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4-Hydroxy-2(*E*)-Nonenal Inhibits CNS Mitochondrial Respiration at Multiple Sites

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Abstract: A destructive cycle of oxidative stress and mitochondrial dysfunction is proposed in neurodegenerative disease. Lipid peroxidation, one outcome of oxidative challenge, can lead to the formation of 4-hydroxy-2(*E*)-nonenal (HNE), a lipophilic alkenal that forms stable adducts on mitochondrial proteins. In this study, we characterized the effects of HNE on brain mitochondrial respiration. We used whole rat brain mitochondria and concentrations of HNE comparable to those measured in patients with Alzheimer's disease. Our results showed that HNE inhibited respiration at multiple sites. Complex I-linked and complex II-linked state 3 respirations were inhibited by HNE with IC_{50} values of ~200 μM HNE. Respiration was apparently diminished owing to the inhibition of complex III activity. In addition, complex II activity was reduced slightly. The lipophilicity and adduction characteristics of HNE were responsible for the effects of HNE on respiration. The inhibition of respiration was not prevented by *N*-acetylcysteine or aminoguanidine. Studies using mitochondria isolated from porcine cerebral cortex also demonstrated an inhibition of complex I- and complex II-linked respiration. Thus, in neurodegenerative disease, oxidative stress may impair mitochondrial respiration through the production of HNE. **Key Words:** Mitochondria—Neurodegeneration—4-Hydroxy-2(*E*)-nonenal—Complex III—Peroxidation—Adduct.
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Several neurodegenerative diseases are associated with increased oxidative damage (Jenner, 1991; Coyle and Puttfarcken, 1993; Beal, 1995*a*,*b*; Schapira, 1996; Markesbery, 1997). Although there are several manifestations of oxidative damage to tissue, lipid peroxidation is thought to be especially harmful because it is a self-propagating process. Moreover, lipid peroxidation may be a particularly important mode of oxidative damage in the CNS, which is enriched in polyunsaturated fatty acids (T. J. Montine et al., 1998; Roberts et al., 1998). A major deleterious outcome of lipid peroxidation is generation of reactive aldehydes, the most cytotoxic of which is 4-hydroxy-2(*E*)-nonenal (HNE) (Esterbauer et al., 1991; T. J. Montine et al., 1996; Kruman et al., 1997; K. Montine et al., 1997*b*; Sayre et al., 1997).

HNE is an α,β unsaturated alkenal that adducts nucleophilic groups such as thiols and amines to form Michael, pyrrole, and imine adducts (Sayre et al., 1993, 1996; Nadkarni and Sayre, 1995; Amarnath et al., 1998). HNE participates in protein cross-linking chemistry via pyrrole formation and Michael adduct–imine formation (Cohn et al., 1996; Nadkarni and Sayre, 1995; Montine et al., 1997*a*). HNE may exert its neurotoxicity through several different pathways, but all share protein modification and subsequent dysfunction as the fundamental mechanism. Cell culture experiments have implicated cytoskeletal proteins, glutamate transporters, glucose transporters, ion transporters, and guanylate cyclase as cellular targets for HNE adduction (Montine et al., 1996; Blanc et al., 1997; Keller et al., 1997*a*,*b*; Kruman et al., 1997; Mark et al., 1997; Mattson et al., 1997; M. D. Neely et al., manuscript submitted for publication). Some of these experiments have suggested that HNE also may lead to disrupted mitochondrial function in neuronal culture; however, this has not been fully characterized (Keller et al., 1997*b*; Kruman et al., 1997).

Postmortem studies of patients with Alzheimer's disease (AD), Parkinson's disease, and amyotrophic lateral sclerosis have identified immunochemically HNE–protein adducts in the neuronal cytoplasm in affected regions; however, the subcellular localization of these adducts has not been characterized (Yoritaka et al., 1996; K. Montine et al., 1997*a*,*b*, 1998; Sayre et al., 1997; Pedersen et al., 1998). Because HNE is highly reactive and extensively metabolized, it is difficult to quantify the production of HNE in diseased tissue. Nevertheless, concentrations of free HNE in the CSF are elevated in AD, Parkinson's disease, and amyotrophic lateral sclerosis

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Abbreviations used: AD, Alzheimer's disease; AMG, aminoguanidine; *m*-CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HNA, 4-hydroxynonanal; HNE, 4-hydroxy-2(*E*)-nonenal; HPE, 4-hydroxy-2(*E*)-pentenal; NAC, *N*-acetylcysteine.

patients compared with controls. Indeed, in CSF from AD patients, HNE concentrations as high as 180 μM have been reported (Lovell et al., 1998).

Mitochondrial respiratory dysfunction and oxidative stress have been associated with many neurodegenerative diseases (Beal, 1992, 1995*a*,*b*; Schapira et al., 1992; Schapira, 1996). Oxidative stress is hypothesized to contribute to mitochondrial impairment through the generation of free radical species, particularly superoxide, hydroxyl radical, and peroxynitrite, that inactivate mitochondrial proteins. Yet, in this environment of oxidative stress, relatively little is known about the mechanisms by which lipid peroxidation products may inhibit mitochondrial respiration. It is known, however, that in nonneuronal systems oxidative challenge can lead to the formation of HNE adducts on isolated mitochondria and on mitochondria isolated from hearts following ischemia–reperfusion injury (Cohn et al., 1996; Lucas and Szweda, 1998).

We hypothesize that HNE may be a mediator between oxidative damage and neurotoxicity in part by inhibiting mitochondrial respiration. Evidence suggests that the mitochondrion may be a sensitive site for HNE toxicity, and several studies have shown that mitochondrial respiration is reduced by thiol alkylating agents (Fonyo and Bessman, 1966; Haugaard et al., 1969; Chude and Boyer, 1974; LeQuoc et al., 1976). Studies using isolated mitochondria from kidney and heart have shown that HNE can inhibit complex I-linked respiration in part by impairing NADH production at the level of α-ketoglutarate dehydrogenase activity (Ullrich et al., 1996; Humphries et al., 1998). In addition, HNE can affect other mitochondrial functions such as inhibiting ADP transport via the adenine dinucleotide transporter, inducing permeability transition pore opening, and reducing mitochondrial DNA transcription (Kristal et al., 1994, 1996; Chen et al., 1995).

In contrast to these studies about the effects of HNE on mitochondria derived from peripheral organs, we are unaware of any study that has examined the actions of HNE on brain mitochondria. This may be important because mitochondria from different tissues may have varying responses to HNE. For example, HNE can cause an increase in respiratory uncoupling in renal but not cardiac mitochondria (Ullrich et al., 1996; Humphries et al., 1998). Furthermore, a related lipid peroxidation product, 4-hydroxy-2-hexenal, can induce permeability transition pore opening in liver mitochondria at femtomolar concentrations, whereas the same alkenal at 125 μM is ineffective in brain mitochondria (Kristal et al., 1996; Kristal and Dubinsky, 1997). In this report, we have examined the functional respiratory targets of HNE in mitochondria isolated from the brains of rats and pigs.

MATERIALS AND METHODS

Chemical syntheses

HNE. HNE was synthesized as previously reported (Amarnath et al., 1998).

4-Hydroxynonanal (HNA). A solution of 4-oxohexanal 1-(1,2-ethanediyl acetal) (3 g, 15 mmol) in ethanol (100 ml) was cooled in ice and treated with sodium borohydride (300 mg, 7.5 mmol). After 2 h the reaction mixture was acidified with 1 *M* HCl, ethanol was removed, and the residual solution was extracted with ethyl acetate (3 × 20 ml) (Ballini and Petrini, 1986). The combined extracts were dried, concentrated [MS *m*/*z* 210 (1, M − 1), 101 (100, hexanal and 1,3-dioxolan-2-ylethyl)], and dissolved in acetone (45 ml). Formaldehyde (37% solution, 11 ml) and *p*-toluenesulfonic acid (0.75 g) were added, and the reaction mixture was stirred at room temperature for 5 h before mixing with 1 *M* phosphate buffer (pH 7.5, 250 ml). Ethanol was removed, and the aqueous solution was extracted with chloroform (4 × 15 ml). The organic extracts were combined, dried, and evaporated. The crude aldehyde was purified by column chromatography (9:1 hexane/ethyl acetate) with a yield of 0.8 g (34%) {MS *m*/*z* 158 (1, M − 1), 140 (1, M − 18), 87 [100, $HO(CH_2)_3CO$]}.

4-Hydroxy-2(E)-pentenal (HPE). A solution of fumaraldehyde mono(dimethylacetal) (2.1 g, 16 mmol) in tetrahydrofuran (30 ml) was cooled to −40°C under an atmosphere of dry argon, and methylmagnesium chloride (7 ml of 3.0 *M* solution in tetrahydrofuran) was added over 20 min (Gree et al., 1986). The reaction mixture was warmed to −15°C and stirred at that temperature for 90 min. It was quenched with saturated ammonium chloride solution (100 ml) and extracted with ether (3 × 25 ml). The extracts were combined, dried, and evaporated. The residue containing 5,5-dimethoxypent-3-en-2-ol [MS *m*/*z* 145 (1, M − 1), 115 (100, M − OCH_3)] was dissolved in tetrahydrofuran (30 ml) and stirred with 2.5% sulfuric acid for 1 h. Saturated ammonium chloride (75 ml) was added, and the mixture was extracted with ether (3 × 30 ml). The crude HPE in the combined extracts was purified by flash chromatography (1:2 ethyl acetate/hexane) with a yield of 390 mg (25%) [MS *m*/*z* 85 (1, M − CH_3), 71 (80, M − CHO), 57 (100)].

Isolation of brain mitochondria

Total rat brain mitochondria were isolated from adult male Sprague–Dawley rats (Harlan). Rats were anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (13 mg/kg, i.p.), and the brains were removed. Mitochondria were isolated using the method of Clark and Nicklas (1970). Protein concentration was assayed using the Bio-Rad protein assay with bovine serum albumin as the standard. For isolation of porcine cerebral cortical mitochondria, male Yorkshire domestic pigs were anesthetized with ketamine (0.22 mg/kg, i.m.) and xylazine (0.22 mg/kg, i.m.). Animals were maintained on an inhalation of nitrous oxide, halothane, and oxygen. Cortex was removed, and mitochondria were isolated in the same manner as the rat mitochondria.

Mitochondrial respiration

Mitochondrial respiration, using freshly isolated mitochondria, was measured by oxygen consumption using an oxygen electrode (YSI International) and oxygen consumption chamber (Gilson). Assay medium contained 40 m*M* KCl, 50 m*M* mannitol, 50 m*M* sucrose, 0.5 m*M* disodium EDTA, 10 m*M* HEPES, 5 m*M* $MgCl_2$, and 10 m*M* K_2HPO_4, pH 7.4 (Dow et al., 1970). In these respiratory studies, control brain mitochondria routinely had respiratory control ratios of ≥4 with glutamate and malate as substrates. In these experiments, mitochondria (0.1 mg/ml) were suspended in medium (25°C) followed by addition of substrate (10 m*M* glutamate and malate for complex I and 10 m*M* succinate for complex II) followed by HNE or other test compound. After 5 min, 0.4 μmol of ADP

was added. A 5-min incubation with HNE was used to make comparisons with previous work with isolated rat cardiac mitochondria (Humphries et al., 1998). State 4 respiration was taken as the rate of oxygen consumption 1 min before addition of ADP (Tzagoloff, 1983; Davey and Clark, 1996). State 3 respiration was measured as the mean rate of oxygen consumption for 2 min following addition of ADP. Cytochrome *c* oxidase (complex IV) activity was measured by addition of 1 m*M* ascorbate and 0.2 m*M* tetramethylphenylene diamine (Sigma) to reduce cytochrome *c* and produce oxygen consumption at a similar rate to the state 3 rate. Uncoupling was effected by addition of 2 μM carbonyl cyanide *m*-chlorophenylhydrazone (*m*-CCCP; Sigma). Following the respiratory assay, mitochondria were frozen on dry ice and stored at −80°C for assays of complex I, II, and III activities.

NADH:ubiquinone oxidoreductase (complex I) activity

Complex I activity was measured for samples following oxygen consumption assays using the procedure of Birch-Machin et al. (1993) except that *n*-decylubiquinone (60 μM; Sigma) was used instead of ubiquinone$_1$. Assays were performed at 30°C. NADH consumption was inhibited by rotenone. Results are expressed as percentages of the control mitochondrial rate. NADH activity was measured as the loss of absorbance at 340 nm ($\epsilon = 6{,}200\ M^{-1} \times cm^{-1}$).

Succinate:ubiquinone oxidoreductase (complex II) activity

Complex II activity was assayed by measuring 2,6-dichloroindophenol (Sigma) reduction according to Birch-Machin et al. (1993) except that *n*-decylubiquinone (60 μM; Sigma) was used instead of ubiquinone$_1$. Assays were performed at 25°C. Activity was measured as the loss of absorbance at 600 nm ($\epsilon = 19{,}100\ M^{-1} \times cm^{-1}$). Activity was completely inhibited by malonate (10 m*M*).

Ubiquinol:cytochrome *c* oxidoreductase (complex III) activity

Complex III assays were performed using *n*-decylubiquinol (Krahenbuhl et al., 1994). *n*-Decylubiquinol was synthesized by reducing *n*-decylubiquinone (Sigma) with $NaBH_4$ (Krahenbuhl et al., 1994). Assays were performed at 25°C. The background nonenzymatic rate of cytochrome *c* reduction in the presence of decylubiquinol without mitochondria was subtracted from all values. Nonenzymatic rates were equal to the antimycin-inhibited rate. Activity was measured as the increase in absorbance at 549 nm ($\epsilon = 18{,}000\ M^{-1} \times cm^{-1}$).

Dehydrogenase assays

Mitochondria (0.1 mg/ml) in respiration buffer were exposed to HNE (0–300 μM) for 5 min at 25°C. A one-tenth volume of 30 m*M* *N*-acetylcysteine (NAC) was added, and mitochondria were pelleted by centrifugation (14,000 *g* for 4 min at 4°C). The pellets were resuspended in 50 m*M* potassium phosphate and frozen at −80°C. Freeze–thawing under these conditions retained enzyme activities, whereas sonication of dilute mitochondrial solutions (100 μg/ml) destroyed activity. Protein content determinations were made on all samples. Malate dehydrogenase was assayed according to the procedure of Ochoa (1955) except that the buffer contained 50 m*M* potassium phosphate, pH 7.4. The reaction was started with addition of oxalacetate, and the decrease in absorbance at 340 nm was measured. α-Ketoglutarate dehydrogenase was assayed based on the procedure of Brown and Perham (1976). Purified porcine α-ketoglutarate dehydrogenase was purchased from Sigma. Assay buffer contained 50 m*M* potassium phosphate (pH 7.4), 8 m*M* α-ketoglutarate, 130 μM CoASH, 200 μM thiamine pyrophosphate, and 2.5 m*M* NAD^+. The reaction was started with addition of α-ketoglutarate, and the increase in absorbance at 340 nm was measured. Rotenone (2 μg/ml) was added to both assay buffers to prevent complex I-mediated consumption of NADH.

HNE–protein adduct immunoreactivity

The formation of reducible HNE–protein adducts (Michael or imine adducts) was measured using an ELISA method developed in our laboratory (K. S. Montine et al., 1998). Mitochondria were thawed, diluted to 0.02 mg/ml in 50 m*M* phosphate buffer (pH 7.4), and boiled for 5 min. Fifty microliters of each sample was added to the well of a 96-well microtiter plate, and the protein was allowed to bind for 1 h at 37°C. Wells were washed with phosphate buffer and then treated with 100 μl of 50 m*M* $NaBH_4$ for 1 h at 37°C to reduce the HNE–protein adduct. The well was blocked for 30 min and then incubated for 1 h at 37°C with rabbit antibody 672, which recognizes the reduced HNE–protein adduct (K. S. Montine et al., 1998). The well was washed and incubated with an alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad). The well was then developed using *p*-nitrophenyl phosphate (Sigma).

RESULTS

The first set of experiments examined the effects of HNE on the respiration of mitochondria isolated from whole rat brain. Complex I-linked state 3 respiration was inhibited by HNE with an IC_{50} of 195 ± 35 μM HNE (Fig. 1A). This finding is consistent with the work of others in renal and cardiac mitochondria showing that complex I-linked state 3 respiration is vulnerable to inhibition by HNE (Ullrich et al., 1996; Humphries et al., 1998). The IC_{50} in rat brain mitochondria was approximately five times that of cardiac mitochondria and less than one-half that of renal mitochondria (Ullrich et al., 1996; Humphries et al., 1998). These discrepancies may be due in part to the substrates used and the time of incubation with HNE. At the highest concentration of HNE (300 μM) a significant increase in complex I-linked state 4 respiration (43.8 ± 4.6 nmol of O_2/min/mg) over control (30.2 ± 1.9 nmol of O_2/min/mg) was observed, indicating a slight uncoupling of respiration. NADH-CoQ reductase (complex I) activity was unchanged between controls and 300 μM HNE-treated samples (Fig. 1B), supporting the previous finding that complex I activity is unaffected by HNE. α-Ketoglutarate and malate dehydrogenase activities were not diminished in whole mitochondria treated with HNE (0–300 μM).

HNE also inhibited complex II-linked state 3 respiration in whole rat brain mitochondria with an IC_{50} of 206 ± 21 μM HNE (Fig. 1A). This is in contrast to rat cardiac and renal mitochondria, in which little or no HNE-mediated inhibition of complex II-linked respiration is seen (Ullrich et al., 1996; Humphries et al., 1998). Although succinate-linked state 3 respiration was not inhibited by 100 μM HNE, the time to onset of the respiratory rate was ~1 min slower than control mito-

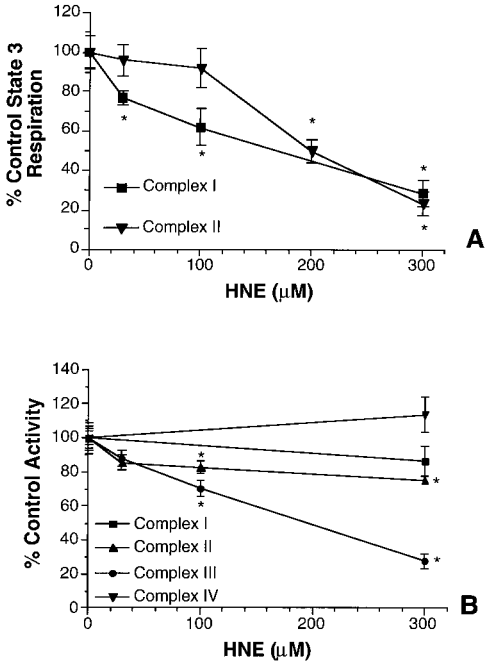

FIG. 1. HNE inhibition of respiration and respiratory complexes in rat brain mitochondria. **A:** Dose–response curve for HNE inhibition of complex I-linked (malate and glutamate) and complex II-linked (succinate) state 3 respiration. HNE was incubated with mitochondria (0.1 mg/ml) for 5 min with substrates present. ADP (0.4 μmol) then was added to induce state 3 respiration. Control complex I-linked respiration was 147 $\pm$ 8.3 nmol of O_2/min/mg, and complex II-linked respiration was 232 $\pm$ 8.0 nmol of O_2/min/mg (mean $\pm$ SEM; n = 5 or 6). **B:** HNE inhibits complex II and complex III activities. Activities are expressed as percentages of control (vehicle-treated) activity. Control activities were for 164 $\pm$ 15 nmol of NADH/min/mg for complex I, 122 $\pm$ 6.6 nmol of 2,6-dichloroindophenol/min/mg for complex II, 331 $\pm$ 18 nmol of cytochrome *c*/min/mg for complex III, and 114 $\pm$ 7.9 nmol of O_2/min/mg for complex IV (mean $\pm$ SEM; n = 3–6). *$p < 0.05$ using Student's *t* test.

chondria (Fig. 2A). At 300 μM HNE, there was a complete suppression of complex II-linked state 3 respiration even up to 5 min following addition of ADP. The decrease in state 3 respiration was not due to decreased cytochrome oxidase (complex IV) activity (Fig. 1B), nor was the inhibition relieved by addition of 2 μM *m*-CCCP, a proton ionophore (Fig. 2B). Thus, inhibition of complex II-linked state 3 respiration was not related to dissipation of the proton motive force, suggesting that a site between succinate dehydrogenase and cytochrome oxidase was affected.

To elucidate further how electron flow was inhibited proximal to cytochrome oxidase, complex II and complex III activities were measured. Complex II activity was reduced ~25% at 300 μM HNE, whereas complex III activity was inhibited ~70% (Fig. 1D). Thus, of the respiratory complexes I–IV, complex III was most susceptible to inactivation by HNE with an IC_{50} of 190 $\pm$ 12.5 μM. This value was similar to that obtained for the inhibition of complex I- and complex II-linked respiration and suggested that complex III inhibition may be a key component in the reduction of oxygen consumption. Complex III has been reported to be inactivated by thiol adducting agents, and an inhibition of complex III may result in a reduction in oxygen consumption or may cause a delay in the onset of state 3 respiration (Leung and Hinkle, 1975; Gellefors et al., 1976; Taylor et al., 1994).

We next tested whether we could prevent the inhibitory effects of HNE with NAC and aminoguanidine (AMG), two compounds that can act as scavengers of free HNE (Al-Abed and Bucala, 1997). In vitro experiments showed that equimolar amounts of NAC and AMG were able to block the formation of Michael and pyrrole adducts by HNE on bovine serum albumin (data not shown). However, a 10-fold excess of NAC or AMG added to mitochondria before addition of HNE did not prevent the suppression of complex I-linked state 3 respiration (Fig. 3A). However, preincubation of HNE with a 10-fold excess of NAC for 30 min before addition to mitochondria did prevent respiratory inhibition (data not shown). Under these conditions, <0.1% of the free HNE remained when added to the mitochondria. Thus, HNE and NAC were not reacting to form a novel respiratory inhibitor. These results suggested that in the presence of brain mitochondria, addition of exogenous NAC was not able to compete adequately with mitochondrial nucleophiles for HNE. For these reasons, we determined relative levels of reducible HNE–protein adduct (thiol and amine adducts) in mitochondria (Fig. 3B). The amount of HNE adducts to mitochondrial proteins increased in a

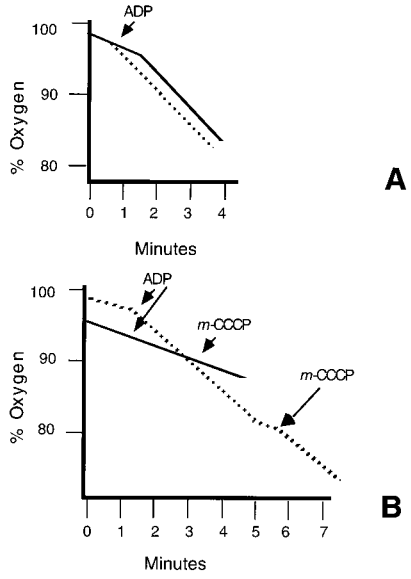

FIG. 2. HNE can delay or block the onset of complex II-linked state 3 respiration. **A:** HNE (100 μM for 5 min) pretreatment of rat brain mitochondria delays the onset of state 3 respiration by ADP (0.4 μmol). **B:** HNE at 300 μM eliminates complex II-linked state 3 respiration induced by ADP or uncoupler (*m*-CCCP). The solid lines are the HNE-treated samples, and the controls are shown by the dashed lines. Succinate was used as substrate for these experiments.

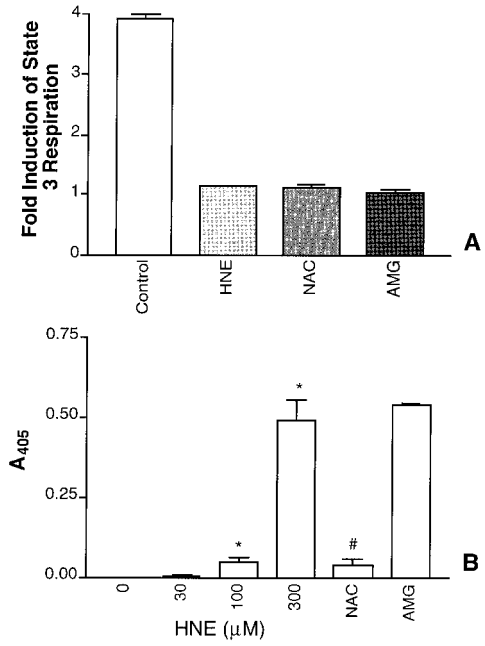

FIG. 3. Inhibition of respiration by HNE is not prevented by NAC or AMG. **A:** NAC or AMG (3 m*M*) does not prevent the inhibition of complex I-linked state 3 respiration when added before HNE (300 μM). **B:** HNE causes a dose-dependent increase in content of reducible HNE–protein adducts. NAC but not AMG (3 m*M*) prevented the formation of HNE–protein adducts by HNE (300 μM). Data are mean ± SEM (bars) values (n = 3). $^{*}p < 0.05$ versus 0 μM HNE; $^{\#}p < 0.05$ versus 300 μM HNE using Student's *t* test.

dose-dependent manner, and NAC, but not AMG, decreased the amount of reducible HNE–protein adducts. In combination, these results suggested that NAC was not able to block adduction by HNE to sites related to respiration when incubated and that HNE at the highest concentration was modifying proteins other than those related to respiration.

The next series of experiments were designed to discriminate whether the effects of HNE were due to protein adduction or altering the mitochondrial membrane environment unrelated to protein adduction. To test this, we synthesized HNA, which has a greater lipophilicity (octanol:water = ∞) than HNE (octanol:water = 16.4) and is identical to HNE in structure except HNA lacks the C2 double bond and hence is unable to form Michael or pyrrole adducts. HNA did not significantly affect complex I-linked state 3 respiration (Fig. 4). This result indicated that inhibition of respiration by HNE was likely due to the ability of HNE to form adducts with nucleophiles.

Although lipophilicity alone did not determine the activity of HNE, the lipophilicity of alkylating agents may influence their effects on respiration (Haugaard et al., 1969; LeQuoc et al., 1976; Chen and Yu, 1994). HPE was used to assess the role of lipophilicity in the effects of HNE on mitochondrial respiration. HPE is a 4-hydroxy-2-alkenal and has been widely used as a less

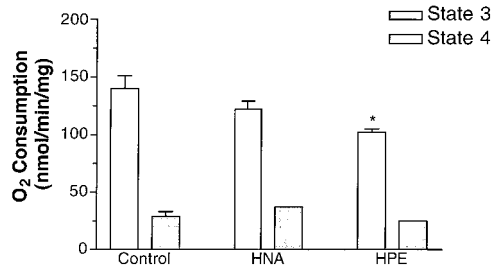

FIG. 4. Nonadducting and less lipophilic analogues of HNE show reduced inhibition of state 3 respiration. HNA (300 μM) or HPE (300 μM) was incubated with rat brain mitochondria (0.1 mg/ml), and the substrates glutamate and malate were added for 5 min before addition of ADP (0.4 μmol). Data are mean ± SEM (bars) values (n = 3). $^{*}p < 0.05$ using Student's *t* test.

lipophilic (octanol:water = 0.6) analogue that pursues the same chemical reactions as HNE (Esterbauer et al., 1991). HPE was ~10-fold less potent than HNE; 300 μM HPE inhibited complex I-linked respiration by only 27% (Fig. 4). Thus, although the adduction chemistry was essential for the inhibitory effects of HNE, the lipophilicity of the adductor had a major modifying effect.

Finally, we tested the effects of HNE on mitochondria isolated from porcine cerebral cortex (Fig. 5). Similar to rat brain mitochondria, HNE suppressed complex I- and complex II-linked state 3 respiration (Fig. 5B). The increased sensitivity of porcine cortical mitochondria to HNE may represent a species difference, a difference between regions of the brain (porcine cerebral cortex vs. whole rat brain), or both. These results corroborated that HNE inhibited complex I- and complex II-linked respiration in brain mitochondria.

DISCUSSION

HNE is an abundant lipid peroxidation product that is neurotoxic largely through the modification of cellular proteins. Defects in mitochondrial respiration are linked

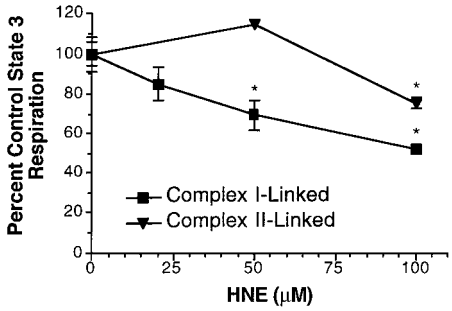

FIG. 5. HNE inhibits respiration in mitochondria isolated from porcine cerebral cortex. Complex I- and complex II-linked respirations were measured as described for rat brain mitochondria. Control complex I-linked respiration was 119 ± 5.7 nmol of O_2/min/mg, and complex II-linked respiration was 136 ± 8.8 nmol of O_2/min/mg. Data are mean ± SEM (bars) values (n = 3–6). $^{*}p < 0.05$ using Student's *t* test.

to several neurodegenerative diseases (Beal, 1995*b*), and in vivo and in vitro studies have shown that mitochondrial proteins are targets of HNE adduction following different models of oxidative stress (Cohn et al., 1996; Lucas and Szweda, 1998). Studies using neuronal cell culture systems and synaptosomes have demonstrated that plasma membrane proteins and their activities can be modified by HNE following extracellular exposure (Keller et al., 1997*a*,*b*). These investigations also indicated that mitochondrial function was impaired; however, this impairment was not characterized. Other laboratories have examined the effects of direct exposure of renal and cardiac mitochondria to HNE; however, we are unaware of any reports examining the effects of HNE on isolated brain mitochondria (Ullrich et al., 1996; Humphries et al., 1998). Because tissue-specific differences are known to exist in the response of mitochondria to HNE, it was important to assess the effect of this lipid peroxidation product on mitochondria isolated from brain tissue.

Our results showed different levels of sensitivity to HNE in brain mitochondria. Consistent with previous reports, complex I-linked state 3 respiration was more sensitive to inhibition by HNE than complex II-linked state 3 respiration, but complex I activity was not compromised (Humphries et al., 1998). Unlike mitochondria isolated from rat heart, α-ketoglutarate dehydrogenase activity in rat brain mitochondria was not inhibited by HNE. Thus, the mechanisms of inhibition of mitochondrial respiration by HNE appear to be different in brain mitochondria versus cardiac mitochondria. We used a range of concentrations of HNE, comparable to those found in the ventricular fluid (up to 180 μM HNE) of some AD patients (Lovell et al., 1998). At these concentrations, we observed an inhibition of complex II and complex III activities, as well as possibly complex V activity. Indeed, the inhibition of complex I- and complex II-linked respiration in rat brain mitochondria by HNE may be through impairment of complex III activity because the IC_{50} values of HNE for these components were nearly identical. However, others have shown that up to 80% reduction in complex III activity alone by myxothiazol may not inhibit complex I-linked state 3 respiration (Davey and Clark, 1996; Davey et al., 1998). Possible explanations for this discrepancy are that HNE may be inhibiting complex III different from myxothiazol or inhibiting other mitochondrial sites in addition to complex III, with the net result of making complex III activity rate-limiting. Our experiments cannot distinguish between the possibilities that HNE may have directly modified complex III proteins or that HNE, through modification of other targets, secondarily reduced complex III activity.

Complex III inhibition can cause severe neurological and physical defects, and a reduction in complex III activity has been demonstrated in the caudate nucleus in Huntington's disease patients and in some regions of the cerebral cortex in AD patients (Reichmann et al., 1993; Mutisya et al., 1994; Beal, 1995*b*). In addition to contributing to a reduction in respiration, the inhibition of complex III also may lead to generation of reactive oxygen species (Boveris and Chance, 1973). Moreover, studies in tissue culture have shown that partial inhibition of complex III by antimycin treatment makes cells more vulnerable to damage from organic hydroperoxides (Guidarelli et al., 1997). Thus, in a neuronal environment of oxidative stress and lipid peroxidation, HNE may amplify oxidative damage by reducing complex III activity.

NAC was ineffective in blocking the inhibitory effects of HNE on mitochondrial respiration. However, NAC significantly reduced the amount of HNE adducts formed on mitochondria. These data suggest that many of the HNE adducts formed on mitochondria are not relevant to the inhibition of respiration. Treatment of mitochondria with pathologically relevant concentrations of HNE most likely will cause the impairment of several mitochondrial functions in addition to respiration. Our data cannot assess the potential effects of NAC as a protectant of these other nonrespiratory functions that might be altered by HNE exposure.

These results with NAC suggest either that HNE modifies relevant respiratory targets that are inaccessible to NAC or that the rate of reaction between HNE and these target nucleophiles exceeds the rate of reaction between HNE and NAC. Our data with HPE, a less lipophilic and significantly less active analogue of HNE, suggested that mitochondrial nucleophiles in lipophilic microenvironments may be the relevant targets for HNE. Indeed, others have shown that thiols in lipid environments are essential for respiration. Moreover, lipophilic thiols have enhanced reactivity to alkenals owing to the stabilization of the thiolate anion (Lindkvist et al., 1997). These data suggest that more lipophilic scavengers may be needed to protect mitochondrial function from exposure to HNE.

Several neurodegenerative disorders have been associated with oxidative damage and mitochondrial dysfunction. These two characteristics may work in a destructive cycle in which free radical species inhibit mitochondrial function, which in turn leads to increased free radical production or energy depletion, culminating in neuron death. Several studies have shown that elements of the tricarboxylic acid cycle and the electron transport chain are inactivated by free radical species such as superoxide, hydroxyl radical, and peroxynitrite (Beal, 1995*b*). However, relatively little is known about the exact role that lipid peroxidation products play in causing mitochondrial dysfunction in neurodegenerative disease. Our studies indicate that only a relatively small amount of HNE adduction may be needed to reduce respiration in isolated brain mitochondria. Taken together with the fact that HNE adducts are highly stable, it is possible that HNE-induced mitochondrial defects accumulate over time, leading to decreased cellular energy levels and contributing to the pathogenesis of neurodegenerative disease. Reactive oxygen species may therefore cause mitochondrial damage directly or indirectly through lipid peroxidation products such as HNE.

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REFERENCES

Al-Abed Y. and Bucala R. (1997) Efficient scavenging of fatty acid oxidation products by aminoguanidine. *Chem. Res. Toxicol.* **10,** 875–879.

Amarnath V., Valentine W. M., Montine T. J., Patterson W. H., Amarnath K., Bassett C. N., and Graham D. G. (1998) Reactions of 4-hydroxy-2(*E*)-nonenal and related aldehydes with proteins studied by carbon-13 nuclear magnetic resonance spectroscopy. *Chem. Res. Toxicol.* **11,** 317–328.

Ballini R. and Petrini M. (1986) Facile and inexpensive synthesis of 4-oxoalkanoic acids from primary nitroalkanes and acrolein. *Synthesis,* 1024–1026.

Beal M. F. (1992) Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illnesses? *Ann. Neurol.* **31,** 119–130.

Beal M. F. (1995*a*) Aging, energy, and oxidative stress in neurodegenerative diseases. *Ann. Neurol.* **38,** 357–366.

Beal M. F. (1995*b*) *Mitochondrial Dysfunction and Oxidative Damage in Neurodegenerative Diseases.* Neuroscience Intelligence Unit, R. G. Landes Co., Austin, Texas.

Birch-Machin M., Jackson S., Kler R., and Turnbull D. (1993) Study of skeletal muscle mitochondrial dysfunction, in *Methods in Toxicology, Vol. 2* (Hash L. and Jones D., eds), pp. 51–57. Academic Press, San Diego.

Blanc E. M., Kelly J. F., Mark R. J., Waeg G., and Mattson M. P. (1997) 4-Hydroxynonenal, an aldehydic product of lipid peroxidation, impairs signal transduction associated with muscarinic acetylcholine and metabotropic glutamate receptors: possible action on $G\alpha_{q/11}$. *J. Neurochem.* **69,** 570–580.

Boveris A. and Chance B. (1973) The mitochondrial generation of hydrogen peroxide. *Biochem. J.* **134,** 707–716.

Brown J. P. and Perham R. N. (1976) Selective inactivation of the transacylase components of the 2-oxo acid dehydrogenase multienzyme complexes of *Escherichia coli. Biochem J.* **155,** 419–427.

Chen J. J. and Yu B. P. (1994) Alterations in mitochondrial membrane fluidity by lipid peroxidation products. *Free Radic. Biol. Med.* **17,** 411–418.

Chen J. J., Bertrand H., and Yu B. P. (1995) Inhibition of adenine nucleotide translocator by lipid peroxidation products. *Free Radic. Biol. Med.* **19,** 583–590.

Chude O. and Boyer P. D. (1974) Protein-bound sulfhydryl groups and thiolesters in mitochondria and submitochondrial particles and their relationship to oxidative phosphorylation. *Arch. Biochem. Biophys.* **160,** 366–371.

Clark J. and Nicklas W. (1970) The metabolism of rat brain mitochondria. *J. Biol. Chem.* **245,** 4724–4731.

Cohn J. A., Tsai L., Friguet B., and Szweda L. I. (1996) Chemical characterization of a protein–4-hydroxy-2-nonenal cross link: immunochemical detection in mitochondria exposed to oxidative stress. *Arch. Biochem. Biophys.* **328,** 158–164.

Coyle J. T. and Puttfarcken P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. *Science* **262,** 689–695.

Davey G. P. and Clark J. B. (1996) Threshold effects and control of oxidative phosphorylation in nonsynaptic rat brain mitochondria. *J. Neurochem.* **66,** 1617–1624.

Davey G. P., Peuchen S., and Clark J. B. (1998) Energy thresholds in brain mitochondria. *J. Biol. Chem.* **273,** 12753–12757.

Dow D. S., Walton K. G., and Fleischer S. (1970) Control of mitochondrial swelling by Mg^{2+}: the relation of ion transport to structural change. *Bioenergetics* **1,** 247–271.

Esterbauer H., Schaur R. J., and Zollner H. (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic. Biol. Med.* **11,** 81–128.

Fonyo A. and Bessman S. P. (1966) The action of oligomycin and of *para*-hydroxymercuribenzoate on mitochondrial respiration stimulated by ADP, arsenate and calcium. *Biochem. Biophys. Res. Commun.* **24,** 61–66.

Gellefors P., Lunden M., and Nelson B. D. (1976) Evidence for a function of core protein in complex III from beef-heart mitochondria. *Eur. J. Biochem.* **67,** 463–468.

Gree R., Tourbah H., and Carrie R. (1986) Fumaraldehyde mono(dimethyl acetal): an easily accessible and versatile intermediate. *Tetrahedron Lett.* **27,** 4983–4986.

Guidarelli A., Clementi E., Brambilla L., and Cantoni O. (1997) Mechanism of the antimycin A-mediated enhancement of *t*-butylhydroperoxide-induced single-strand breakage of DNA. *Biochem. J.* **328,** 801–806.

Haugaard N., Lee N. H., Kostrzewa R., Horn R. S., and Haugaard E. S. (1969) The role of sulfhydryl groups in oxidative phosphorylation and ion transport by rat liver mitochondria. *Biochim. Biophys. Acta* **172,** 198–204.

Humphries K. M., Yoo Y., and Szweda L. I. (1998) Inhibition of NADH-linked mitochondrial respiration by 4-hydroxy-2-nonenal. *Biochemistry* **37,** 552–557.

Jenner P. (1991) Oxidative stress as a cause of Parkinson's disease. *Acta Neurol. Scand.* **84,** 6–15.

Keller J. N., Mark R. J., Bruce A. J., Blanc E., Rothstein J. D., Uchida K., Waeg G., and Mattson M. P. (1997*a*) 4-Hydroxynonenal, an aldehydic product of membrane lipid peroxidation, impairs glutamate transport and mitochondrial function in synaptosomes. *Neuroscience* **80,** 685–696.

Keller J. N., Pang Z., Geddes J. W., Begley J. G., Germeyer A., Waeg G., and Mattson M. P. (1997*b*) Impairment of glucose and glutamate transport and induction of mitochondrial oxidative stress and dysfunction in synaptosomes by amyloid β-peptide: role of the lipid peroxidation product 4-hydroxynonenal. *J. Neurochem.* **69,** 273–284.

Krahenbuhl S., Talos C., Weissman U., and Hoppel C. (1994) Development and evaluation of a spectrophotometric assay for complex III in isolated mitochondria, tissues and fibroblasts from rats and humans. *Clin. Chim. Acta* **230,** 177–187.

Kristal B. S. and Dubinsky J. M. (1997) Mitochondrial permeability transition in the central nervous system: induction by calcium cycling-dependent and -independent pathways. *J. Neurochem.* **69,** 524–538.

Kristal B. S., Chen J. J., and Yu B. P. (1994) Sensitivity of mitochondrial transcription to different free radical species. *Free Radic. Biol. Med.* **16,** 323–329.

Kristal B. S., Park B. K., and Yu B. P. (1996) 4-Hydroxyhexenal is a potent inducer of the mitochondrial permeability transition. *J. Biol. Chem.* **271,** 6033–6038.

Kruman I., Bruce-Keller A. J., Bredesen D., Waeg G., and Mattson M. P. (1997) Evidence that 4-hydroxynonenal mediates oxidative stress-induced neuronal apoptosis. *J. Neurosci.* **17,** 5089–5100.

LeQuoc D., LeQuoc K., and Gaudemer Y. (1976) Energy-dependent variation of thiol groups reactivity or accessibility in rat mitochondria, revealed by measurements of labelled thiol reagents incorporation. *Biochem. Biophys. Res. Commun.* **68,** 106–113.

Leung K. H. and Hinkle P. C. (1975) Reconstitution of ion transport and respiratory control in vesicles formed from reduced coenzyme Q–cytochrome *c* reductase and phospholipids. *J. Biol. Chem.* **250,** 8467–8471.

Lindkvist B., Weinander R., Engman L., Koetse M., Engberts J., and Morgenstern R. (1997) Glutathione transferase mimics micellar catalysis of an enzymatic reaction. *Biochem. J.* **323,** 39–43.

Lovell M. A., Ehmann W. D., Mattson M. P., and Markesbery W. R. (1998) Elevated 4-hydroxynonenal in ventricular fluid in Alzheimer's disease. *Neurobiol. Aging* **18,** 457–461.

Lucas D. and Szweda L. (1998) Cardiac reperfusion injury: aging, lipid peroxidation, and mitochondrial dysfunction. *Proc. Natl. Acad. Sci. USA* **95,** 510–514.

Mark R. J., Lovell M. A., Markesbery W. R., Uchida K., and Mattson M. P. (1997) A role for 4-hydroxynonenal, an aldehydic product of lipid peroxidation, in disruption of ion homeostasis and neuronal death induced by amyloid β-peptide. *J. Neurochem.* **68,** 255–264.

Markesbery W. R. (1997) Oxidative stress hypothesis in Alzheimer's disease. *Free Radic. Biol. Med.* **23,** 134–137.

Mattson M. P., Fu W., Waeg G., and Uchida K. (1997) 4-Hydroxynonenal, a product of lipid peroxidation, inhibits dephosphorylation of the microtubule-associated protein tau. *Neuroreport* **8,** 2275–2281.

Montine K., Kim P., Olson J., Markesbery W., and Montine T. (1997*a*) 4-Hydroxy-2-nonenal pyrrole adducts in human neurodegenerative disease. *J. Neuropathol. Exp. Neurol.* **56,** 866–871.

Montine K., Olson S., Amarnath V., Whetsell W., Graham D., and Montine T. (1997*b*) Immunochemical detection of 4-hydroxynonenal adducts in Alzheimer's disease is associated with APOE4. *Am. J. Pathol.* **150,** 437–443.

Montine K. S., Reich E., Neely M. D., Sidell K. R., Olson S. J., Markesbery W. R., and Montine T. J. (1998) Distribution of reducible 4-hydroxnonenal adduct immunoreactivity in Alzheimer disease is associated with APOE genotype. *J. Neuropathol. Exp. Neurol.* **57,** 415–425.

Montine T. J., Amarnath V., Martin M. E., Strittmatter W. J., and Graham D. G. (1996) *E*-4-Hydroxy-2-nonenal is cytotoxic and cross-links cytoskeletal proteins in P19 neuroglial cultures. *Am. J. Pathol.* **148,** 89–93.

Montine T. J., Markesbery W. R., Morrow J., and Roberts L. J. II (1998) F2-isoprostane levels are elevated in ventricular fluid of patients with Alzheimer's disease. *Ann. Neurol.* **44,** 410–413.

Mutisya E. M., Bowling A. C., and Beal M. F. (1994) Cortical cytochrome oxidase activity is reduced in Alzheimer's disease. *J. Neurochem.* **63,** 2179–2184.

Nadkarni D. V. and Sayre L. M. (1995) Structural definition of early lysine and histidine adduction chemistry of 4-hydroxynonenal. *Chem. Res. Toxicol.* **8,** 284–291.

Ochoa S. (1955) Malic dehydrogenase from pig heart. *Methods Enzymol.* **1,** 735–739.

Pedersen W. A., Weiming F., Keller J. N., Markesbery W. R., Appel S., Smith R. G., Kasarski E., and Mattson M. P. (1998) Protein modification by the lipid peroxidation product 4-hydroxynonenal on the spinal cords of amyotrophic lateral sclerosis patients. *Ann. Neurol.* **44,** 819–824.

Reichmann H., Florke S., Hebenstreit G., Schrubar H., and Riederer P. (1993) Analyses of energy metabolism and mitochondrial genome in post-mortem brain from patients with Alzheimer's disease. *J. Neurol.* **240,** 377–380.

Roberts L. J. II, Montine T. J., Markesbery W. R., Tapper A. R., Hardy P., Chemtob S., Dettbarn W. D., and Morrow J. D. (1998) Formation of isoprostane-like compounds (neuroprostanes) in vivo from docosahexaenoic acid. *J. Biol. Chem.* **273,** 13605–13612.

Sayre L. M., Arora P. K., Iyer R. S., and Salomon R. G. (1993) Pyrrole formation from 4-hydroxynonenal and primary amines. *Chem. Res. Toxicol.* **6,** 19–22.

Sayre L. M., Sha W., Xu G., Kaur K., Nadkarni D., Subbanagounder G., and Salomon R. G. (1996) Immunochemical evidence supporting 2-pentylpyrrole formation on proteins exposed to 4-hydroxy-2-nonenal. *Chem. Res. Toxicol.* **9,** 1194–1201.

Sayre L. M., Zelasko D. A., Harris P. L. R., Perry G., Salomon R. G., and Smith M. A. (1997) 4-Hydroxynonenal-derived advanced lipid peroxidation end products are increased in Alzheimer's disease. *J. Neurochem.* **68,** 2092–2097.

Schapira A. H. V. (1996) Oxidative stress and mitochondrial dysfunction in neurodegeneration. *Curr. Opin. Neurol.* **9,** 260–264.

Schapira A. H. V., Mann V. M., Cooper J. M., Krige D., Jenner P. J., and Marsden C. D. (1992) Mitochondrial function in Parkinson's disease. *Ann. Neurol.* **32** (Suppl.), S116–S124.

Taylor R. W., Birch-Machin M. A., Bartlett K., Lowerson S. A., and Turnbull D. M. (1994) The control of mitochondrial oxidations by complex III in rat muscle and liver mitochondria. *J. Biol. Chem.* **269,** 3523–3528.

Tzagoloff A. (1983) *Mitochondria,* p. 132. Plenum Press, New York.

Ullrich O., Henke W., Grune T., and Siems W. (1996) The effect of the lipid peroxidation product 4-hydroxynonenal and of its metabolite 4-hydroxynonenoic acid on respiration of rat kidney cortex mitochondria. *Free Radic. Res.* **24,** 421–427.

Yoritaka A., Hattori N., Uchida K., Tanaka M., Stadtman E. R., and Mizuno Y. (1996) Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson's disease. *Proc. Natl. Acad. Sci. USA* **93,** 2696–2701.