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Acrolein Inhibits NADH-Linked Mitochondrial Enzyme Activity: Implications for Alzheimer's Disease

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In Alzheimer's disease (AD) brain increased lipid peroxidation and decreased energy utilization are found. Mitochondria membranes contain a significant amount of arachidonic and linoleic acids, precursors of lipid peroxidation products, 4-hydroxynonenal (HNE) and 2-propen-1-al (acrolein), that are extremely reactive. Both alkenals are increased in AD brain. In this study, we examined the effects of nanomolar levels of acrolein on the activities of pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH), both reduced nicotinamide adenine dinucleotide (NADH)-linked mitochondrial enzymes. Acrolein decreased PDH and KGDH activities significantly in a dose-dependent manner. Using high performance liquid chromatography coupled to mass spectrometry (HPLC-MS), acrolein was found to bind lipoic acid, a component in both the PDH and KGDH complexes, most likely explaining the loss of enzyme activity. Acrolein also interacted with oxidized nicotinamide adenine dinucleotide (NAD^+) in such a way as to decrease the production of NADH. Acrolein, which is increased in AD brain, may be partially responsible for the dysfunction of mitochondria and loss of energy found in AD brain by inhibition of PDH and KGDH activities, potentially contributing to the neurodegeneration in this disorder.

Keywords: Acrolein; Pyruvate dehydrogenase; Lipoic acid; α-Ketoglutarate dehydrogenase; Mitochondria

INTRODUCTION

There is increasing evidence that oxidative stress plays a role in Alzheimer's disease (AD) brain (Butterfield *et al.*, 2001; 2002; Butterfield, 2002; Butterfield and Lauderback, 2002). An increase in protein oxidation (Hensley *et al.*, 1995), lipid peroxidation (Lovell *et al.*, 1995; Markesbery and Lovell, 1998), DNA oxidation (Lyras *et al.*, 1997; Lovell *et al.*, 2001), widespread peroxynitrite reactivity and redox reactive metals (Smith *et al.*, 1997) are present in the AD brain. Peroxidation of lipids leads to the formation of highly reactive α,ß-unsaturated aldehydes such as 4-hydroxynonenal (HNE) (Sayre *et al.*, 1997) and 2-propen-1-al (acrolein) (Calingasan *et al.*, 1999; Lovell *et al.*, 2000), both found to be elevated in AD brain (Markesbery and Lovell, 1998; Lovell *et al.*, 2001), particularly in neurofibrillary tangles, one of the major hallmarks of AD.

The strongly electrophilic, α,ß-unsaturated aldehyde acrolein is formed *in-vivo* by oxidation of polyunsaturated fatty acids including arachidonic acid (Uchida *et al.*, 1998). Acrolein has been shown to react rapidly by Michael addition with cysteine, histidine, and lysine residues, and deplete cellular glutathione (Ohno and Ormstad, 1985; Horton *et al.*, 1997; Butterfield and Stadtman, 1997). Upon covalent binding to proteins, acrolein introduces a carbonyl group (Pocernich *et al.*, 2001), a marker for protein oxidation (Butterfield and Stadtman, 1997; Uchida *et al.*, 1998). Likewise, acrolein can modify DNA bases with the formation of exocyclic adducts (Chung *et al.*, 1984; Marnett, 1994). Acrolein has been shown to interact with plasma-membrane specific Na/K-ATPase and inhibit glucose transport and glutamate uptake (Lovell *et al.*, 2000).

Mitochondrial dysfunction and loss of energy has also been associated with AD (Mattson, 2000). Pyruvate dehydrogenase (PDH) and α-ketoglutarate dehydrogenase (KGDH) are mitochondrial enzymes

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that convert oxidized nicotinamide adenine dinucleotide (NAD^+) to the reduced form (NADH), which is vital to mitochondrial respiration and oxidative phosphorylation. In AD brain, KGDH is oxidatively modified by reactive alkenals (Gibson *et al.*, 1988). Lipoic acid is one part of the PDH and KGDH complexes and is susceptible to oxidative modification because of the two sulfhydryl groups contained in this cofactor. In the current study, the effect of acrolein on mitochondrial function was assessed by the activities of NADH-linked mitochondrial enzymes PDH and KGDH. The high affinity of acrolein to sulfhydryl groups makes lipoic acid susceptible to binding by acrolein and thus may render PDH and KGDH inactive.

METHODS

Pyruvate and α-Ketoglutarate Dehydrogenase Enzyme Activity

PDH and KGDH were obtained from Sigma. Enzyme activity was assayed by measuring the production of NADH (Brown and Perham, 1976), followed spectrophotometrically at 340 nm, upon the addition of 200 μM TPP, 0.5 mM NAD^+, 50 μM CoA, 10 μM $CaCl_2$, 0.5 mM pyruvate or α-ketoglutarate dehydrogenase and 16 mUnits of purified PDH or KGDH enzyme at 37°C for times indicated. All solutions were prepared in a buffer containing 120 mM KCl, 5 mM KH_2PO_4, and 5 mM MOPS at pH 7.25. The assay was performed in the presence of 2.5 μM rotenone to prevent NADH consumption by complex I. To react with excess acrolein, 0.4 mM cysteine was added as indicated.

High Performance Liquid Chromatography (HPLC)

Reduced lipoic acid was reacted with acrolein for 30 min at 25°C in 5 mM KH_2PO_4 pH=7.2. At 30 min, the reaction was quenched by addition of 15 mM $NaBH_4$ in 0.1 mM NaOH. Reduction was allowed to proceed for 10 min at 25°C. Excess $NaBH_4$ was quenched by the addition of HCl. The products were purified by reverse phase HPLC (Waters C18 column, 0-100% acetonitrile, 0.8 mL/min, for 30 min). The product was collected in peak two (retention time = 15.5 min) as monitored at 210 nm and then analyzed by ESI-mass spectrometry. The ESI-MS observed mass/charge (*m/z*) ratio was 265, the same calculated *m/z* ratio for lipoic acid conjugated to one acrolein molecule.

RESULTS AND DISCUSSION

In this study, we evaluated whether acrolein, at levels found in the AD brain (Lovell *et al.*, 2001), would affect the enzyme activity of pyruvate dehydrogenase or α-ketoglutarate dehydrogenase. Figures 1 and 2 demonstrate that increasing nanomolar levels of acrolein significantly inhibit PHD and KGDH enzyme activity ($p < 0.0006$ and $p < 0.001$, respectively). We further investigated if acrolein were interacting with any of the coezymes of the PDH complex, which would lead to an inhibition of the enzyme activity. Acrolein (5 nM) was first incubated with each of the PDH coenzymes separately for 2 min at room temperature followed by 10 mM cysteine to scavenge any unreacted acrolein. The PDH enzyme assay shows that only incubation with NAD^+ produced a significant inhibition of PDH activity (FIG. 3). All other coenzymes activities were unaffected by acrolein. The 12% decrease in PDH activity due to interaction of acrolein with NAD^+ does not account for the 50% reduction in activity observed when 5 nM acrolein is incubated with all of the coenzymes and PDH (FIG. 1). This finding suggests that acrolein interacts directly with PDH, thereby causing the majority of the enzyme activity inhibition.

A main component of the PDH and KGDH complexes is lipoic acid. Lipoic acid uses its two sulfhydryl groups to transfer an acetyl group from TPP to CoA to form acetyl-CoA and leaving a reduced dihydrolipoamide. Dihydrolipoamide is re-oxidized to the disulfide form, resulting in the reduction of NAD^+ to NADH. Thus the sulfhydryl groups, which are extremely reactive with acrolein (Butterfield and Stadtman, 1997; Pocernich *et al.*, 2001), play a vital role in the PDH and KGDH enzyme activities. Lipoic acid was incubated with acrolein and separated by reverse-phase HPLC. Figure 4 shows that after the addition of acrolein to lipoic acid two new peaks appear in the HPLC spectrum. Analysis with electrospray ionization mass spectrometry (ESI-MS) determined an *m/z* ratio of 265 in peak 2 (FIG. 4B), the same mass as that calculated for lipoic acid conjugated to one acrolein molecule. Thus, using HPLC-MS it is suggested that acrolein does bind lipoic acid, which likely results in the observed decrease in PDH and KGDH enzyme activities.

Other researchers (Lovell *et al.*, 2001) demonstrated significant elevations of extractable acrolein in AD amygdala (2.5 ± 0.9 nmol/mg of protein) and hippocampus and parahippocampal gyrus (5.0 ± 1.6

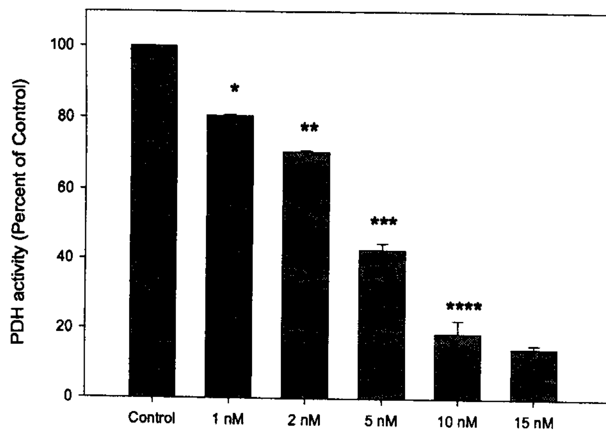

FIGURE 1 Effect of acrolein on purified pyruvate dehydrogenase. Purified PDH enzyme was incubated with varying concentrations of acrolein for 2 min at room temperature and then assayed as described. Acrolein inhibited PDH activity in a dose-dependent manner. (*,**,***,****) $p < 0.0006$, ANOVA for comparison between doses.

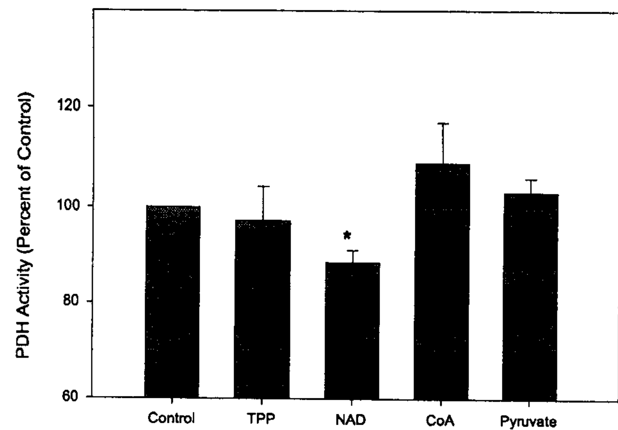

FIGURE 3 Coenzymes of PDH were incubated separately with acrolein. PDH enzyme activity was assayed as described. 200 μM TPP, 0.5 mM NAD^+, 50 μM CoA, 0.5 mM pyruvate were incubated with 5 nM acrolein for 2 min at room temperature and the remaining acrolein was scavenged by 0.4 mM cysteine. Incubation with NAD^+ demonstrated a significant decrease in PDH enzyme activity. (*) $p < 0.001$, ANOVA for comparison to control.

nmol/mg of protein) compared to age-matched controls (0.3 ± 0.5 and 0.7 ± 0.1 nmol/mg of protein, respectively). Acrolein, which was shown to inhibit state 3 respiration, did not reduce the activity of complexes I-V in the mitochondria (Picklo and Montine, 2001).

Mitochondrial dysfunction and loss of energy has been implicated in several neurodegenerative disorders, including AD (Blass, 2000; Mattson, 2000). A reduced rate of brain metabolism (Blass, 2000) and deficient activities of three mitochondrial enzyme complexes, PDH, KGDH, and cytochrome oxidase are documented abnormalities in AD (Gibson *et al.*, 1998). Oxidative stress, which is implicated in AD (Butterfield *et al.*, 2001; 2002; Butterfield, 2002; Butterfield and Lauderback 2002), and diminished glutathione levels, also found in AD, can damage PDH (Tirmenstein *et al.*, 1997) and KGDH (Gibson *et al.*,

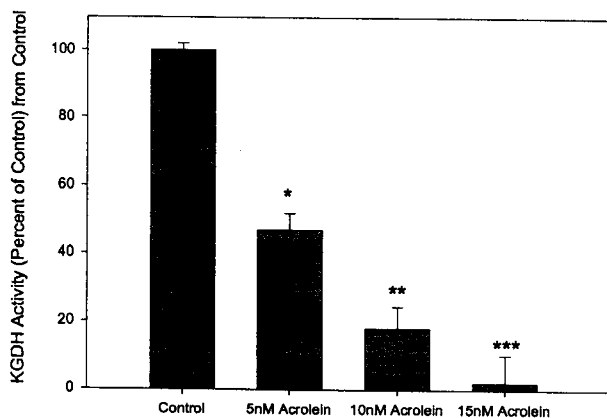

FIGURE 2 KGDH activity was inhibited in a dose-dependent manner by nanomolar levels of acrolein. (*,**,***) $p < 0.001$, ANOVA for comparison between doses.

1998), possibly leading to even more generation of ROS. Amyloid-ß-peptide, the main component of plaques in AD, induces inhibition of PDH leading to accumulation of pyruvate or lactate and energy failure, which is characteristic of AD brain (Gibson *et al.*, 1988). Aß has also been shown to generate ROS production and induce lipid peroxidation (Lauderback *et al.*, 2001; Butterfield *et al.*, 2002). Mitochondria are a site of free radical generation (Boveris *et al.*, 1972; Turrens, 1997) and mitochondrial membranes contain a significant amount of arachidonic and linoleic acids, precursors of HNE and acrolein (Ruggiero *et al.*, 1992; Uchida *et al.*, 1998). Production of acrolein in the mitochondria and subsequent inhibition of enzymes such as PDH and KGDH is most likely possible under oxidative stress.

This study demonstrates that acrolein inhibits PDH and KGDH enzyme activities, thereby potentially reducing the production of NADH. This loss of energy production, coupled with oxidative modification of other energy related enzymes in AD brain as assessed by proteomics (Castegna *et al.*, 2002; 2003), may be responsible in part for the loss of energy associated with mitochondrial dysfunction and AD. Such loss of ATP production or loss of the redox status of mitochondria could contribute to the neurodegeneration in AD brain.

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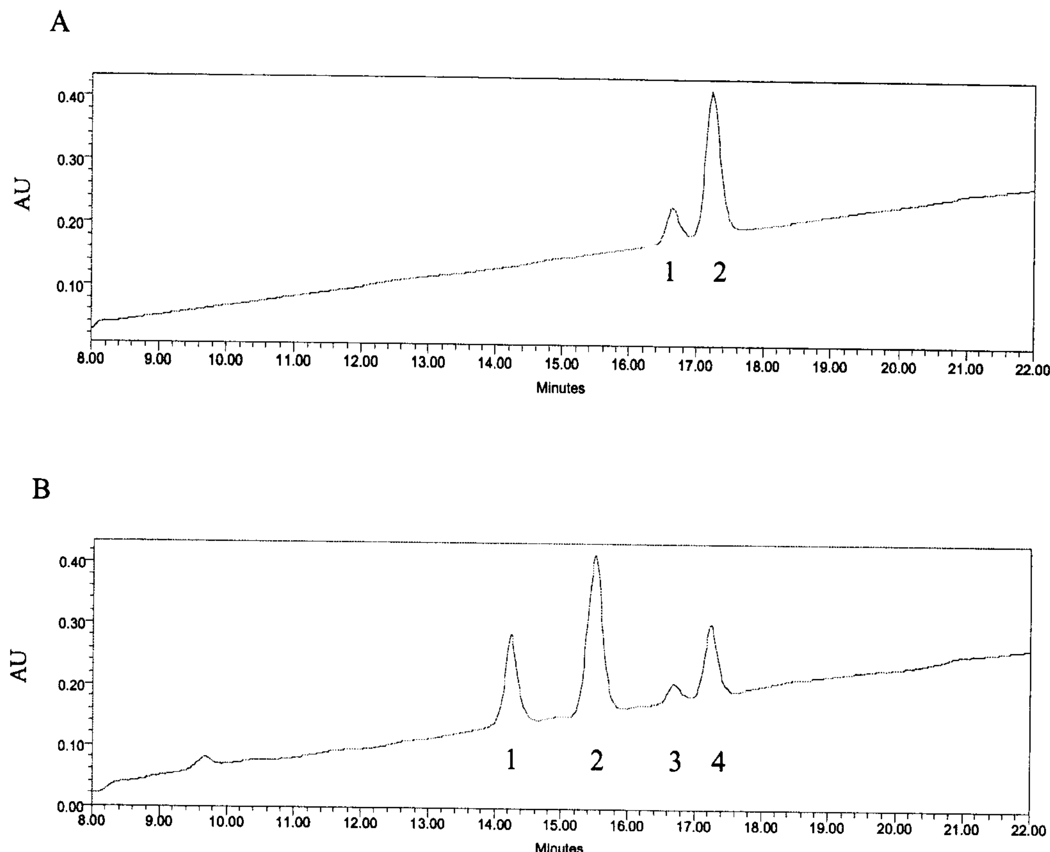

FIGURE 4 A: HPLC spectrum of lipoic acid. B: HPLC spectrum of 8 mM lipoic acid and 8 mM acrolein. Peak 2 is the product of lipoic acid conjugated to acrolein as determined by mass spectrometry.

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