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Acrolein inhibits respiration in isolated brain mitochondria

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Abstract

Lipid peroxidation is elevated in diseased regions of brain in several neurodegenerative diseases. Acrolein (2-propenal) is a major cytotoxic product of lipid peroxidation and its adduction to neuronal proteins has been demonstrated in diseased brain regions from patients with Alzheimer's disease. Mitochondrial abnormalities are implicated in several neurodegenerative disorders, and mitochondria are targets of alkenal adduction in vivo. We examined the effects of acrolein upon multiple endpoints associated with the mitochondrial involvement in neurodegenerative disease. Acrolein inhibited state 3 respiration with an IC_{50} of approx. 0.4 μmol/mg protein; however, there was no reduction in activity of complexes I–V. This inhibition was prevented by glutathione and *N*-acetylcysteine. Acrolein did not alter mitochondrial calcium transporter activity or induce cytochrome *c* release. These studies indicate that acrolein is a potent inhibitor of brain mitochondrial respiration.

Keywords: Acrolein; Lipid peroxidation; Mitochondria; Neurodegeneration; Alzheimer's disease; Respiration

1. Introduction

Lipid peroxidation, resulting from free radical damage to polyunsaturated fatty acids, occurs in Alzheimer's disease, Parkinson's disease, and in acute cerebral cell death following ischemia [1–4]. Lipid peroxidation generates cytotoxic alkenals such as 4-hydroxynonenal (HNE) and 2-propenal, commonly known as acrolein. These alkenals exert cytotoxicity by adducting cellular nucleophilic groups found on proteins, nucleic acids, or aminophospholipids [5–8]. The levels of unreacted HNE and HNE-protein adducts as well as acrolein-protein adducts are elevated in diseased regions of brain from patients with Alzheimer's disease and Parkinson's disease [3,9–13]. Acrolein formation from arachidonic acid in vitro has been reported to be approx. 40-fold greater than that of HNE [14]. Given that acrolein is estimated to be approx. 100-fold more reactive than HNE towards nucleophiles, acrolein may be of particular importance in neurodegenerative disease [7,15].

Mitochondria are implicated in several neurodegenerative disorders and are targets of alkenal adduction in vivo [16–19]. Mitochondria are a site of free radical generation, and mitochondrial membranes contain significant amounts of arachidonic

Abbreviations: HNE, 4-hydroxynonenal; PCA, perchloric acid; P_i, inorganic phosphate; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); *m*-CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid

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and linoleic acids, precursors of HNE and acrolein [20–22]. Mitochondria oxidized in vitro form HNE-protein adducts, and cardiac mitochondria isolated from rodent hearts following ischemia-reperfusion injury contain HNE-protein adducts [18,23]. Following cerebral ischemia in rodents, HNE-protein adduct immunoreactivity co-localized with that of Bcl-2, suggesting the presence of HNE adducts on central nervous system (CNS) mitochondrial proteins [19].

Because of the relevance of lipid peroxidation products and mitochondria to neurodegeneration, we studied whether acrolein modified the physiology of mitochondria isolated from brain by examining several mitochondrial endpoints associated with neurodegenerative disease. Our data show that acrolein primarily inhibits mitochondrial respiration at several levels but does not compromise complexes I–V. Cytochrome *c* release and calcium transporter function were not altered by acrolein exposure.

2. Materials and methods

2.1. Reagents

Acrolein (Aldrich) was freshly distilled the day of use. HNE was synthesized according to methods used in this laboratory [5]. Propionaldehyde, glutathione (reduced form), and *N*-acetylcysteine were purchased from Sigma. Ruthenium(III) chloride oxide (ruthenium red) was purchased from Alfa Aesar Chemicals.

2.2. Isolation of mitochondria

Whole brain mitochondria (synaptic and non-synaptic) were isolated from adult, male, Sprague-Dawley rats (Harlan) based on the procedure of Rosenthal et al. [24]. Isolation buffer consisted of mannitol (0.21 M), sucrose (70 mM), HEPES (5 mM), ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA; 1 mM), and bovine serum albumin (BSA; 1 mg/ml) with a final pH of 7.4 at 4°C. Rat were deeply anesthetized with ketamine (100 mg/ml, i.p.) and xylazine (13 mg/ml, i.p.) [25]. The brains were removed and homogenized in 10 ml per brain of ice cold buffer. The homogenates were then further diluted to 40 ml per brain and centrifuged at $3000 \times g$ for 2 min (4°C) to pellet nuclei and cellular debris. The supernatant was then centrifuged at $12\,000 \times g$ (4°C) for 10 min. The pellet was resuspended in 10 ml of homogenization buffer and 20 μl of 10% digitonin was added to disrupt the synaptosomes. The suspension was then centrifuged as above. The pellet was resuspended in buffer (10 ml) without BSA or EGTA and centrifuged. This final pellet was resuspended in 0.5 ml of buffer without BSA or EGTA. Typically, the brain of one animal was used per preparation per day. Protein concentration was measured using Protein Assay Reagent (Bio-Rad) with BSA as the standard.

2.3. Respiratory measurements

Respiration was measured using an oxygen electrode (YSI International) and oxygen consumption chamber (Gilson Medical Electronics). Experiments were performed at 37°C. Assay medium contained 125 mM KCl, 5 mM $MgCl_2$, 2 mM KPO_4 and 5 mM HEPES-KOH (pH 7.4). Mitochondria were diluted to 0.1 mg/ml. Complex I-linked substrates (5 mM glutamate and malate) or complex II-linked substrate (5 mM succinate with 1 μg/ml rotenone) were used. State 3 respiration was measured for 2 min following ADP (1 mM) addition [25]. Specific experimental protocols are given in the figure legends.

2.4. NADH oxidase:cytochrome c reductase measurement

Electron transport function from complex I to complex III was analyzed by determining NADH oxidase:cytochrome *c* reductase activity in a microwell format [26]. Mitochondria (0.1 mg/ml final concentration) were incubated in the respiratory medium (1.2 ml) for 5 min with acrolein, pelleted by centrifugation ($14\,000 \times g$ for 5 min), resuspended in 0.3 ml of oxidase assay buffer (25 mM potassium phosphate, pH 7.4, with 5 mM $MgCl_2$), and frozen at −80°C. Assays were performed at 37°C. Freeze-thawed mitochondria (40 μg of protein) were added to assay buffer (0.16 ml) containing oxidized cytochrome *c* (50 μM; Sigma) and 0.5 mM KCN and incubated for 5 min. NADH (0.24 mM, 10 μl) was

added to start the reaction and the increase in absorbance at 548 nm measured using an adjustable wavelength plate reader (SpectraMax). Rotenone (1 μg/ml) and antimycin A (1 μg/ml) were added to determine the nonenzymatic rate of reduction that was subtracted from all samples.

2.5. Assessment of complex V activity

Complex V activity was measured as oligomycin-sensitive, Mg^{2+}-ATPase activity [26]. Mitochondria (50 μg) in respiration buffer (0.5 ml) were exposed to acrolein for 5 min at 37°C, pelleted by centrifugation, resuspended in 0.1 ml of 25 mM HEPES (pH 7.4), and frozen at −80°C. Mitochondria were thawed on ice and diluted in 0.4 ml 25 mM HEPES (pH 7.4) containing 5 mM $MgCl_2$ at 37°C. Mitochondria were incubated at 37°C for 5 min to induce swelling and breaking, followed by the addition with 2.5 mM ATP for 5 min. The reaction was terminated by the addition of 1/10 volume of 3 N perchloric acid (PCA), and the inorganic phosphate (P_i) content was measured [27]. Background P_i controls were taken to be the P_i content of samples which were treated with PCA prior to addition of ATP. Oligomycin (2 μg/ml) was added to parallel samples to determine the oligomycin-insensitive rate. These rates were subtracted from the total rate to determine the oligomycin-sensitive Mg^{2+}-ATPase activity.

2.6. Measurement of cytochrome c release

Isolated mitochondria were exposed to acrolein in respiration buffer containing substrates and ATP (3 mM). Incubations were terminated by filtration through a 0.2 μm spin filter (Costar). The filtrate was stored at −80°C until analyzed. Alamethicin (4 μg/ml; Sigma) exposure was used as a positive control for mitochondrial release of cytochrome *c* [28]. Analysis of cytochrome *c* in the mitochondrial medium was performed using an HPLC method developed in our laboratory using a Jupiter 5 micron C4 reverse phase column (150×4.6 mm; Phenomenex) with pre-column filter on a Waters 2690 HPLC system with diode array capability (Waters) [29]. Absorption at 393 nm was used for detection. Cytochrome *c* isolated from rat heart (Sigma) was used as a standard.

2.7. Measurements of acrolein and HNE reactivity with mitochondria

Adduction reactivities of acrolein and HNE were estimated by measuring the amount of acrolein and HNE remaining after 5 and 10 min incubations with mitochondria in the respiratory buffer at 37°C. Mitochondria were heated at 100°C for 10 min to inactivate any metabolizing enzymes prior to mixing with the alkenal. Acrolein content was assayed using a procedure in which acrolein reacts with *m*-aminophenol in the presence of 1.5 N HCl to form the fluorescent 7-hydroxyquinoline (350 nm excitation/500 nm emission) that was quantitated using reverse phase HPLC [30]. Addition of the *m*-aminophenol derivatization solution was used to stop the reaction of acrolein with the mitochondrial solution. Zero time points were samples in which the derivatization reagent was added to the mitochondrial solution prior to addition of acrolein. Samples were centrifuged through a 0.2 μm spin filter (Costar) prior to injection.

HNE content was measured by reverse phase HPLC analysis using 220 nm detection [31]. Reactions were terminated with the addition of 1/10 volume of 3 N PCA and acetonitrile (1:1 v/v). The zero time points were samples in which PCA was added to the mitochondrial solution prior to addition of HNE. Samples were centrifuged through a 0.2 μm spin filter (Costar) prior to injection. Thiol content of mitochondrial samples was determined by incubating the samples with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB; Sigma) [32].

2.8. Statistical analyses

Statistical comparisons were performed using Student's *t*-test or two-way analysis of variance (ANOVA) where appropriate using Prism software (GraphPad) Results are expressed as the mean ± S.E.M.

3. Results and discussion

The first set of experiments were designed to analyze the effects of acrolein on the respiratory parameters of isolated rat CNS mitochondria. Acrolein

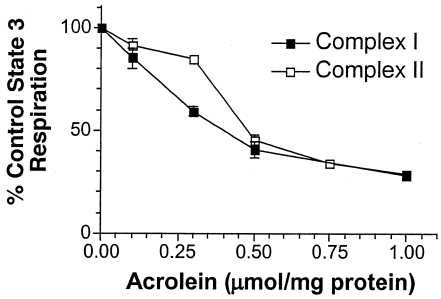

Fig. 1. Acrolein inhibits ADP-induced respiration. Mitochondria were incubated with substrates for 5 min with increasing doses of acrolein followed by the addition of ADP (1 mM) to induce state 3 respiration. Glutamate and malate were used as complex I-linked substrates. Succinate with rotenone was used as the complex II substrate. Results are the mean ± S.E.M. of five to eight samples per data point of experiments using mitochondria isolated from two different animals on two separate days for each substrate. Control complex I-linked state 3 respiration was 166 ± 4 nmol O_2/min/mg and 160 ± 4 nmol O_2/min/mg for two separate experiments. Control complex II-linked respiration was 460 ± 16 nmol O_2/min/mg and 338 ± 5 nmol O_2/min/mg for two separate experiments. Two-way ANOVA indicated that the use of substrate influenced the effect of acrolein ($P < 0.05$).

inhibited ADP-induced state 3 respiration with complex I-linked substrates, IC_{50} of approx. 0.4 μmol/mg protein, and with complex II-linked substrates, IC_{50} of approx. 0.5 μmol/mg protein (Fig. 1). State 4 respiration was not altered by the concentrations of acrolein tested. Addition of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (*m*-CCCP; 0.2 μM) restored succinate-linked respiration to the ADP-induced, control state 3 rate (Fig. 2) and indicated that complex II through complex IV is not a target of acrolein. These results are similar to the effects of acrolein upon isolated rat hepatic mitochondria [33]. In contrast, acrolein inhibits complex II-linked state 3 and uncoupled respiration in mitochondria isolated from rat heart [34]. Indeed, we and others have shown that mitochondria isolated from rat brain, rat heart, or rat liver may have differing responses to exposure to other α,β unsaturated aldehydes such HNE and 4-hydroxyhexenal [35–37]. Under these experimental conditions, *m*-CCCP did not stimulate complex I-linked uncoupled respiration with glutamate and malate as substrates in control samples. Propionaldehyde, an analogue of acrolein lacking the C2 double bond, was ineffective even at 1.5 μmol/mg, indicating that acrolein caused respiratory inhibition through Michael adduction [33].

Acrolein (0.75 μmol/mg) did not inhibit NADH: cytochrome *c* oxidoreductase activity (complexes I–III) or oligomycin-sensitive, Mg^{2+}-stimulated ATPase (complex V) activity. In total, the data from the oxygen consumption measurements, showing no loss of electron transport between complex II and complex IV, and the individual complex assays demonstrated that no rate-limiting inhibition of the respiratory complexes I–V occurred. These findings are in contrast to the complex III inhibition caused by HNE, and indicate that the structure of the alkenal influences the adduction target [25].

Potential remaining targets of acrolein-mediated inhibition of state 3 respiration include P_i transport, substrate transport, the adenine nucleotide translocase, or citric acid cycle enzymes. Respiratory impairment by acrolein in hepatic mitochondria occurs in part by interrupting P_i transport, analogous to the thiol alkylating agent *N*-ethylmaleimide and formaldehyde [33,38–40]. Attempts to measure P_i-induced swelling (based on the procedures of Johnson and Chappell) under the same conditions used in our experiments were not successful [39]. Malate transport into the mitochondria is dependent on matrix P_i content and hence would also be decreased with inhibition of the P_i transporter [39]. In addition, acrolein inhibits glutamate transport in hepatic mitochondria [33]. Thus, respiratory inhibition in brain mitochondria by acrolein is likely the result of acrolein adduction to multiple targets.

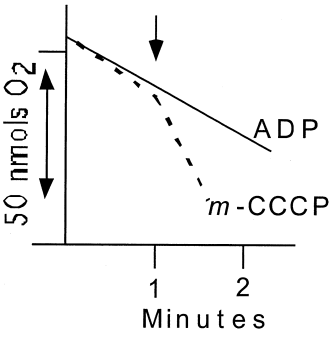

Fig. 2. Uncoupled complex II-linked respiration is not inhibited by acrolein. Mitochondria were incubated with acrolein (0.75 μmol/mg) and substrate (succinate plus rotenone) for 5 min prior to the addition (denoted by the arrow) of ADP (1 mM) or the uncoupler *m*-CCCP (0.2 μM).

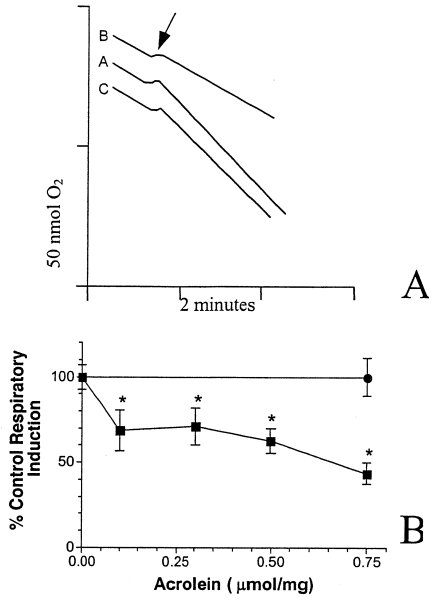

Fig. 3. Acrolein inhibits calcium-induced respiration but not the calcium transporter activity. (A) Increase in respiration by calcium addition. In trace A, mitochondria were incubated in respiratory buffer containing succinate with rotenone and ATP (3 mM). After 5 min (shown by the arrow), the ability of the mitochondria to increase respiration because of uptake of calcium was tested by the addition of $CaCl_2$ (1 μmol/mg). In trace B, the calcium transporter inhibitor ruthenium red (0.2 μM) was included. In trace C, acrolein (0.75 μmol/mg) was added. The ratio of respiratory rates before and after calcium addition was used to calculate the percent induction by calcium in panel B. (B) Acrolein inhibits respiration induced by glutamate and malate (shaded squares) but not by succinate with rotenone (shaded circles). Each point represents the mean ± S.E.M.; $n = 3$ or 4 from a single preparation of mitochondria. $^*P < 0.05$ using Student's t-test to compare each concentration of acrolein to the control (0 μmol/mg acrolein).

Mitochondrial uptake of calcium, particularly following the opening of ionotropic glutamate receptors, can lead to the generation of reactive oxygen species with ensuing cell death [41,42]. Thus, we determined whether acrolein altered mitochondrial calcium transporter activity by measuring calcium-induced increases in oxygen consumption, an effect blocked by an inhibitor of the mitochondrial calcium transporter, ruthenium red (0.2 μM) [41,43]. Calcium-induced respiration was not diminished by acrolein with succinate as substrate, and only partially inhibited with glutamate and malate (Fig. 3). Basal respiratory rates (with substrates and ATP present) were not altered. These data suggest that the calcium transporter activity is not compromised by acrolein. Similar to data in Fig. 1, these data also pointed to an increased sensitivity of complex I-linked respiration to acrolein exposure.

The release of cytochrome c from the mitochondrial intermembrane space is one trigger for apoptosis, and is proposed as a major signaling pathway in neurodegenerative disease [44,45]. This release may occur independently of respiratory inhibition or of transition pore opening [28,46]. Acrolein, at a concentration that inhibited ADP-induced respiration, did not induce cytochrome c release (Fig. 4).

Prior work from our laboratory shows that the thiol scavenger N-acetylcysteine does not prevent HNE-mediated inhibition of respiration, possibly because of the hydrophobic nature of HNE versus the hydrophilicity of the thiol scavengers [25]. In contrast, pretreatment of mitochondrial with a 2-fold excess glutathione, N-acetylcysteine, or the methyl ester of N-acetylcysteine significantly attenuated the effects of acrolein (Fig. 5). These data suggested that the structure of the toxic alkenal is a factor when determining the efficacy of thiol-based protectants.

Lastly, we compared the reactivities of acrolein and HNE with mitochondrial nucleophiles by measuring the loss of alkenal over time when incubated with heat-inactivated mitochondria (Fig. 6). Acrolein

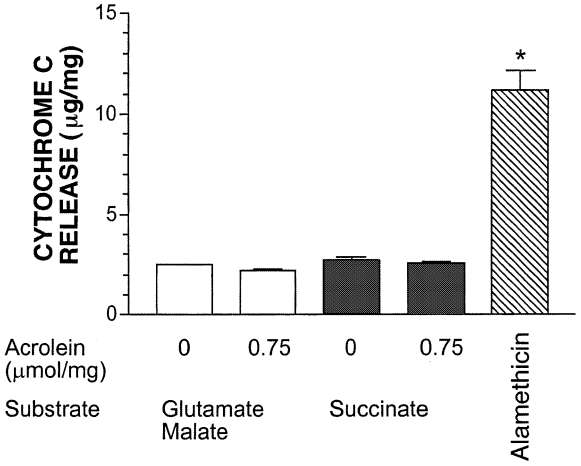

Fig. 4. Acrolein does not cause cytochrome c release. Mitochondria were incubated in respiratory buffer containing substrates, ATP (3 mM), and acrolein for 5 min. The incubation was terminated by filtration to remove mitochondria. The filtrate was analyzed by HPLC for cytochrome c content. Each point represents the mean ± S.E.M.; $n = 3$ from a single preparation of mitochondria $^*P < 0.05$ using Student's t-test.

loss was rapid compared to HNE. Indeed, loss of HNE was negligible at 10 min as compared to the 30% loss of acrolein. The 30% loss of acrolein represented 0.150 μmol of acrolein/mg protein; however, brain mitochondrial preparations contained only approx. 0.05 μmol of thiols/mg protein as measured by incubation with DTNB [32]. These data indicated that acrolein alkylated mitochondrial nucleophiles other than thiols.

Lipid peroxidation and mitochondrial dysfunction have been observed in chronic neurodegenerative disease and acute cerebral insult. Exactly how these two phenomena interact is not known. In this work, we studied multiple endpoints of mitochondrial pathophysiology relevant to neurodegeneration and found that respiratory function is compromised by acrolein exposure. The brain has shallow energy reserves such that increases in energy demand must be met by increases in energy production by the mitochondria [47]. This environment is complicated by cytosolic calcium present following ionotropic glutamate receptor opening [42]. The failure of the mitochondrion to increase neuronal energy requirements in the face of increased ADP may lead to neuronal toxicity. Our data indicated that acrolein did not affect basal levels of respiration but rather impeded increases in respiration by elevated ADP levels. The fact that acrolein did not inhibit calcium transporter activity suggests that calcium-induced free radical generation can still occur in mitochondria exposed to acrolein [41]. We hypothesize that the accumulation of alkenal adducts on the mitochondrion may render the neuron vulnerable to energy stress under conditions where an increase in mitochondrial respiration is needed.

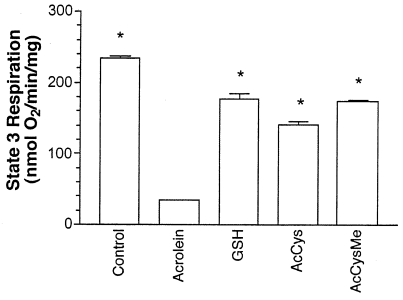

Fig. 5. Thiol scavengers prevent acrolein-mediated respiratory inhibition. Mitochondria, with glutamate and malate as substrates, were exposed to acrolein (1.0 μmol/mg) for 5 min with and without the thiol scavengers (2.0 μmol/mg) glutathione (GSH), *N*-acetylcysteine (AcCys), or the methyl ester of *N*-acetylcysteine (AcCysMe) followed by addition of ADP. Results are the mean ± S.E.M. for three or four samples from a single preparation of mitochondria. $^{*}P < 0.05$ (using Student's *t*-test) compared to acrolein alone.

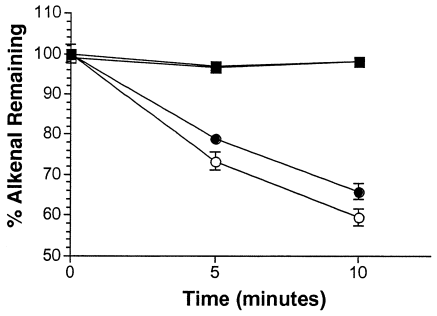

Fig. 6. Acrolein alkylates mitochondrial and BSA nucleophilic groups more rapidly than HNE. Heat-inactivated mitochondria (0.1 mg/ml) or BSA (0.1 mg/ml) were exposed to either HNE or acrolein (0.5 μmol/mg protein) at 37°C in respiration buffer. Remaining alkenal at the time points was determined by HPLC. ○, acrolein treated; □, HNE treated. Shaded samples, mitochondria; open symbols, BSA. Values shown are the mean ± S.E.M. $n = 4$ for BSA samples and $n = 6$ for mitochondrial samples.

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