Increased F_2-isoprostanes in Alzheimer's disease: evidence for enhanced lipid peroxidation *in vivo*

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ABSTRACT **Alzheimer's disease (AD) includes a group of dementing neurodegenerative disorders that have diverse etiologies but the same hallmark brain lesions. Since oxidative stress may play a role in the pathogenesis of AD and isoprostanes are chemically stable peroxidation products of arachidonic acid, we measured both $iPF_{2\alpha}$-III and $iPF_{2\alpha}$ -VI using gas chromatography-mass spectrometry in AD and control brains. The levels of both isoprostanes, but not of 6-keto $PGF_{1\alpha}$, an index of prostaglandin production, were markedly elevated in both frontal and temporal poles of AD brains compared to the corresponding cerebella. Levels were also elevated compared to corresponding areas of brains from patients who had died with schizophrenia or Parkinson's disease or from nonneuropsychiatric disorders. $iPF_{2\alpha}$ -IV, but not $iPF_{2\alpha}$-III, levels were higher in ventricular CSF of AD brains relative to the non-AD brains. These data suggest that specific isoprostane analysis may reflect increased oxidative stress in AD.—Praticò, D., Lee, V. M.-Y., Trojanowski, J. Q., Rokach, J., FitzGerald, G. A. Increased F_2-isoprostanes in Alzheimer's disease: evidence for enhanced lipid peroxidation *in vivo*. *FASEB J.* 12, 1777–1783 (1998)**

Key Words: oxidative stress · schizophrenia · temporal lobes · neurofibrillary tangles

ALZHEIMER'S DISEASE (AD)[2] is a neurodegenerative disorder characterized by a progressive decline in cognitive function as well as by numerous amyloid plaques, neurofibrillary tangles (NFTs), and extensive neuronal loss in the brains of AD patients (1). Although epidemiologic studies have failed to identify a single cause of AD (2), genetic studies have implicated several mutations in three separate genes on different chromosomes that encode the amyloid-β (Aβ) precursor proteins (APP), presenilin-1 (PS-1), and presenilin-2 (PS-2) as the cause of autosomal dominantly inherited AD in a subset of kindreds with familial AD (FAD) (3, 4). In addition, the ε4 allele of the apolipoprotein E (APOE) gene has been shown to be a genetic risk factor for AD (4). However, all of the known FAD mutations account for fewer than 5% of affected patients, since the majority of AD cases are sporadic and there is only modest evidence in support of familial clustering (5).

Despite this heterogeneity, common factors may be involved in the pathogenesis of both hereditary and sporadic AD. These factors may promote the formation of Aβ deposits and NFTs, as well as the massive degeneration of neurons in selected regions of all AD brains (1). It has been suggested that the abnormal processing or production of Aβ and plaque formation are pivotal events in the pathogenesis of the disease (6, 7). Aggregated, but not monomeric, species of Aβ are hypothesized to induce the dysfunction and death of neurons *in vitro* by a range of mechanisms (8–10). It has been proposed that AD brain regions with accumulations of numerous Aβ-rich senile plaques (SPs) are loci of elevated oxidative stress (11), perhaps reflective of an inflammatory reaction. Furthermore, it has been suggested that oxidant stress may be of functional importance in the pathogenesis of AD and that the production of reactive oxygen species (ROS) in the brain leads to lipid peroxidation and neuronal degeneration in AD (12). Efforts to elucidate the role of lipid peroxidation and oxidative stress *in vivo* have been hampered by the availability of markers of limited value due to their chemical instability or lack sensitivity or specificity (13). Although there are few studies of lipid peroxidation in the AD brain, they have provided evidence for increased lipid peroxidation by measuring levels

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[2] Abbreviations: Aβ, amyloid-β; AD, Alzheimer's disease; Apo, apolipoprotein; APOE, ε4 allele of the apolipoprotein E; APP, Aβ precursor proteins; BHT, butylated hydroxytoluene; COX, cyclooxygenase; CSF, cerebrospinal fluid; FAD, familial AD; FPs, frontal poles; iP, isoprostanes; NFTs, neurofibrillary tangles; 8-OhdG, 8-hydroxy-2-deoxyguanosine; PD, Parkinson's disease; PG, prostaglandin; PMI, postmortem interval; PS-1, presenilin-1; ROS, reactive oxygen species; SCHI, schizophrenia; SPs, senile plaques; TBARS, thiobarbituric acid reactive substances; TPs, temporal poles.

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TABLE 1. *Characterization of study groups by number, male-to-female ratio, age, postmortem interval*[a]

Study group	*n*	Gender (M/F)	Age (year)	PMI (h)
AD	19	10/9	79 ± 2.1 (56–92)	10.3 ± 1.4 (4–17)
PD	6	6/0	68 ± 8 (34–80)	8.7 ± 1.6 (5–15)
SCHI	10	5/5	75 ± 1.2 (69–82)	11.8 ± 1.1 (7–16.5)
CONTROLS	8	5/3	76 ± 4.8 (60–98)	11.4 ± 1.4 (5–16)

[a] Age, postmortem interval (PMI) are reported as means ± SEMs; ranges are given in parentheses. M/F, male-to-female ratio.

of thiobarbituric acid reactive substances (TBARS) (14–17). However, the validity of this method is limited because it measures other aldehydes conjugated to TBARS, as well as nonlipid related chromogens. Recently, two separate groups of investigators reported no difference in the level of TBARS and lipid hydroperoxides in AD vs. control brains (18, 19). Immunohistochemical data suggesting the presence of by-products of lipid peroxidation (i.e., 4-hydroxynonenal) in AD brain have also been reported (20, 21), but only one study has reported quantitative estimate of 4-hydroxynonenal levels in postmortem cerebrospinal fluid (CSF) of AD brains (22).

Isoprostanes (iP) are prostaglandin (PG) isomers that are produced by free radical attack on arachidonic acid in situ in membrane phospholipids (23). They offer advantages over other indices of lipid peroxidation. For instance, in contrast to lipid hydroperoxides, which rapidly decompose, isoprostanes are chemically stable end-products of lipid peroxidation that are released by phospholipases, circulate in plasma, and excreted in urine (24). While isomers of prostaglandins, leukotrienes, and epoxyeicosatrienoic acids may be formed in this manner, attention has been focused on isomers of $PGF_{2\alpha}$, the F_2-isoprostanes ($F_{2\alpha}$-iPs). We have developed specific and sensitive methods to measure two distinct F_2-isoprostanes (25, 26): 8-iso-$PGF_{2\alpha}$, now known as $iPF_{2\alpha}$-III, and $IPF_{2\alpha}$-I, now known as $iPF_{2\alpha}$-VI (27). Analysis of these compounds has shown that they reflect lipid peroxidation *in vitro* (28) and *in vivo* (29, 30). Given the mechanism of their formation and clearance, isoprostanes may reflect lipid peroxidation at the tissue site of free radical generation or in body fluids like plasma and urine. For example, we have shown that $iPF_{2\alpha}$-III is elevated in human atherosclerotic plaques, where it is localized to monocyte/macrophages and smooth muscle cells (31), and in circulating low density lipoprotein as well as in the urine from hypercholesterolemic subjects (32).

In the present study, we demonstrate that the levels of both these F_2-isoprostanes were increased selectively in affected regions of AD brains, i.e., in frontal and temporal cortex, but not in the cerebellar cortex. Furthermore, we detected higher levels of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI in samples of AD frontal and temporal cortex compared to those from patients lacking evidence of central inflammation: those with Parkinson's disease (PD), schizophrenia (SCHI) and neuropathologically normal controls. Levels of $iPF_{2\alpha}$-VI were also elevated in AD CSF. Our results are consistent with the hypothesis that oxidative stress is a feature of AD, and that the determination of isoprostane levels in CSF or other body fluids could be exploited to develop novel tests to reflect disease activity in AD in living patients. Finally, the mechanism of oxidative damage in the AD brain could become a target for the design of new therapeutic agents to arrest or slow the progression of AD.

MATERIALS AND METHODS

Brain tissue samples

Brain specimens were obtained at autopsy from 19 patients with AD, 6 with PD, 10 with SCHI, and 8 control subjects. Postmortem diagnostic evaluation of the patients and controls studied here was performed in accordance with previously described procedures and criteria that have been used in earlier studies from our laboratory (33–35). The control subjects had no history of dementia, other neurological diseases, and systemic illnesses affecting the brain, and neuropathologic examination did not reveal any significant abnormalities in these control brains. Frozen (−80°C), unfixed samples (1–5 mg) of frontal pole (FP), temporal pole (TP), and cerebellum were used for isoprostane analysis,. Clear, blood-free CSF was obtained at autopsy from the lateral ventricle of additional AD (n=15) (10 males, 5 females, age 65–85 years old) and non-AD (n=10) (7 males, 3 females, age 67–86 years old) control brains. The CSF was centrifuged at 1500 rpm for 10 min, aliquoted, and stored at −80°C until analysis. All of the CSF and brain samples were coded, so subsequent analysis of these samples was performed without knowledge of the age or diagnosis of the individuals from whom they were obtained or the regional identity of the brain tissues.

Brain sample extracts

The samples of FP, TP, and cerebellum were minced and resuspended in phosphate buffered saline containing 10 mM EDTA and 1 mM butylated hydroxytoluene (BHT) to prevent auto-oxidation. During sample extraction and processing, 10 μg of deuterium-labeled 2-arachidonyl phosphatidylcholine and 10 μg of [$^2H_8$]arachidonic acid were also added to the samples to monitor the artifactual formation of F_2-isoprostanes and 6-keto $PGF_{1\alpha}$, respectively. After homogenization with a blade homogenizer, total lipids were extracted with 20 ml ice-cold Folch solution, chloroform/methanol (2:1, v/v) . The solution was then vortexed and centrifuged at 800 × g for 15 min at 4°C. The organic phase containing the extracted lipids was dried under nitrogen; then, 5 ml aqueous KOH (15%) was added and the mixture was incubated at 45°C for 1 h to effect hydrolysis and release of total (esterified plus free) $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI. Finally, 6-keto $PGF_{1\alpha}$ levels were assayed in the same specimens as outlined below.

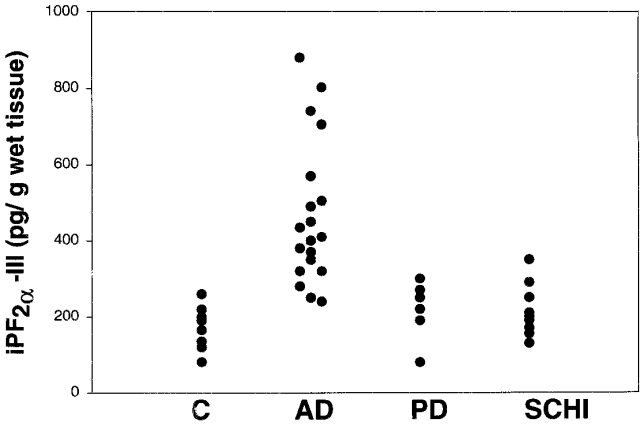

Figure 1. $iPF_{2\alpha}$-III levels in brain frontal pole from normal controls (C), Alzheimer's disease (AD), Parkinson's disease (PD), and schizophrenia (SCHI) patients.

Biochemical analysis

The levels of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI and 6-keto $PGF_{1\alpha}$ were assayed by gas chromatography/mass spectrometry assay as previously described (25, 26), . Briefly, known amounts of the internal standards [$^{18}O_2$]-$iPF_{2\alpha}$-III, [$^2H_4$]-$iPF_{2\alpha}$-VI, or [$^2H_4$]-6-keto $PGF_{1\alpha}$ were added to the samples, which were subjected to solid phase extraction, then derivatized and purified by two thin-layer chromatography steps. Finally, each sample was analyzed on a Fisons MD-800 (Fisons Instruments, Milan, Italy) gas chromatography/mass spectrometer, and quantification was performed using peak ratios. The linearity of these assays over the detection range has previously been described (26, 29). The limit of detection is 0.5–1 pg/mg brain tissue and 0.5–1.0 pg/ml CSF for both isoprostanes.

Statistical analysis

Data are presented as means ±SEM. Median values and range are given for $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-VI, and 6-keto $PGF_{1\alpha}$. Statistical analysis was performed by analysis of variance with subsequent pairwise comparison by two-tailed *t* test, as appropriate. Only *P* values lower than 0.05 were regarded as statistically significant. Correlations between the two isoprostanes, the isoprostanes and the postmortem interval (PMI), age, or the duration of the disease in AD patients were examined by using linear regression.

RESULTS

The clinical and autopsy data on the patient groups are shown in **Table 1**. There were no significant differences in age and PMI between the groups. The level of $iPF_{2\alpha}$-III in the FP of AD brains was markedly elevated [median(range) pg/g wet tissue, 410 (240–880) pg/g wet tissue] compared to the brains of PD [230 (80–300) pg/g wet tissue, $P=0.004$], SCHI [280 (130–380) pg/g wet tissue, $P<0.001$], or normal control [200 (81–260) pg/g wet tissue, $P=0.002$] subjects (**Fig. 1**). Similar increases in the levels of $iPF_{2\alpha}$-III were seen in the TP from AD relative to the controls. The levels were [median(range) pg/g wet tissue] as follows: AD [445(250–685)], PD [303(160–350)], SCHI [223(130–300)], and normal controls [205(110–300)]. No statistically significant correlation was observed between age, PMI, or disease duration and the levels of $iPF_{2\alpha}$-III in the FP or TP samples from AD brains (data not shown).

Levels of $iPF_{2\alpha}$-VI in the AD brains were higher than those for $iPF_{2\alpha}$-III and ranged from 605 to 1790, with a median of 950 pg/g wet tissue in the FP of AD brains. The corresponding values were 650(400–894), $P = 0.004$ in PD brains; 400(290–510), $P < 0.001$ in SCHI brains; and 460(300–700) ($P<0.001$) pg/g wet tissue in the normal control brains (**Fig. 2**). Similar results were obtained from assays of the levels of $iPF_{2\alpha}$-VI in the TP samples, where values [median(range) pg/g wet tissue)] were [1100 (700–1880)] for AD, [700 (500–950)] for PD, [355 (220–420)] for SCHI, and [480 (320–650)] for normal controls. Once again, there was no significant correlation between age, PMI, or disease duration and $iPF_{2\alpha}$-VI levels in any of these AD patients (data not shown).

However, there was a significant correlation between the levels of these two isoprostanes in both FP brain cortex (r=0.73; **Fig. 3**) and TP samples from AD patients (data not shown). To determine whether the postmortem procedures for isoprostane analysis artifactually produced $iPF_{2\alpha}$-III or $iPF_{2\alpha}$-VI from arachidonyl-containing phospholipids in these brain samples, we incubated deuterated phosphatidylcholine (10 μg) with the tissue at the time of the processing. No artifactual formation of $iPF_{2\alpha}$-III or $iPF_{2\alpha}$-VI was detected during the processing procedure used here (data not shown).

We also measured the levels of a product of cyclooxygenase (COX) -dependent metabolism of arachidonic acid, the hydrolysis product of prostacyclin, prostaglandin 6-keto $PGF_{1\alpha}$ in the same specimens. In sharp contrast to the isoprostanes, there was no difference in the levels of 6-keto $PGF_{1\alpha}$

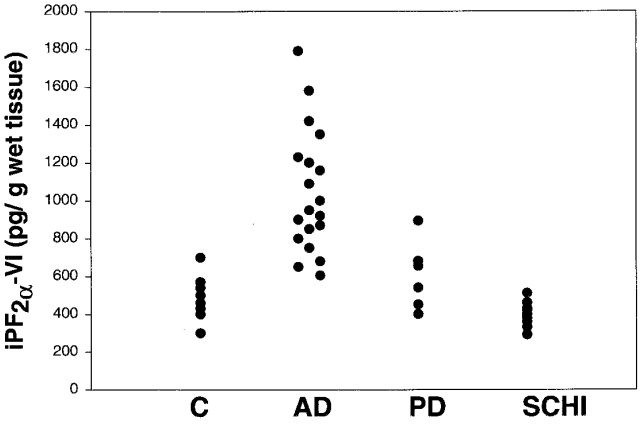

Figure 2. $iPF_{2\alpha}$-VI levels in brain frontal pole from normal controls (C), Alzheimer's disease (AD), Parkinson's disease (PD), and schizophrenia (SCHI) patients.

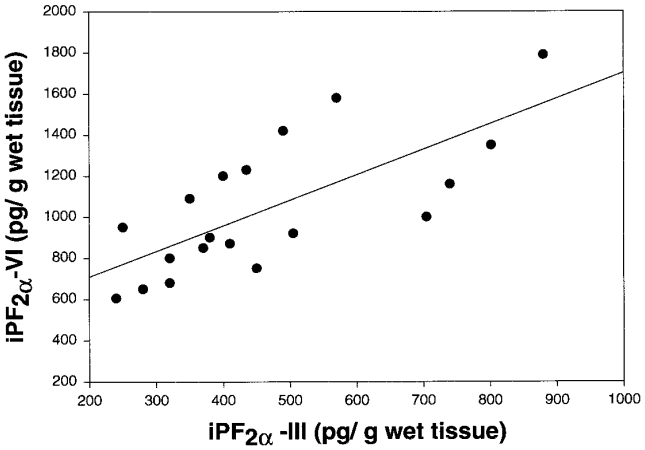

Figure 3. Correlation between $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI levels in brain Frontal Pole from Alzheimer's disease (AD) patients (P=0.0005).

between AD and non-AD control groups studied here (**Table 2**). No formation of $[^2H_8]$-6-keto $PGF_{1\alpha}$ was detected when specimens were processed in the presence of $[^2H_8]$arachidonic acid (data not shown). Next, the levels of the two F_2-isoprostanes were investigated in cerebellar samples from the same subjects, since the cerebellum is almost devoid of AD lesions. We observed that the levels of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI were lower in cerebellum than in neocortex. Furthermore, there were no significant differences in the levels of the F_2-isoprostanes when the AD cerebella were compared to those of the non-AD patients (**Table 3**).

Postmortem ventricular CSF obtained from additional AD (n=15) and non-AD controls (n=10) was assayed for the two F_2-isoprostanes. $iPF_{2\alpha}$-III levels tended to be higher in AD than in non-AD patients, but the difference was not statistically significant [49(3084) vs. 41(22–60) pg/ml, P=0.14]. By contrast, $iPF_{2\alpha}$-VI levels were significantly higher in AD CSF relative to non-AD CSF [102(33–220) vs. 38(22–80) pg/ml; P=0.009]. No correlation was found between CSF levels of the two isprostanes and age, PMI, or disease duration (data not shown).

DISCUSSION

AD is a heterogeneous group of dementias characterized by loss of cognitive abilities (1). The pathological features of the disease include SPs, NFTs, and extensive neuronal loss (2, 3). Although there has been much speculation that ROS may play an important role in AD, few data support this hypothesis. The study of oxidant stress has been constrained by the paucity of reliable quantitative markers of oxidant stress *in vivo* (13), which may explain the conflicting results in the literature. Thus, whereas some authors showed an increase in lipid peroxidation in AD brain by measuring TBARS at baseline or after incubation with oxidants (14–17), others have not confirmed this observation (18, 19). Immunohistochemical data suggest the presence in AD brain of stable by-products of lipid peroxidation (20, 21). Although Lovell et al. (22) have recently reported increased levels of 4-hydroxynonenal in postmortem CSF of AD patients, no such quantitative data are available for this compound in AD brains. Evidence of oxidative damage to proteins is also limited. Thus, although a tendency toward an increase in carbonyl groups in AD frontal cortex has been reported, the difference did not attain statistical significance (36). The presence of protein adducts in postmortem AD brains has also been reported (37). Mitochondrial 8-hydroxy-2-deoxyguanosine (8-OHdG), an index of oxidative damage to DNA, is elevated in cerebral cortex and cerebellum of AD patients compared to controls. However, nuclear 8-OHdG levels are not altered in AD (38).

Taken together, these studies suggest that increased oxidative stress and lipid peroxidation may play a role in the pathogenesis of AD, and recent work suggests that oxidative stress leads to protein cross-linking and aggregation of Aβ *in vitro* (39). The link between AD and oxidative stress has also been suggested by the observation that apolipoprotein E4 (ApoE4), the allele associated with an increased risk for the disease, has decreased antioxidant activity when compared with other ApoE alleles (40) .

Isoeicosanoids are prostaglandin isomers formed by a direct ROS attack on arachidonic acid. Unlike conventional, enzymatically derived prostaglandins, which are formed intracellularly and immediately released, isoprostanes are formed initially in situ in the cell membranes from which they are cleaved out by phospholipases, circulate in plasma, and are excreted in urine (24). Formation of these stable compounds *in vivo* can be reliably monitored through noninvasive analytical approaches that can afford a sensitive and specific quantitative index of lipid peroxidation (41, 42) . Thus, for these reasons, we have focused on F_2-isoprostanes by developing specific and sensitive methods, using gas chromatography/mass spectrometry to measure two specific F_2-isoprostanes, $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI. We have previously reported that $iPF_{2\alpha}$-III generation is elevated in several syndromes putatively associated with oxidant stress *in vivo*, including cigarette smoking (29) and coronary reperfusion (30, 43). Both F_2-isoprostanes are elevated in situ at the site of their formation in human (31) and murine (44) atherosclerotic plaques, where lipid peroxidation is thought to occur *in vivo*.

These observations prompted us to address the hypothesis that the formation of F_2-isoprostanes might be elevated in AD. Indeed, we show here that the levels of two F_2-isoprostanes were significantly higher in the frontal and temporal pole samples of AD

TABLE 2. *6-Keto $PGF_{1\alpha}$ levels in frontal pole cortex of normal controls (C), Alzheimer's disease (AD), Parkinson's disease (PD), and schizophrenia (SCHI) patients*[a]

	C ($n = 8$)	AD ($n = 19$)	PD ($n = 6$)	SCHI ($n = 10$)
6-Keto $PGF_{1\alpha}$ (pg/g wet tissue)	960 ± 196 (145–2300)	703 ± 170 (100–2090)	716 ± 171 (190–1800)	1040 ± 230 (260–2400)

[a] Results are expressed as means ± SEMs. Range values are given in parentheses.

brains compared to their cerebella, a region rarely affected by AD (1). Levels were also higher than in the cortices of brains from patients with Parkinson's disease or schizophrenia, diseases in which inflammation is not a prominent component compared to neuropathologically normal controls. Even though it is unknown whether isoprostane generation is altered in other neurodegenerative disorders, it is likely that these markers reflect the process of lipid peroxidation rather than a pathology peculiar to AD. The quantity of either isoprostane was apparently independent of the age of the patients, duration of disease, or PMI. Tissue levels of the two isoprostanes were highly correlated, consistent with the hypothesis that formation of both compounds reflects a common free radical-dependent mechanism of formation. Although conventional prostaglandins are not stored in cells after their formation, others have reported various disturbances of their biosynthetic enzymes amongst regions in the brain (45). In the present study, ex vivo generation of prostacyclin, as reflected by its hydrolysis product 6-keto $PGF_{1\alpha}$, was not altered in involved areas of the AD brain. Given the different mechanisms of prostaglandin and isoprostane formation, experiments with labeled precursors suggest that an actual difference in prostacyclin formation was not obscured by a large artifactual increase in generation of the prostanoid during sample preparation.

Levels of the more abundant $iPF_{2\alpha}$-VI were higher in ventricular CSF of AD patients. Although CSF levels of $iPF_{2\alpha}$-III tended to be higher in AD, this difference did not attain statistical significance. Given that this is the less abundant isoprostane, the failure to differentiate the groups may be an issue of relative signal to noise. Since free radicals may increase production of APP and Aβ (46), which itself may generate ROS as a product of its neurotoxicity (10), these data raise the possibility that isoprostane analysis in CSF, or perhaps in plasma or urine, might reflect the activity or progression of AD in living patients. For example, we have recently demonstrated that urine, plasma, and vascular levels of $iPF_{2\alpha}$-VI rise as atherosclerosis develops in the hypercholesterolemic mouse (44). The lack of correlation between isoprostanes and age or PMI in these subjects further suggests that the increase reflects neuropathological changes in the AD brain rather than a nonspecific effect of aging. Our findings are consistent with the hypothesis that elevated levels of isoprostanes reflect an increase in lipid peroxidation in AD.

In summary, we have used chemically stable products of lipid peroxidation to provide evidence for oxidant stress in affected brain regions of patients with AD. It is interesting that the same biochemical markers tend to be elevated in the CSF of AD patients. While our compounds reflect peroxidation of arachidonic acid, preliminary evidence that isomers derived from docosahexaenoic acid may also be elevated in the CSF of four postmortem patients with AD has recently been provided (47). We have not addressed the mechanisms that result in oxidant stress in AD here, but there is increasing interest in the role of COX activation in the inflammatory component of AD (48), and epidemiological studies have raised the possibility that COX inhibitors may delay the progression of the disease (49, 50). The radicals that lead to isoprostane generation in AD may well have derived from COX, as well as other sources. Analysis of one prostaglandin product did not provide evidence for discriminant COX activation in the AD brain regions in which the isoprostane content was elevated. However, a more comprehensive evaluation of prostaglandin formation in AD seems timely. Given the preliminary evidence that ROS may directly exacerbate lesion formation (10, 46), our observations raise the possibility that isoprostane analysis, though unlikely to be specific for AD, may reflect disease progression and provide a rational basis for

TABLE 3. *$iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI levels in cerebellum from Alzheimer's disease (AD), Parkinson's disease (PD), and schizophrenia (SCHI) patients*[a]

	AD ($n = 19$)	PD ($n = 6$)	SCHI ($n = 10$)
$iPF_{2\alpha}$-III (pg/g wet tissue)	100 ± 10 (50–141)	118 ± 12 (60–170)	95 ± 11 (40–135)
$iPF_{2\alpha}$-VI (pg/g wet tissue)	128 ± 9.9 (95–180)	126 ± 10 (90–175)	119 ± 8.7 (96–165)

[a] Results are expressed as means ± SEMs. Ranges are given in parentheses.

the selection, evaluation and monitoring of antioxidant strategies for clinical trials in AD (51). FJ

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