Original Contribution

Trans-4-oxo-2-nonenal potently alters mitochondrial function

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ABSTRACT

Alzheimer disease elevates lipid peroxidation in the brain and data indicate that the resulting lipid-aldehydes are pathological effectors of lipid peroxidation. The disposition of 4-substituted nonenals derived from arachidonate (20:4; \textit{n}-6) and linoleate (18:2; \textit{n}-6) oxidation is modulated by their protein adduction targets, their metabolism, and the nature of the 4-substituent. Trans-4-oxo-2-nonenal (4-ONE) has a higher toxicity in some systems than the more commonly studied trans-4-hydroxy-2-nonenal (HNE). In this work, we performed a structure-function analysis of 4-hydroxy/oxoalkenal upon mitochondrial endpoints. We tested the hypotheses that 4-ONE, owing to a highly reactive nature, is more toxic than HNE and that HNE toxicity is enantioselective. We chose to study freshly isolated brain mitochondria because of the role of mitochondrial dysfunction in neurodegenerative disorders. Whereas there was little effect related to HNE chirality, our data indicate that in the mitochondrial environment, the order of toxic potency under most conditions was 4-ONE > HNE. 4-ONE uncoupled mitochondrial respiration at a concentration of 5 \textmu M and inhibited aldehyde dehydrogenase 2 (ALDH2) activity with an \textit{IC}_{50} of approximately 0.5 \textmu M. The efficacy of altering mitochondrial endpoints was ALDH2 inhibition > respiration = mitochondrial swelling = ALDH5A inhibition > GSH depletion. Thiol-based alkenal scavengers, but not amine-based scavengers, were effective in blocking the effects of 4-ONE upon respiration. Quantum mechanical calculations provided insights into the basis for the elevated reactivity of 4-ONE > HNE. Our data demonstrate that 4-ONE is a potent effector of lipid peroxidation in the mitochondrial environment.

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than HNE in RKO cells [10]. This inconsistency indicates that chemical reactivity is not the sole predictor of aldehyde toxicity and that biological effects are environment-dependent.

In this work, we performed a structure–function analysis of 4-hydroxy/oxoalkenal neurotoxicity. We tested the hypotheses that 4-ONE, owing to its highly reactive nature, is more neurotoxic than HNE and that HNE toxicity is enantioselective. We chose to study freshly isolated brain mitochondria because of the role of mitochondrial dysfunction in neurodegenerative disorders such as AD. Our data indicate that in the mitochondrial environment, the order of toxic potency under most conditions was 4-ONE > HNE. Enantioselective effects of HNE were observed only in mitochondria at high concentrations of S-HNE.

Materials and Methods

Chemicals

The individual R and S enantiomers of HNE and racemic HNE were synthesized as previously described [17]. 4-ONE was purchased from Cayman Chemical (Ann Arbor, MI, USA). Acetaldehyde, pyridoxamine, succinic semialdehyde, NAD+, NADH, GSH, L-carnosine, and trans-2-hexenal were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Quantitation of HNE and 4-ONE

Analysis of HNE content was performed by HPLC with 220 nm detection using benzaldehyde as an internal standard as previously described [18]. Analysis of 4-ONE content was performed by HPLC using a C8 column (4.6 × 250 mm; Phenomenex, Torrance, CA, USA) and 230 nm detection. Trans-2-hexenal was added as an internal standard. An isotropic solvent system of 30:50 (vol/vol) ACN:H2O was used. Under these conditions 4-ONE eluted at approximately 6 min. A Shimadzu HPLC system with photodiode array detector was used as described previously.

Determination of 4-ONE and HNE reaction rate constants

Reactions were performed with 50 μM 4-ONE or 50 μM racemic HNE with NAC (1 mM) at 37 °C in sodium phosphate buffer (50 mM, pH 7.4). The reaction was started with the addition of NAC and aliquots of the reactions were taken at increasing time points. The reaction was stopped by addition of a 1/10 volume of 6% phosphoric acid. Data were fit according to a one-site loss model with Prism 4.0 software (GraphPad, San Diego, CA, USA). The calculated half-life (t1/2 in seconds) was used to compute the first-order rate constant (k) in which k = ln(2)/t1/2.

Isolation of rat brain mitochondria

All experimental protocols were in accordance with the NIH guidelines for use of live animals and were approved by the University of North Dakota Institutional Animal Care and Use Committee. Sprague–Dawley rats (male, 250 g) were purchased from Charles River Laboratories (Wilmington, MA, USA). Rats were deeply anesthetized with ketamine (100 mg/kg) and xylazine (13 mg/kg), the brain was removed, and mitochondria were isolated as described previously [19,20].

Measurement of respiration

Respiration was measured using an oxygen electrode (YSI International) and oxygen consumption chamber (Gilson Medical Electronics). Experiments were performed with stirring at 37 °C. Respiration assay medium contained 40 mM KCl, 50 mM mannitol, 50 mM sucrose, 1 mM EGTA, 10 mM Hepes free acid, 5 mM MgCl2, and 10 mM K2HPO4, pH 7.2 [22]. The complex I-linked substrates pyruvate (10 mM) and malate (5 mM) and ADP (1 mM) were used to determine the respiratory control ratio. State 4 respiration was taken as the respiratory rate after the addition of pyruvate and malate 1 min before the addition of ADP to stimulate State 3 respiration. Mitochondria were used only if the respiratory control ratio was 5 or higher. m-CCCP (1 μM) was used to effect a State 3-like rate of respiration [22]. Mitochondria were used at a final concentration of 0.1 mg/ml unless otherwise specified.

Measurement of mitochondrial swelling

Mitochondrial swelling was measured by monitoring the change in absorbance at 540 nm over time. Assays were performed in 37 °C respiration buffer with mitochondria at 0.1 mg/ml in a 1-ml volume. Pyruvate and malate were added as substrates followed by addition of the aldehyde.

Complexes I, II, III, and IV

Mitochondria were exposed to increasing concentrations of 4-ONE for 5 min followed by quenching of the reaction by the addition of 2 mM l-mercaptoethanol to quench the aldehyde. Mitochondria were pelleted by centrifugation, washed with respiration buffer, resuspended in 25 mM potassium phosphate (pH 7.2) containing 5 mM MgCl2, and frozen at −80 °C for subsequent analyses. NADH:ubiquinone oxidoreductase (complex I) activity was measured according to the procedure of Kirby and colleagues utilizing NADH and n-decylubiquinone as substrates [21]. Succinate:ubiquinone oxidoreductase (complex II) activity was measured as described by Kirby and colleagues using succinate and dichlorophenyl indolephenol as substrates [21]. Ubiquinol:cytochrome c oxidoreductase...
(complex III) activity was measured as described by Kirby and colleagues using n-decylubiquinol and oxidized cytochrome c as substrates [21]. Assays for complexes I, II, and III, were performed in a microplate format using a SpectraMax 384Plus UV/Vis plate reader (Molecular Devices, Sunnyvale, CA, USA). Cytochrome c oxidase (complex IV) activity was measured by addition of 1 mM ascorbate and 0.2 mM tetramethylphencyl-nenyline diamine (Sigma) to reduce cytochrome c and produce oxygen consumption [22].

**Aldehyde dehydrogenase assays**

Mitochondria (0.2 mg/ml) were exposed to the different alkenals for 5 min in a 2-ml final volume of respiration buffer followed by quenching of the alkenal with the addition of 2.0 mM β-mercaptoethanol. The sample was centrifuged for 10 min at 13,000 g to pellet the mitochondria. The supernatant was removed and the pellet resuspended in 0.2 ml of PBS buffer (pH 7.4, 37 °C) with 1 mM NAD+ present as cofactor [19]. To study the depletion of GSH in mitochondria, mitochondria (0.2 mg/ml) were exposed to the different alkenals for 5 min in a 0.5-ml final volume in respiration buffer. After 5 min, a 50-μl aliquot of 10% sulfosalicylic acid was added. The sample was immediately frozen at −80 °C until analysis for total GSH content as described previously [17].

**Mitochondrial GSH depletion**

To study the depletion of GSH in mitochondria, mitochondria (0.2 mg/ml) were exposed to the different alkenals for 5 min in a 0.5-ml final volume in respiration buffer. After 5 min, a 50-μl aliquot of PBS buffer (pH 7.4, 37 °C) was added. The sample was immediately frozen at −80 °C until analysis for total GSH content as described previously [17].

**Computational chemistry**

Geometry optimizations followed by harmonic vibrational frequency calculations were performed (in the gas phase and in the condensed phase) using the MPW1B95 variant of density functional theory (DFT) with the 6-311G** basis set for 4-ONE and HNE [23–27]. The MPW1B95 variant of DFT is a hybrid meta generalized gradient approximation DFT approach developed by Zhao and Truhlar based on the modified Perdew and Wang exchange functional and Becke’s 1995 one-parameter gradient-corrected correlation functional [26]. Condensed-phase calculations were carried out using water as the solvent and employing the self-consistent reaction field polarizable continuum model based on the integral equation formalism approach [23–27]. The Gaussian03 suite of programs was used for all quantum mechanical calculations reported herein [24].

All gas-phase geometry optimizations were performed with a parameter convergence tolerance of 10−3 a.u. and subsequent harmonic vibrational frequency calculations showed no imaginary frequencies, which confirms the equilibrium structures as true minima. When calculations that take into account solvent effects were performed, there was one small imaginary frequency for HNE. Considering that the low frequency mode due to rotations and translations, which should be 0 in the absence of numerical noise, were substantially larger (i.e., in the range −40 to 12 cm−1 for HNE), it should be concluded that the observed negative frequency was due to numerical artifact. An analysis of the low-frequency normal mode showed that the imaginary mode for HNE was due to soft rotations of the aliphatic tail of the molecule. Hence, it is appropriate to consider the structures obtained in the solvent calculations as equilibrium structures corresponding to the true minimum seen for the gas-phase calculations.

To further investigate the differences between 4-ONE and HNE in relation to their chemical reactivity, several molecular properties were calculated. These include dipole moment, molecular hardness (\(\eta\)), molecular softness (\(\sigma\)), chemical potential (\(\upsilon\)), and electrophilicity index (\(\omega\)). These properties were computed according to procedures described in the literature [28,29], where \(\eta = \frac{\epsilon_{\text{LUMO}} - \epsilon_{\text{HOMO}}}{2}\); \(\sigma = \frac{1}{\eta\upsilon}\); \(\upsilon = \frac{\epsilon_{\text{LUMO}} + \epsilon_{\text{HOMO}}}{2}\); and \(\omega = \upsilon/2\eta\). All calculations were carried out using the Gaussian03 software package [24].

**Statistical analyses**

Statistical comparisons were performed with one-way ANOVA and two-way ANOVA, as appropriate, with Bonferroni’s posttests using Prism 4.0 software (GraphPad). All data are presented as the means ± SD with significance obtained when \(p<0.05\).

**Results**

Previous kinetic reaction data indicate that 4-ONE reacts at a faster rate than HNE with simple nucloephiles and with some model proteins [16]. To confirm this elevated reactivity, we examined the reactions of 4-ONE and HNE with the simple nucleophilic reactant NAC to calculate the first-order rate constants. The first-order rate constant of 4-ONE was 7.2×10−2 s−1, whereas for HNE the first-order rate constant was 4.6×10−3 s−1, a difference of approximately 160-fold (Fig. 1). This difference in the first-order rate constants for NAC is nearly identical to the results obtained by other investigators [15,16].

To better understand the nature of the intermolecular interactions and/or chemical reactivity vis-à-vis molecular properties of 4-ONE and HNE, several molecular properties were calculated using quantum chemical methods. These include dipole moment, molecular hardness, molecular softness, chemical potential, and electrophilicity index (see Table 1), as well as plots of electrostatic potential surfaces (ESPs) and highest occupied molecular orbital (HOMO) densities (Fig. 2). The ESPs demonstrate that 4-ONE possesses an area of larger positive potential (concentrated blue color) surrounding the C3 carbon atom compared to HNE (Fig. 2). 4-ONE has two large negative potentials (red) around the 4-oxo group and the aldehyde group; whereas HNE has only one highly negative potential area on the aldehyde functional group. The HOMO electron densities show extensive electron conjugation in 4-ONE, but in HNE the HOMO electron density is more localized. The separation of the molecular electron density into areas of positive potential and areas of negative potential leads to net molecular dipoles of slightly different magnitudes for 4-ONE (6.5 D) and HNE (5.9 D; Table 1). The results obtained from theoretical calculations also show that 4-ONE is a softer molecule (\(\sigma = 0.355\) eV) than HNE (\(\sigma = 0.301\) eV), which correlates well with the calculated molecular hardness of 2.816 and 2.913, respectively.

**Table 1**

<table>
<thead>
<tr>
<th>Property</th>
<th>4-ONE</th>
<th>HNE</th>
</tr>
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<tbody>
<tr>
<td>(\mu_s) (D)</td>
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<td>3.976</td>
</tr>
<tr>
<td>(\mu_s) (D)</td>
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<td>0.672</td>
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<tr>
<td>(\epsilon_{\text{HOMO}})</td>
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<td>5.893</td>
</tr>
<tr>
<td>(\epsilon_{\text{LUMO}})</td>
<td>2.816</td>
<td>3.323</td>
</tr>
<tr>
<td>(\epsilon_{\text{HOMO}})</td>
<td>0.355</td>
<td>0.301</td>
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<tr>
<td>(\epsilon_{\text{LUMO}})</td>
<td>−4.919</td>
<td>−4.405</td>
</tr>
<tr>
<td>(\sigma) (eV)</td>
<td>4.296</td>
<td>2.913</td>
</tr>
</tbody>
</table>

\(\mu_s\) is the molecular dipole moment computed in the x, y, and z directions and the total; \(\eta\) is the molecular hardness; \(\sigma\) is the molecular softness; \(\epsilon\) is the chemical potential; and \(\omega\) is the electrophilicity index.
being present during the ALDH assays. Comparison of the two aldehydes for inhibition of ALDH2 demonstrated no significant difference with IC50 of approximately 0.4 to 0.6 μM for 4-ONE and HNE (Fig. 5A). In contrast, 4-ONE inhibited ALDH5A activity with an IC50 of 9 μM and HNE (100 μM) inhibited ALDH5A activity by 20% (Fig. 5B). The differences in effects of ALDH2 versus ALDH5A may be the result of the lower Km of HNE for ALDH2 (1 μM) versus its Km for ALDH5A (30 μM) and the fact that ALDH2 contains three vicinal thiol groups (including the catalytic cysteine at its active site) that may participate in adduction [9,14,19]. These data demonstrate that ALDH2 is the most sensitive to inactivation by these aldehydes in situ compared to the other mitochondrial parameters tested. We investigated the ability of these compounds to deplete mitochondrial GSH content. Similar to the respiration studies, 4-ONE depleted GSH with the highest efficacy, with an IC50 of approximately 35 μM, whereas HNE did not deplete 50% of the mitochondrial GSH even at 100 μM HNE, the highest concentration used (Fig. 5C). Previous work from this laboratory demonstrated there was not an enantioselective effect of R- or S-HNE upon mitochondrial GSH depletion [17].

A potential cause of uncoupling is swelling of the mitochondria with subsequent leakage of protons across the inner membrane. By monitoring the changes in ASO4 over time, we observed that 4-ONE caused mitochondrial swelling that was blocked by the addition of GSH (Fig. 6A). Significant swelling occurred in a concentration-dependent manner with a plateau obtained at 10 μM 4-ONE (Fig. 6B). These data are synchronous with the increase in uncoupling induced by 4-ONE (see Fig. 3).

Because 4-ONE altered mitochondrial respiration, we tested the ability of carbonyl scavengers (1 mM each) to prevent respiratory changes induced by the addition of 20 μM 4-ONE. GSH and NAC effectively blocked the respiratory changes caused by 4-ONE, whereas l-carnosine and pyridoxamine provided no protection (Fig. 7A). Because previous data have demonstrated that l-carnosine and pyridoxamine are scavengers of carbonyls, and particularly pyridoxamine toward 1,4-dicarbonyls, we tested whether the scavengers depleted 4-ONE content under the same conditions as the respiration experiments without mitochondria present [30–32]. GSH and NAC reduced 4-ONE content after a 5-min incubation to below detectable levels by HPLC–UV. On the other hand, l-carnosine depleted 4-ONE content only 20% and pyridoxamine was ineffective (Fig. 7B).

Discussion

Reactive lipid-aldehyde species are implicated in the pathogenesis of neurodegenerative diseases as well as other diseases such as atherosclerosis and diabetes. Of these lipid-aldehydes, the n-6 derived HNE has been studied extensively, whereas the biological effects of 4-ONE are only now being uncovered. In vitro cell culture and biochemistry studies indicate that the toxicity of these compounds is influenced by HNE chirality, electrophilic strength, and the target endpoints studied. Using freshly isolated rat brain mitochondria, we performed a structure–function analysis of HNE, its enantiomers, and 4-ONE to test the hypotheses that 4-ONE, owing to its highly reactive nature, is more toxic than HNE and that HNE toxicity is enantioselective. While there was little difference regarding HNE enantiomers, our data indicate that 4-ONE potently alters mitochondrial function. However, the sensitivity to 4-ONE versus HNE was target-dependent with the order of sensitivity being ALDH2 > respiration = swelling = ALDH5A > GSH depletion. Our data also indicate that thiol-based, but not amine-based, carbonyl scavengers were effective protectants against 4-ONE.

Respiratory changes were one of the most sensitive effects of 4-ONE treatment. Uncoupling was 20-fold more sensitive with 4-ONE than with HNE. Although we did not use over 100 μM HNE in our current experiments, we have previously shown that HNE inhibits State 3 respiration with an IC50 of 200 μM, 20-fold less potent than the 4-ONE effect observed [22]. Inhibition of the respiratory complexes I–V did not

**Fig. 2.** Molecular electrostatic surface potential (ESP) and highest occupied molecular orbital (HOMO) models for 4-ONE and HNE obtained from quantum mechanical density functional theory calculations. The ESP models (isodensity = 0.0004 e/Å3) provide the electrostatic charge in a color scale, with the blue showing the area with the most positive charge and red showing areas with the most negative charge. In the HOMO models (isodensity = 0.02 e/Å3), green and red represent the α and β electron densities, respectively. The structures of the corresponding aldehydes shown as skeletons within the mesh ESP models are oriented in the same way as in the HOMO models. Note the larger positive charge (dark blue area) at C3 for 4-ONE compared to lower positive charge (light blue area) at C3 for HNE. Also, the HOMO models show extensive electron delocalization in 4-ONE, which facilitates electron movement toward the carbonyl groups and leads to greater charge separation; such electron delocalization is absent in HNE.
4-ONE potently alters mitochondrial respiration. Rat brain mitochondria were incubated with increasing concentrations 4-ONE with (A) pyruvate and malate (complex I-linked substrates) or (B) succinate (complex II-linked substrate) for 5 min before the addition of ADP to stimulate State 3 respiration. State 4 respiration was calculated 1 min before the addition of ADP. Significant increases in State 4 respiration were observed at 5 μM 4-ONE with all substrates. (C, D) Uncoupled respiration in the presence of m-CCCP, pyruvate, and malate was also inhibited by 4-ONE to a similar extent at State 3 respiration. Complex IV through complex IV activity was not inhibited by 4-ONE. Data are the means ± SD and were all compared (4-ONE concentrations versus control, 0 μM 4-ONE) using a one-way ANOVA with Bonferroni’s posttest. *p<0.05. Data in (A) was obtained from experiments performed in triplicate on three separate days (n = 3); for data in (B), n = 2, with assays performed in triplicate or quadruplicate. Complex IV and uncoupling assays n = 3, with assays performed in triplicate. Complex I, II, and III assays, n = 3 in triplicate with activities of 171 ± 14, 79 ± 7, and 797 ± 49 nmol/min/mg, respectively. Control pyruvate/malate-linked respiration in (A) was State 3, 348 ± 60 nmol O₂/min/mg, and State 4, 60 ± 10 nmol O₂/min/mg. Controls for succinate-linked respiration in (B) were, for both days, State 3, 214 and 448 nmol O₂/min/mg, and State 4, 88 ± 14 and 91 ± 10 nmol O₂/min/mg. Control complex IV activities were 724 and 790 nmol O₂/min/mg. Control uncoupled respiration was 307 ± 49 nmol O₂/min/mg.

Fig. 3. 4-ONE potently alters mitochondrial respiration. Rat brain mitochondria were incubated with increasing concentrations 4-ONE with (A) pyruvate and malate (complex I-linked substrates) or (B) succinate (complex II-linked substrate) for 5 min before the addition of ADP to stimulate State 3 respiration. State 4 respiration was calculated 1 min before the addition of ADP. Significant increases in State 4 respiration were observed at 5 μM 4-ONE with all substrates. (C, D) Uncoupled respiration in the presence of m-CCCP, pyruvate, and malate was also inhibited by 4-ONE to a similar extent at State 3 respiration. Complex IV through complex IV activity was not inhibited by 4-ONE. Data are the means ± SD and were all compared (4-ONE concentrations versus control, 0 μM 4-ONE) using a one-way ANOVA with Bonferroni’s posttest. *p<0.05. Data in (A) was obtained from experiments performed in triplicate on three separate days (n = 3); for data in (B), n = 2, with assays performed in triplicate or quadruplicate. Complex IV and uncoupling assays n = 3, with assays performed in triplicate. Complex I, II, and III assays, n = 3 in triplicate with activities of 171 ± 14, 79 ± 7, and 797 ± 49 nmol/min/mg, respectively. Control pyruvate/malate-linked respiration in (A) was State 3, 348 ± 60 nmol O₂/min/mg, and State 4, 60 ± 10 nmol O₂/min/mg. Controls for succinate-linked respiration in (B) were, for both days, State 3, 214 and 448 nmol O₂/min/mg, and State 4, 88 ± 14 and 91 ± 10 nmol O₂/min/mg. Control complex IV activities were 724 and 790 nmol O₂/min/mg. Control uncoupled respiration was 307 ± 49 nmol O₂/min/mg.

The finding that 4-ONE at low concentrations induces mitochondrial uncoupling may have physiologic significance as a response to oxidative damage. Previous research proposes that HNE may have a protective effect through activation of uncoupling proteins [33]. The uncoupling of mitochondrial respiration in some cases is seen as a protective response by allowing for utilization of NADH and electrons in the electron transport chain to prevent superoxide generation [34,35]. Thus, the uncoupling by 4-ONE at a concentration 20-fold less than observed for HNE indicates that 4-ONE, not HNE, may be a more important oxidative damage signaling molecule. The mechanisms underlying this effect of 4-ONE clearly warrant more study.

The fact that ALDH2 was similarly inhibited by 4-ONE and HNE gives insight into the in situ environment of ALDH2 in the mitochondrial matrix and demonstrates that chemical reactivity is not the sole determinant of biological efficacy. ALDH2 metabolizes short- and long-chain aliphatic aldehydes. Our laboratory has shown that HNE has a Km of approximately 1 μM for ALDH2 [9]. Thus, it is not surprising that inhibition of ALDH2 activity by HNE in this system occurred with an IC50 of a similar concentration. The finding that both 4-ONE and HNE inhibit ALDH2 at similar concentrations indicates that the initial noncovalent interaction of the aldehydes in the active site of ALDH2 in situ is of greater importance than the chemical reactivity of 4-ONE>HNE.

It is surprising, however, that HNE led to a loss of ALDH2 activity. Other investigators, using purified enzyme, have demonstrated that HNE inhibits ALDH2, in a competitive, mixed-type manner [36]. In our experimental design, intact mitochondria were treated with HNE followed by disruption of the mitochondria and assay of ALDH2 activity. We limited the possibility of unreacted HNE being present in the system by quenching the reaction with a large excess of free thiol, pelleting the sample, and then diluting the sample. Thus, the inhibition of ALDH2 by HNE observed in this mitochondrial system is likely to be irreversible. Whereas it is known that ALDH2 is covalently modified by HNE in the absence of the NAD⁺ cofactor, ALDH2 in the presence of its cofactor NAD⁺ readily catalyzes the formation of the acid product trans-4-hydroxy-2-nonenonic acid [9,14]. Our current data suggest that in the mitochondrial environment, the ALDH2 active site exists without NAD⁺ present and support the observation that NAD⁺ content in the mitochondrial matrix is a rate-
limiting factor for NAD+-dependent enzymes such as ALDHs [37]. The data have implications for pathological states such as cardiac ischemia–reperfusion injury in which ALDH2 has recently been shown to play a protective role [38–40].

Characterization of the reaction rate of 4-ONE and HNE with NAC along with the determination of quantum mechanical parameters provides molecular insight into alkenal chemistry. The faster reaction rate of 4-ONE with NAC compared to HNE is not surprising given the 4-oxo electron-withdrawing group present on 4-ONE. Electrostatic potential surface models demonstrated that 4-ONE has a greater positive charge surrounding the unsaturated C2–C3 bond than HNE, a finding supported by the HOMO model in which the electrons from C4 to C1 of 4-ONE are delocalized, leading to an electron-poor, and hence more electrophilic, C3 carbon. Whereas it is assumed that the aldehyde of HNE also leads to the withdrawal of electrons from the C2–C3 double bond, the formation of a delocalized electron system from C1 to C3 was minimal. A comparison of calculated overall dipole moments was not a valid predictor of reactivity because the overall dipole moment for 4-ONE was only slightly greater (6.479 versus 5.893) than that for HNE. On the other hand, reactivity can be explained according to the hard and soft acid and base theory in which hard (Lewis) acids prefer to react with hard bases and soft acids with soft bases. A hard Lewis acid is a weak electrophile and a soft Lewis acid is a strong electrophile. Given that the electrophilicity index for 4-ONE is larger than that for HNE, 4-ONE is a softer acid than HNE. Because nucleophilic targets are soft bases, our calculations predict that the softer acid, 4-ONE (the stronger electrophile versus HNE), will probably react preferentially over HNE with the soft-acid, nucleophilic targets such as thiolates. Similar quantum mechanical calculations have been performed to evaluate the reactivity HNE with thiols versus amines [41]. These computational data will be useful for understanding the physical interaction of these aldehydes with their biological targets.

**Fig. 4.** S-HNE preferentially uncouples mitochondrial respiration. Mitochondria with pyruvate and malate as substrates were incubated with increasing concentrations of either (A) racemic HNE or (B) R-HNE or S-HNE for 5 min before the addition of ADP. There was no effect upon ADP-stimulated respiration. The uncoupling effect of S-HNE upon State 4 respiration (B) was significantly different from that of R-HNE. Data are the means ± SD. Assays were performed on 3 days (n = 3; A) or 4 days (n = 4; B) in triplicate. Data were compared in (A) using a one-way ANOVA with Bonferroni’s posttest to examine the effects of State 4 or State 3 respiration; *p < 0.05 comparing individual concentrations of HNE to control (0 μM HNE). The effects of R-HNE versus S-HNE (B) were compared by two-way ANOVA with Bonferroni’s posttest; *p < 0.05 for a significant difference in R-HNE versus S-HNE. Control (no HNE) State 3 respiration was 308 ± 34 nmol/min/mg and control State 4 respiration was 62 ± 10 nmol/min/mg.

**Fig. 5.** ALDH2 is an equally highly sensitive target for 4-ONE and HNE. Mitochondria were incubated with 4-ONE or racemic HNE in increasing concentrations for 5 min prior preparation for (A) ALDH2 activity and (B) ALDH5A activity or (C) GSH depletion. Samples for ALDH activities were processed to quench any remaining alkenal before determination of activity. Note that 4-ONE and HNE were equally inhibitory toward ALDH2, whereas 4-ONE was more potent at blocking ALDH5A and depleting GSH than HNE. 40 μM 4-ONE was the highest concentration used because this concentration completely inhibited respiration. All data are n = 3, means ± SD. One-way ANOVA with Bonferroni’s posttest was used to compare the effect of each alkenal upon each endpoint. *p < 0.05 for concentrations of the alkenals that were significantly different from controls (0 μM alkenal). A two-way ANOVA was used to determine that the effects of HNE and 4-ONE upon ALDH2 activity were not significantly different. Control ALDH2 activity was 405 ± 54 nmol/min/mg and control ALDH 5A activity was 114 ± 7 nmol/min/mg. Control GSH content was 14.4 ± 0.9 nmol/mg.
effectively scavenged 4-ONE and L-carnosine and pyridoxamine had an explanation is supported by our results showing that NAC and GSH are considered to be hard nucleophiles [44]. This an anion, will have a faster reaction rate than a primary or secondary group of NAC, upon the basis of being a soft nucleophile and reacting as such system [30,42]. L-Carnosine forms adducts with HNE and is used to scavenge 1,4-dicarbonyls such as isoketals yet was ineffective in this system [22]. This difference may be explained by the higher reactivity of 4-ONE with NAC. Our data clearly show that i-carnosine and pyridoxamine were ineffective in blocking the effects of 4-ONE. Pyridoxamine has been with NAC. Our data clearly show that L-carnosine and pyridoxamine [22]. This difference may be explained by the higher reactivity of 4-ONE with NAC. Our data clearly show that i-carnosine and pyridoxamine were ineffective in blocking the effects of 4-ONE. Pyridoxamine has been used to scavenge 1,4-dicarbonyls such as isoketals yet was ineffective in this system [30,42]. i-Carnosine forms adducts with HNE and is predicted to react similarly with 4-ONE [31,43]. However, the thiolate group of NAC, upon the basis of being a soft nucleophile and reacting as an anion, will have a faster reaction rate than a primary or secondary amine, which are considered to be hard nucleophiles [44]. This explanation is supported by our results showing that NAC and GSH effectively scavenged 4-ONE and i-carnosine and pyridoxamine had little or no ability to react with 4-ONE.

In summary, our data demonstrate that 4-ONE potently alters mitochondrial function as assessed by multiple endpoints. Although in most cases, 4-ONE was more potent than HNE, noncovalent molecular interactions of the alkenals with target molecules also influence their reactivity with these targets. As opposed to the well-characterized effects of HNE, our data and those of others indicate that more research is needed into the cellular effects of 4-ONE, particularly with respect to neurodegenerative disease.

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