1 and 2 (Table 3), suggesting that differential mitochondrial expression might be related to molecular defects of OXPHOS genes rather than RNA degradation because of long postmortem delays of brain specimens.

Immunofluorescence Analysis of Mitochondrial Genes and Oxidative Damage in the Brain Specimens of AD Patients and Control Subjects

We found an increased expression of cytochrome oxidase and ATPase in the brain sections of early AD patients, suggesting that cytochrome oxidase, ATPase mRNAs, and proteins are elevated in AD patients. Further, increased cytochrome oxidase and ATPase expressions were found mainly in the cytoplasm of pyramidal neurons in the cortex (Figs. 1 and 2), suggesting that these overexpressions are neuronal-specific. Our findings are in agreement with recent findings of increased mtDNA and cytochrome oxidase in the pyramidal neurons of brain sections of AD patients by Hirai et al. (2001).

We also found increased oxidative damage marked by 8-OHG in the brain sections of AD patients compared to those of control subjects (Fig. 4A,B). However, we also observed immunoreactive-positive neurons for 8-OHG in the brain sections of control subjects, suggesting that oxidative damage is related to the aging process.

Our double-labeling immunofluorescence analysis revealed that oxidative damage, marked by 8-OHG, increased mainly in the same neurons showing increased cytochrome oxidase, suggesting that mitochondrial dysfunction might lead to oxidative damage in AD brains. We also noted that the intensity of immunoreactivity of 8-OHG was less than cytochrome oxidase immunoreactivity in the neurons of AD patients (Fig. 5A–C). Further, not all overexpressed cytochrome neurons were positive for 8-OHG immunoreactivity, suggesting that neurons undergo oxidative damage at different stages in AD patients (Fig. 5A–C).

In previous gene expression analyses of the cerebral cortex of APP transgenic mice (Tg2576), which was compared to age-matched, wild-type mice at ages long before (2 mo), immediately before (5–6 mo), and after (18 mo) the appearance of amyloid plaques and cognitive impairment, we found at 2 mo of age that upregulated genes were associated with mitochondrial genes of oxidative phosphorylation of electron transport and oxidative stress, and these same genes were progressively upregulated in 5- and 18-mo-old APP mice (Reddy, unpublished observations). These findings suggest that mutant APP and soluble Aβ peptides may target mitochondria in the cell cytoplasm, leading to mitochondrial dysfunction. To compensate for the deficiency of OXPHOS electron transport, mitochondrial genes might be activated in APP mice early in life (Reddy, unpublished observations). Our observations are also supported by increased cytochrome oxidase immunoreactivity in selective brain regions, including the hippocampus and the anterior cingulated and primary visual cortex in APP23 transgenic mice, which overexpressed the 751 amino acid isoform of the β-amyloid precursor protein with the Swedish mutation (Strazielle et al., 2003).

In summary, our quantitative real-time PCR analysis revealed the downregulation of mitochondrial-encoded genes in complex I of OXPHOS in both early and definite AD brain specimens. Contrary to this downregulation, complexes III and IV showed increased mRNA expression in both early and definite AD brain specimens, suggesting a great demand for ATP production in OXPHOS, probably the result of energy deficits in AD brains. Further, we also observed a large patient-to-patient mitochondrial gene expression variation, suggesting that mitochondrial DNA defects are responsible for the heterogeneity of the phenotype in AD brain specimens. Our immunofluorescence analysis suggests that only a subpopulation of neurons is affected by mitochondrial abnormalities in AD patients. Our double-labeling immunofluorescence analysis of 8-OHG and cytochrome oxidase suggests that in AD brains, only selective, overexpressed neurons with cytochrome oxidase undergo oxidative damage. Based on these results, we propose that an increase in cytochrome oxidase gene expression could be the result of functional compensation by the surviving neurons or an early mitochondrial alteration related to increased oxidative damage.

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