Acrolein induces oxidative stress in brain mitochondria

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Received 14 April 2004; received in revised form 21 June 2004; accepted 8 September 2004

Abstract

Acrolein, a byproduct of lipid peroxidation, has been shown to inflict significant structural and functional damage to isolated guinea pig spinal cord. Reactive oxygen species (ROS) are thought to mediate such detrimental effects. The current study demonstrates that acrolein can directly stimulate mitochondrial oxidative stress. Specifically, exposure of purified brain mitochondria to acrolein resulted in a dose-dependent increase of ROS and decreases in glutathione content and aconitase activity. This effect was not accompanied by significant intramitochondrial calcium influx or mitochondrial permeability transition, but rather by impaired function of the mitochondrial electron transport system. As well, we detected a significant inhibition of mitochondrial adenine nucleotide translocase (ANT) in the presence of acrolein. This inhibition of ANT likely contributes to acrolein-induced ROS elevation since application of atractyloside, a specific ANT inhibitor, induced significant increase of ROS. We hypothesize that inhibition of ANT may mediate, in part, the acrolein-induced ROS increase in mitochondria.

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Keywords: 2-Propenal; Lipid peroxidation; Mitochondria; Reactive oxygen species; Adenine nucleotide translocase

1. Introduction

Oxidative stress has been implicated in central nervous system (CNS) injury and neurodegenerative diseases (Coyle and Puttfarcken, 1993; Gotz et al., 1994; Simonian and Coyle, 1996; Lewen et al., 2000). Free radical-induced lipid peroxidation serves to propagate and amplify oxidant-mediated damage. It is currently believed that the byproducts of lipid peroxidation reactions, such as α,β-unsaturated aldehydes, which include 4-hydroxynonenal (HNE) and 2-propenal (acrolein), mediate many detrimental effects associated with oxidative stress (Esterbauer et al., 1991; Kehrer and Biswal, 2000; Uchida, 2003). Tissue levels of acrolein and HNE are elevated in neurodegenerative diseases, such as Alzheimer’s disease (Markesbery and Lovell, 1998; Calingasan et al., 1999; Lovell et al., 2001), while HNE has also been shown to be increased after CNS injury (Springer et al., 1997; Baldwin et al., 1998). Recently, we have noted that acrolein is also increased in mammalian spinal cord following a compression injury (Luo et al., 2003). Since acrolein is the most reactive of the α,β-unsaturated aldehydes (Esterbauer et al., 1991; Kehrer and Biswal, 2000; Uchida, 2003), it may play a particularly important role in inflicting cellular damage.

Previous investigations suggest that acrolein, a byproduct of free radical-mediated lipid peroxidation itself, may exert its detrimental effect through ROS generation and lipid peroxidation (Adams and Klaidman, 1993; Uchida, 1999; Kehrer and Biswal, 2000; Nardini et al., 2002). Recent observations in this laboratory support this hypothesis. We found that exposure of acrolein leads to time- and dose-dependent ROS generation and lipid peroxidation in spinal cord tissue (Luo and Shi, 2004). We have also found that antioxidants can reduce acrolein-induced membrane damage and cell death (Luo and Shi, 2004), which further suggests a role of ROS in acrolein toxicity. However, the mechanisms by which acrolein induces ROS generation are still unknown. Specifically, to
our knowledge, the possibility that acrolein may directly interact with mitochondria leading to overproduction of ROS has not been documented.

Mitochondria are one of the most important cellular sources of ROS production and are particularly susceptible to oxidative stress (Cadenas and Davies, 2000; Lenaz et al., 2002). Specifically, the mitochondrial respiratory chain represents a major source of ROS production. It has been estimated that during normal cellular metabolism, 1–2% of the electrons which flow into the electron transport chain catalyze the incomplete reduction of O2 to superoxide radical (Boveris and Chance, 1973). It is well known that the generation of ROS will significantly increase when the function of the electron transport chain is compromised (Cadenas and Davies, 2000; Lenaz et al., 2002). Since acrolein has been shown to impair the function of the respiratory chain in mitochondria isolated from heart and brain tissue (Biagini et al., 1990; Picklo and Montine, 2001), it is possible that acrolein may encourage ROS generation through such inhibition in injured neuronal tissues as well, where acrolein has been shown to be increased. However, the direct relationship between acrolein and ROS elevation in mitochondria has not been established, nor has the specific molecular target of acrolein been identified.

In the current study, we plan to first establish the relationship between acrolein exposure and oxidative stress in brain mitochondria by directly exposing acrolein to purified mitochondria extracted from guinea pig brain tissue. We also intend to test our hypothesis that acrolein compromises mitochondrial function by inhibiting adenine nucleotide translocase (ANT), an enzyme associated with the respiratory chain. Such hypothesis is based on the observation that acrolein does not inhibit respiratory complexes I–V (Picklo and Montine, 2001), indicating that some mitochondrial components other than the respiratory complexes, such as ANT, could be potential targets of acrolein attack. Other oxidants, such as nitric oxide (NO), peroxynitrite, HNE, and 4-hydroxyhexenal (HHE), have been shown to inhibit ANT (Chen et al., 1995; Vieira et al., 2001).

2. Experimental procedures

2.1. Isolation of mitochondria from guinea pig brain

Non-synaptic mitochondria were isolated from guinea pig brain by a standard procedure (Lai and Clark, 1979). Briefly, brains from adult guinea pigs were rapidly removed according to the Purdue Animal Care and Use Committee-approved protocol and immediately put into an ice-cold isolation medium containing 0.25 M sucrose, 0.5 mM K+-EDTA, 10 mM Tris–HCl at pH 7.4. The tissue was washed with the isolation medium, and homogenized with 30 ml of the isolation medium in a Dounce-type homogenizer. The homogenate was then diluted with isolation medium to a final volume of 60 ml. All of the following steps were performed at 4°C. The homogenate was centrifuged at 2000 × g for 3 min. The supernatant was collected and centrifuged again at 2000 × g for 3 min. The supernatant of the second centrifugation was centrifuged at 12 500 × g for 8 min to obtain the crude mitochondria pellet. The pellet was suspended in 12 ml of the 3% Ficoll medium, and 6 ml of this suspension was layered onto 25 ml of the 6% Ficoll medium and centrifuged at 11 500 × g for 30 min. The supernatant from the last spin was sharply decanted so that the loose, fluffy, white top layer of the pellet was removed. The resultant brown pellet was then resuspended with 5 ml of isolation medium and centrifuged at 11 500 × g for 10 min. The pellet was resuspended to a final volume of 1 ml in the isolation medium. The mitochondria from the first three isolations were examined by electron microscope to ensure purity. The mitochondria were well coupled as demonstrated by the high respiratory control ratio (RCR) = 5.16 ± 0.28, when glutamate plus malate were used as substrate (see details in section 2.3) and could be kept on ice for multiple experiments if performed within 4 h after isolation. Mitochondrial protein was determined using the bicinchoninic acid method (Pierce), using bovine serum albumin as a standard.

2.2. Incubation of mitochondria with acrolein

All of the experiments except the mitochondrial oxygen consumption measurement were performed in a standard assay buffer containing 215 mM mannitol, 71 mM sucrose, 3 mM HEPES, pH 7.4, and 5 mM succinate (Kristal et al., 1996). For the assays of mitochondrial membrane potential and swelling, aliquots of fresh mitochondria (250 μg/ml) were first suspended in assay buffers and transferred to proper cuvettes. Acrolein was then added to the cuvettes immediately before these assays were begun. For all other assays, aliquots of brain mitochondria were diluted to 250 μg/ml with the standard assay buffer and incubated at room temperature in the presence or absence of acrolein (final concentrations: 1, 10, 100 μM) for 30 min. In the control mitochondria, the RCR remains unchanged after incubation (5.02 ± 0.18), suggesting the 30 min incubation with the standard assay buffer did not non-specifically affect the biochemical integrity of the mitochondria. For some experiments, 0.1–1000 μM of acrolein was used. After incubation, the mitochondria pellet was collected by centrifugation and used for the following biochemical assays. Acrolein solution (10 mM) was made with distilled water immediately before use for every experiment.

2.3. Measurement of mitochondrial oxygen consumption

Mitochondrial oxygen consumption was determined using a Clark-type oxygen electrode (model 203, Instech, Plymouth Meeting, PA). All assays were conducted in a respiration assay medium containing 125 mM KCl, 2 mM KH2PO4, 5 mM HEPES and 1 mM MgCl2 at pH 7.4.
Mitochondria (0.25 mg/ml) were added to the assay medium at 37 °C in a sealed chamber (total volume of 600 μl) equipped with a magnetic stirrer. Glutamate (5 mM) and malate (5 mM) were used as substrates. When used, acrolein was added before the addition of substrates. State three respiration was initiated by the addition of 1 mM ADP. State four respiration was followed 5 min after ADP was used up. The respiratory rates were calculated as nmol of O2 per min and per mg of mitochondrial protein and the RCR calculated as the ratio of the rate of state three respiration to the state four rate.

2.4. Assay for mitochondrial aconitase activity

Aconitase activity was assayed as described by Hausladen and Fridovich (1996). Aliquots of mitochondria (0.1 mg protein) were transferred to an assay medium containing 5.0 mM sodium citrate, 0.6 mM MgCl2, 1.0 U/ml isocitrate dehydrogenase and 0.2% Triton X-100. The reaction was started by addition of 0.2 mM NADP+. The rate of NADP+ reduction was monitored continuously for 5 min at 340 nm with a spectrophotometer. Results were calculated as nmol of O2 per min and per mg of mitochondrial protein and the RCR calculated as the ratio of the rate of state three respiration to the state four rate.

2.5. Assay of mitochondrial glutathione

Total mitochondrial glutathione (GSH) was estimated according to the method described by Tietze (1969). Briefly, to an aliquot of mitochondria suspension, an equal volume of 5-sulfosalicylic acid (1%, w/v) was added, mixed, and centrifuged at 10 000 × g for 10 min. The supernatant was collected and used for assay of GSH by the enzymatic recycling method.

2.6. Measurement of intramitochondrial ROS formation

Formation of intramitochondrial ROS was detected with a fluorescent spectrophotometer using 2',7'-dichlorodihydrofluorescein diacetate (DCFH2-DA) (Cathcart et al., 1983). DCFH2-DA was selected because acrolein itself did not interfere with the fluorescence of DCFH2-DA (Nardini et al., 2002). Mitochondria were incubated with 2 μM DCFH2-DA in the absence or presence of acrolein and other test reagents. Mitochondria were then washed with isolation buffer and the fluorescence was measured at 502 nm excitation and 530 nm emission. For the experiments described in Fig. 5, aliquots of non-synaptic mitochondria were first incubated with cyclosporine A (CsA, 4 μM), EDTA (1.0 mM), allopurinol (100 U/ml) and cyanamide (25 μM) for 10 min. The mitochondria were then exposed to acrolein (10 μM) and kept at 25 °C for 30 min.

2.7. Mitochondrial transmembrane potential measurement

Mitochondrial transmembrane potential (Δψm) is estimated by the quantitation of Rhodamine 123 (Rh 123) quenching (Zamzami et al., 2001). Rh 123 is concentrated in the mitochondria and is quenched at a high Δψm level. As Δψm decreases, Rh 123 is released, causing dequenching and an increase in Rh 123 fluorescence. Thus, a low Δψm level corresponds to a higher value of Rh 123 fluorescence (Zamzami et al., 2001). Mitochondria (1mg/ml) were incubated in the isolation buffer with 5 μM Rh 123 for 5 min. Quenching of Rh 123 fluorescence (excitation 490 nm and emission 535 nm) was measured continuously in a fluorometer.

2.8. Assay of mitochondrial swelling

Changes in the status of mitochondrial permeability transition were assessed spectrophotometrically as described previously (Kristal et al., 1996). One milligram of mitochondria was suspended at room temperature in 1 ml of standard assay buffer. Induction of permeability transition was monitored following exposure to calcium alone or in combination with other inducers, as described in specific figure legends.

2.9. Intramitochondrial calcium measurement

The concentration of intramitochondrial Ca2+ ([Ca2+]i) was measured with Fura-2/AM, a calcium fluorescent indicator (Gryniewicz et al., 1985). Aliquots of brain mitochondria were diluted to 250 μg/ml with the standard assay buffer and challenged with 10 μM Ca2+ in the presence or absence of acrolein. Such calcium concentration (10 μM) did not cause significant mitochondrial permeability transition during incubation, as determined by the mitochondrial-swelling assay (data not shown). After 30 min incubation, mitochondria were collected by centrifugation and resuspended at 1 mg/ml. To determine changes in [Ca2+]i, mitochondria (1 mg protein/ml) were incubated with 1.5 μM Fura 2-AM for 30 min at 30 °C. Fluorescence measurements were performed using Perkin-Elmer L50B Fluorescence Spectrometer. The wavelengths used were 340 and 380 nm for excitation and 510 nm for emission. [Ca2+]i was calculated using the following formula assuming a Ka for Fura-2 of 225 nM (Gryniewicz et al., 1985): [Ca2+]i = Ka × SI2/Sb2 × (Rresting−Rmin)/(Rmax−Rresting). Rresting, the resting Ca2+ ratio; Rmin, the ratio in EGTA (5 mM in 20 mM Tris-base, pH 8.5); Rmax, the maximal ratio with saturating Ca2+ in the presence of 2% Triton X-100, and the constant SI2/Sb2, the ratio of calcium free/calcium bound given by the fluorescence values obtained before and after CaCl2 addition at 380 nm.

2.10. Evaluation of mitochondrial electron transport

After exposure of mitochondria to the various concentrations of acrolein, the mitochondria were collected and the rate of mitochondrial electron transport was measured by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyle-
tra-zolium bromide) reduction assay (Cohen et al., 1997) using succinate (15 mM) as substrate. Results were expressed as the percentage of control (samples not exposed to acrolein, but staying at room temperature for the same periods as those exposed to acrolein).

2.11. Assay of adenine nucleotide translocase activity

After exposure of mitochondria to various experimental conditions, ANT activities were evaluated according to Passarella et al. (1988). Briefly, mitochondria (1 mg protein/ml) were suspended at 25 °C in 1 ml of a standard reaction medium (0.2 M sucrose, mM KCl, 20 mM Hepes-Tris pH 7.20, 1 mM MgCl₂, 1 mM Pi-Tris) in the presence of an ATP detection system (consisting of 2.5 mM glucose, 0.5 E.U. hexokinase, 0.5 E.U. glucose-6-phosphate dehydrogenase and 0.2 mM NADP⁺). Externally added ADP started an ADP exchange with intramitochondrial ATP through ANT. NADPH formation (ε₃₄₀ nm = 6200 M⁻¹ cm⁻¹) (Passarella et al., 1988), which is proportional to ATP efflux, was monitored continuously for 5 min by spectrophotometry at 340 nm. The rate of absorbance increase was used to calculate the amount of exchanged ADP (nmol min⁻¹ mg protein⁻¹).

2.12. Chemicals

Rhodamine 123 and DCFH₂-DA were obtained from Molecular Probes (Eugene, OR). All other chemicals including acrolein (neat), MTT, and Fura-2/AM were purchased from Sigma Chemical Co. (St. Louis, MO).

2.13. Statistical analysis

For most of the experiments, n = 5, i.e., mitochondria were prepared from five separate animals. The assays were run in triplicates and the average was used for each animal. The data was expressed as a mean ± S.D. The data were analyzed by one-way ANOVA using the software package SPSS (version 11.5, SPSS, Chicago, IL, USA). Results showing overall significance were subjected to post-hoc least-significance difference test; P < 0.05 was considered statistically significant.

3. Results

3.1. Acrolein induces oxidative stress on isolated brain mitochondria

For the first part of our study, we tested the effects of acrolein on mitochondrial derived ROS generation using DCFH₂-DA. As shown in Fig. 1A, acrolein causes a dose-dependent increase of ROS generation. Acrolein at concentrations of 1 μM or higher stimulated a significant increase of ROS generation (P < 0.01 for all comparisons of acrolein with control). For example, the ROS generation was doubled after exposure of 50 μM of acrolein.

Mitochondrial aconitase, an enzyme sensitive to superoxide (Hausladen and Fridovich, 1996), was inhibited by acrolein in a concentration-dependent manner (Fig. 1B). The activity of aconitase was significantly reduced to 92.3 ± 3.5% of control (n = 5, P < 0.05) by 1 μM of acrolein. The aconitase activity was reduced by nearly half by 100 μM acrolein.
Exposure of mitochondria to acrolein also resulted in a significant decrease of mitochondrial antioxidants. Glutathione, a major antioxidant in mitochondria, was significantly reduced by acrolein exposure at concentrations of 10 and 100 μM, with a 40% decrease at 100 μM of acrolein (Fig. 1C).

### 3.2. Effect of acrolein on intramitochondrial calcium content

With three concentrations (1, 10, 100 μM) tested, only 100 μM of acrolein caused a significant increase of intramitochondrial calcium content. As shown in Fig. 2, in the control, the intramitochondrial calcium content was 4.19 ± 0.24 μmol/mg protein, while in mitochondria exposed to 1 and 10 μM acrolein, the calcium content was 4.14 ± 0.26 and 4.37 ± 0.36 μmol/mg protein, respectively, which is not significantly different from control (P > 0.05). In the 100 μM of acrolein-exposed group, the calcium content was 5.53 ± 0.48 μmol/mg protein, which was significantly higher than control (P < 0.01).

### 3.3. Effect of acrolein on mitochondrial swelling and membrane potential

Fig. 3A shows the effects of acrolein on mitochondrial swelling. In the absence of calcium, acrolein did not cause a significant change of absorbance at 540 nm (A<sub>540 nm</sub>) (trace b, c). Interestingly, acrolein inhibited calcium-induced mitochondrial swelling in a concentration-dependent manner. As shown in Fig. 3A, while 1 μM of acrolein had only a slightly inhibitory effect on swelling (trace e), 100 μM of acrolein had a more prominent effect (trace f).

Monitoring the mitochondrial membrane potential using Rhodamine 123 discovered slightly different results. As shown in Fig. 3B, 1 μM and 100 μM of acrolein did not cause a significant change of membrane potential, as detected by the constant fluorescent intensity. A positive control, atractyloside (100 μM) caused significant Rhodamine 123 fluorescence increase, suggesting a decrease in Δψ<sub>mt</sub>.

### 3.4. Acrolein inhibits mitochondrial respiration and electron transport

As reported (Picklo and Montine, 2001), acrolein significantly inhibited the state three respiratory rate but did not affect the rate of state four respiration. For example, with the presence of 10 μM acrolein, the mitochondrial state three respiratory rate was 126.6 ± 16.8 nmol O<sub>2</sub>/(min mg) mitochondrial protein, significantly slower than that of the control (190.9 ± 18.3 nmol O<sub>2</sub>/(min mg) mitochondrial protein, P < 0.01, n = 3). However, the state four respiratory rate was not significantly different from that of control (33.6 ± 4.18 versus 38.04 ± 5.23 nmol O<sub>2</sub>/(min mg) mitochondrial protein, P > 0.05). Consequently, the presence of
acrolein led to a significant decrease of RCR (3.76 ± 0.26 versus 5.02 ± 0.18 in the control, \( P < 0.01 \)).

Acrolein caused a dose-dependent inhibition of mitochondrial electron transport, as shown by the MTT reduction test. (Fig. 4A). Mitochondrial electron transport was significantly inhibited by acrolein at concentrations of 1 \( \mu \text{M} \) or higher, and was almost completely inhibited by 1000 \( \mu \text{M} \) of acrolein. The IC\textsubscript{50} was 84 \( \mu \text{M} \).

3.5. Acrolein inhibits adenine nucleotide translocase

The dose-dependent inhibition of mitochondrial ANT activity by acrolein was shown in Fig. 4B. Mitochondrial ANT activity was significantly inhibited by acrolein at concentrations of 5 \( \mu \text{M} \) or higher. The IC\textsubscript{50} was 99 \( \mu \text{M} \).

3.6. Conditions affecting acrolein-induced free radical generation

To further demonstrate the role of ANT in acrolein-induced oxidative stress, the mitochondrial production of ROS was monitored and the effects of different conditions were tested. As expected, acrolein (10 \( \mu \text{M} \)) increased the ROS by 50% over control. Similarly, atracyloside (10 \( \mu \text{M} \)), a specific ANT inhibitor, also increased ROS generation, but with higher efficacy (100% increase) (Fig. 5). EDTA, a calcium chelator, and CsA, a mitochondrial permeability transition inhibitor, did not inhibit acrolein-induced ROS, indicating that calcium and mitochondrial permeability transition were not involved in acrolein-induced oxidative stress. Allopurinol (an inhibitor of xanthine oxidase) did not significantly affect acrolein-stimulated generation of hydrogen peroxide (\( P > 0.05 \) when compared with acrolein only).

4. Discussion

4.1. Acrolein induces oxidative stress in isolated mitochondria

In our previous study, we have shown that acrolein is capable of inflicting significant functional and structural damage in isolated spinal cord tissue (Luo and Shi, 2004). We have suggested that such cellular damage may be mediated by ROS. This is based on the fact that a significant ROS increase accompanied the acrolein-induced cellular damage and ROS scavengers could reduce acrolein-mediated damage (Luo and Shi, 2004). In this study, we have found that acrolein was able to stimulate significant ROS production in isolated brain mitochondria. Taken
together, we hypothesize that mitochondria may be one of the targets that acrolein attacks to generate ROS, which consequently inflict cellular damage. As mitochondria are a major source of cellular reactive oxygen species (ROS), acrolein-induced mitochondrial ROS generation may be an important mechanism of the cellular oxidative stress in live animals and cells after acrolein exposure in specific and in CNS trauma in general.

4.2. Mechanisms of acrolein-induced oxidative stress in mitochondria

The results of the current study demonstrate that acrolein-induced mitochondrial oxidative stress is due to the increased production of ROS and the decrease of antioxidants (glutathione). Glutathione is one of the most important antioxidants in the brain mitochondria. Specifically, glutathione plays a critical role in hydrogen peroxide, since the brain mitochondria lack catalase (Bai and Cederbaum, 2001). In accordance with this notion, acrolein has been shown to be able to rapidly bind to and deplete cellular glutathione (Kehrer and Biswal, 2000).

One relevant finding of this study is that we observed that a significantly impaired adenine nucleotide translocase activity accompanied acrolein-induced mitochondrial oxidative stress and inhibition of electron transport. It is known that ANT deficit can lead to inhibition of the mitochondrial electron transport function, which in turn has two direct effects on oxidative stress: promoting the generation of reactive oxygen species and lowering the resistance of mitochondria to pro-oxidants (Esposito et al., 1999). Therefore, we speculate that ANT inactivation is an important factor contributing to overall ROS increases following acrolein exposure.

The mechanism of ROS elevation due to ANT inactivation is not well established. However, several hypotheses have already been suggested. Wallace (1999, 2001) postulated that deficits in ANT reduce matrix ADP and limit matrix ADP-dependent proton translocation through F1-F0-ATPase. The reduction in proton transport to the mitochondrial matrix yields hyperpolarization of the mitochondrial membrane, which further limits electron transfer through the respiratory chain. Electrons would then accumulate and be available for production of \( \text{O}_2^\cdot^- \) from \( \text{O}_2 \), and render the mitochondria more vulnerable to oxidative damage. In summary, our data support the hypothesis that the inhibition of ANT can lead to oxidative stress, which is consistent with the speculation that ANT inactivation is an intermediate step in acrolein-mediated ROS increase in mitochondria. Since acrolein has been shown to be ineffective in inhibiting the activities of the respiratory complexes I–V (Picklo and Montine, 2001), ANT may be the major, if not the sole, target of acrolein action on the electron transport chain in mitochondria.

The mechanisms by which acrolein inhibits ANT are not known. It is possible that acrolein may directly modify ANT. The location of these two factors certainly favors such speculation. ANT is the most abundant single protein within the inner mitochondrial membrane (Klingenberg and Nelson, 1994), which is where acrolein is produced and may be localized. Furthermore, ANT contains sulphydryl moieties in its cysteine residues (Klingenberg and Nelson, 1994; Fiore et al., 1998), and the sulphydryl moieties are highly reactive toward acrolein (Kehrer and Biswal, 2000), making this group a likely target of acrolein modification.

The relative levels and importance of such modifications may vary depending on the physiological and/or pathophysiological state of the mitochondria. More research is needed to elucidate the molecular mechanism(s) by which acrolein inactivates ANT and the relative contribution of this process to the loss of mitochondrial function.

ANT has two conformational states, c-state and m-state, and ADP/ATP are transported via interconversion of these two states (Le Quoc and Le Quoc, 1988; Brandolin et al., 1993; Bernardi et al., 1994; Fiore et al., 1998). ANT inhibitor bongkrekic acid binds to ANT, fixing its conformation in the m-state, and inhibits permeability transition and the release of cytochrome c. Conversely, atractyloside, also an ANT inhibitor, induces permeability transition and cytochrome c release by changing the ANT conformation to its c-state. The results of the current study suggest that the mechanism by which acrolein inhibits ANT may be similar to that of bongkrekic acid.

4.3. The role of calcium and membrane permeability transition in acrolein-mediated mitochondrial damage

Mitochondrial calcium influx is one of the most common factors of inducing mitochondria-derived ROS generation. Elevated intramitochondrial calcium concentration may inhibit electron transport and oxidative phosphorylation, or activate key enzymes responsible for ROS generation leading to the increased production of ROS (Dykens, 1994; Lewen et al., 2000; Starkov et al., 2002). However, in the present study, acrolein did not cause significant calcium influx at 1 and 10 μM concentrations, while at these concentrations significant ROS generation was observed. Moreover, calcium chelator EDTA did not prevent the acrolein-induced generation of ROS. These results suggest that calcium plays a relatively less important role in acrolein-induced mitochondrial generation of ROS, especially at lower concentrations of acrolein, which is clinically more relevant than higher concentrations.

Another common factor for inducing mitochondrial oxidative stress is mitochondrial membrane permeability transition (Zamzami and Kroemer, 2001). The permeability transition occurs through the opening of a transmembrane pore in the inner mitochondrial membrane. This process collapses ion gradients across the inner mitochondrial membrane, leading to mitochondrial depolarization, reduction of oxidative phosphorylation, and generation of ROS (Zamzami and Kroemer, 2001). However, in the present...
study, no significant mitochondrial permeability transition was observed after acrolein exposure. On the contrary, acrolein had a mild inhibitory effect on calcium-induced permeability transition. This is consistent with previous reports showing that some products of lipid peroxidation such as HNE and aldehydes are potent inhibitors of mitochondrial permeability transition under certain conditions (Kristal et al., 1996; Irwin et al., 2002). This can also explain why acrolein did not cause cytochrome c release in CNS mitochondria preparations (Picklo and Montine, 2001), since the opening of the permeability transition pore is an important pathway through which cytochrome c is released (Zamzami and Kroemer, 2001). Finally, CsA, a classic mitochondrial permeability transition inhibitor, did not prevent the acrolein-induced generation of ROS. These results suggest that mitochondrial permeability transition plays a small role in acrolein-induced mitochondrial generation of ROS. As mentioned above, this observation is also consistent with the idea that acrolein stimulates ROS production by inhibiting ANT at an m-state, similar to bongkrekic acid, which inhibits the formation of the mitochondrial permeability transition pore.

4.4. The role anthine oxidase in acrolein-mediated ROS increase

There are several enzymes within the mitochondria that are involved in the degradation or detoxification of acrolein (Picklo et al., 2002). However, such a degradation process is not completely harmless. For example, it is reported that xanthine oxidase can produce superoxide while metabolizing acrolein (Adams and Klaidman, 1993). Therefore, it is possible that such mechanisms may also play a role in the overall acrolein-mediated ROS elevation. However, based on the data from this investigation, xanthine oxidase-dependent ROS increase contributes very little to the overall ROS level induced by acrolein. We have shown that the inhibition of these two enzymes by allopurinol (an inhibitor of xanthine oxidase) did not significantly prevent acrolein-induced ROS generation, suggesting that different mechanism(s), other than acrolein degradation, is the key in acrolein-induced ROS production.

4.5. Relevance of acrolein concentration in the current study

The acrolein concentrations used in most of the experiments in this study were from 1 to 100 μM. We have found that acrolein at a concentration as low as 1 μM can induce significant mitochondrial ROS generation (Fig. 1A) and functional impairment (Fig. 4A). We believe that the concentrations of acrolein used in the study are achievable in vivo. For example, the 1 μM acrolein used in this study corresponds to 4 nmol acrolein/mg mitochondrial protein (calculated based on the mitochondria being suspended and used at a concentration of 0.25 mg/ml). It has been shown that the level of acrolein in the brain tissue of patients with Alzheimer’s disease reaches 2.5 ± 0.9 nmol/mg protein in amygdale and 5.0 ± 1.6 nmol/mg protein in parahippocampal gyrus (Lovell et al., 2001). The concentration of acrolein–protein adducts in the normal human plasma is equivalent to 30–50 μM (Satoh et al., 1999; Sakata et al., 2003); and in patients with renal failure this concentration can reach as high as 180 μM (Sakata et al., 2003). It is worth noting that the longest duration of acrolein incubation used in the current study was 30 min. In reality, however, acrolein may be present for much longer times. This is due to prolonged elevation of ROS and lipid peroxidation in various disorders (Hall, 1989; Springer et al., 1997; Baldwin et al., 1998). We found that acrolein–protein adducts were significantly increased as early as 4 h following traumatic spinal cord injury and maintained at higher levels up to seven days after injury (Luo et al., 2003). Furthermore, we found that in guinea pig spinal cord tissue, the longer the exposure of acrolein the more severe structural and functional deficits (Luo and Shi, 2004). Therefore, we believe that the toxicity of acrolein in vivo injuries may be more severe than our estimates.

4.6. The possible role of acrolein in neurodegenerative disease and CNS trauma

Acrolein is the most reactive of the α,β-unsaturated aldehydes (Esterbauer et al., 1991; Kehrer and Biswal, 2000; Uchida, 2003). While numerous mediators of oxidative damage may exist in vivo, acrolein may represent an important one, particularly in neurodegenerative disease and CNS trauma. Such hypothesis is based on following observations. First, acrolein has previously been shown to be increased in neurodegenerative diseases (Calingasan et al., 1999; Lovell et al., 2001), and we have recently shown that acrolein was indeed elevated following spinal cord traumatic injury (Luo et al., 2003). Second, we have shown that acrolein can inflict structural and functional damage to isolated spinal cord tissue (Shi et al., 2002; Luo and Shi, 2004), as well as PC 12 cells in culture at a concentration as low as 1 μM (Luo and Shi, unpublished observations). Third, it is estimated from aqueous environments that acrolein has a half-life of 7–10 days (Ghilarducci and Tjeerdema, 1995). Therefore, acrolein is relatively stable, compared with short-lived ROS, which have a half-life of 10–12 s. Fourth, CNS mitochondria may be more vulnerable to acrolein compared to mitochondria from other organs such as liver and heart. For example, in the brain mitochondrial preparation from guinea pig (current paper), the IC50 of the MTT reduction is about 84 μM. This is close to the value of IC50 of state three in rat brain mitochondria (approximately 40 μM) (Picklo and Montine, 2001). In the mitochondria from rat heart, however, this value was 10–20 times higher (800 μM) (Biagini et al., 1990). In addition, the mitochondria isolated from different organs may also have different responses to other α,β-unsaturated aldehydes such
as HNE and HHE (Kristal et al., 1996; Humphries et al., 1998).

The current investigation provides a possible mechanism of acrolein toxicity by demonstrating the ability of acrolein to attack mitochondria directly. Since mitochondria are a major source of ROS and the targets of ROS attack, oxidative damage and mitochondrial dysfunction may work in a destructive, vicious cycle in which increased ROS inhibit mitochondrial function, which leads to a further increase of free radical production, which in turn leads to more mitochondrial damage. The evolving or escalation of such a cycle eventually leads to neuronal cell death. Acrolein may play a critical role in this vicious circle due to its toxicity as well as stability.

In summary, the results of the current study demonstrate that acrolein induces oxidative stress in mitochondria and that acrolein may exert its effects through a decrease in electron transport linked to impaired adenine nucleotide translocase activity. Such acrolein-mediated oxidative stress may not only underlie the neuropathology of CNS trauma, but neurodegenerative disease as well.

References


