Inhibition of NADH-Linked Mitochondrial Respiration by 4-Hydroxy-2-nonenal[†]

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ABSTRACT: During the progression of certain degenerative conditions, including myocardial ischemia-reperfusion injury, mitochondria are a source of increased free-radical generation and exhibit declines in respiratory function(s). It has therefore been suggested that oxidative damage to mitochondrial components plays a critical role in the pathology of these processes. Polyunsaturated fatty acids of membrane lipids are prime molecular targets of free-radical damage. A major product of lipid peroxidation, 4-hydroxy-2-nonenal (HNE), is highly cytotoxic and can readily react with and damage protein. In this study, the effects of HNE on intact cardiac mitochondria were investigated to gain insight into potential mechanisms by which free radicals mediate mitochondrial dysfunction. Exposure of mitochondria to micromolar concentrations of HNE caused rapid declines in NADH-linked but not succinate-linked state 3 and uncoupled respiration. The activity of complex I was unaffected by HNE under the conditions of our experiments. Loss of respiratory activity reflected the inability of HNE-treated mitochondria to meet NADH demand during maximum rates of O_2 consumption. HNE exerted its effects on intact mitochondria by inactivating α-ketoglutarate dehydrogenase. These results therefore identify a potentially important mechanism by which free radicals bring about declines in mitochondrial respiration.

The mitochondrial respiratory chain represents a major subcellular source of oxygen radicals. 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 O_2 to superoxide radical (*1*, *2*). Under these conditions, various antioxidant systems scavenge free radicals and preserve mitochondrial integrity (for review, see ref *3*). During certain degenerative processes however, such as reperfusion of ischemic myocardial tissue (*4*−*6*) and aging (*7*−*9*), evidence suggests that mitochondrial generation of superoxide anion and hydroxyl radical increase. These processes are often associated with declines in mitochondrial respiratory activity (*8*−*12*). It is well established that free radicals have numerous deleterious effects on cells, organelles, and cellular components *in vitro*. Highly reactive and short-lived, oxygen radicals would be expected to cause damage at or near the site of their formation. Oxidative damage is therefore likely to play a role in the loss of mitochondrial function which occurs during certain pathophysiological events.

In vitro studies utilizing submitochondrial particles and intact mitochondria exposed to various forms of oxidative stress reveal a large number of enzymatic functions and proteins susceptible to free-radical damage (*13*−*17*). The mechanisms and relative contributions of numerous free-radical events have not, however, been established. It has therefore been difficult to demonstrate a direct relationship between free-radical damage and alterations in mitochondrial structure and function *in vivo*. Clearly, isolated mitochondria and submitochondrial particles provide simple *in vitro* models for highly-targeted mechanistic studies of oxidative damage. Investigations of specific free-radical events likely to occur will aid subsequent studies utilizing more complex biological systems by defining potential targets and mechanisms of damage.

Polyunsaturated fatty acids of membrane lipids are highly susceptible to free-radical damage (*3*, *18*). Not surprisingly, increased rates of free-radical production are often paralleled by elevated rates of lipid peroxidation (*3*, *14*, *18*). Peroxidation of membrane lipids results in the fragmentation of polyunsaturated fatty acids, giving rise to various aldehydes, alkenals, and hydroxyalkenals such as malonaldehyde (MDA) and 4-hydroxy-2-nonenal (HNE)[1] (*3*, *18*). Many of these products are cytotoxic when introduced into cells in culture and into whole animals, effects believed to be mediated by their reactivity toward cellular components (*18*). Mitochondria, a source of free radicals, are likely targets of lipid peroxidation and the reactive byproducts of this process. It is therefore critical to identify mechanisms by which lipid peroxidation alters mitochondrial function.

4-Hydroxy-2-nonenal (HNE), an α,β-unsaturated aldehyde, is a major product of lipid peroxidation and one of the most reactive under physiological conditions (*18*). HNE readily reacts with cysteine, histidine, and lysine residues on protein which can result in the inactivation of enzymes and in the disruption of protein structure (*18*−*25*). In addition, the

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[1] Abbreviations: HNE, 4-hydroxy-2-nonenal; TFA, trifluoroacetic acid; MOPS, 4-morpholinepropanesulfonic acid; IC_{50}, inhibitory constant; K_{obs}, rate constant for inactivation.

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levels of lipid peroxides, peroxidation products, and HNE increase during myocardial reperfusion and during the progression of other degenerative processes (*3*, *5*, *18*, *26*−*28*). It is therefore proposed that HNE represents an important mediator of free-radical damage and is likely to play a critical role in free-radical-induced mitochondrial dysfunction. Intact mitochondria, isolated from rat heart, were therefore incubated with HNE to determine (1) the effects of HNE on mitochondrial respiration, (2) conditions under which HNE exerts these effects, and (3) specific sites of HNE-mediated dysfunction. Cardiac mitochondria were used in this study because of our interest in myocardial reperfusion injury. We provide evidence that HNE can induce declines in respiration by inhibiting α-ketoglutarate dehydrogenase, an enzyme crucial for the production of NADH. Thus, this study identifies a potential mechanism by which lipid peroxidation gives rise to mitochondrial dysfunction and provides the basis for investigating the role of this process in certain degenerative events.

MATERIALS AND METHODS

4-Hydroxy-2-nonenal Synthesis and Preparation. 4-Hydroxy-2-nonenal dimethylacetal was synthesized as previously described (*29*) and was obtained from Dr. Lin Tsai (National Institutes of Health). Prior to use, HNE was generated by acid treatment of 4-hydroxy-2-nonenal dimethylacetal (1.0 μL into 400 μL of a 1:1 water/acetonitrile mixture containing 0.05% TFA). After 5.0 min the solution was diluted with 400 μL of H_2O, resulting in a final concentration of approximately 7.0 mM HNE. The concentration of HNE was determined by measurement of UV absorbance at 224 nm with a molar absorptivity of 13 750 M^{-1} for HNE.

Isolation of Subsarcolemmal Mitochondria from Rat Heart. Sprague Dawley rats (250−300 g) obtained from Zivic Miller laboratories were euthanized with sodium pentobarbitol, and hearts were immediately removed and immersed and rinsed in cold buffer containing 180 mM KCl, 5.0 mM MOPS, and 2.0 mM EGTA at pH 7.25 (buffer A). Hearts (0.8−1.0 g) were then minced and homogenized in 20 mL of buffer A per gram of heart with a Polytron homogenizer (low setting, 2 s). The homogenate was then centrifuged at 500*g* for 7.5 min at 4 °C. The supernatant was filtered through cheesecloth and centrifuged at 5000*g* for 10 min at 4 °C. The resulting mitochondrial pellet was washed two times and resuspended into 200 μL of buffer A to a final protein concentration of approximately 25 mg/mL. Protein determinations were made using the BCA method (Pierce), with BSA as a standard. Mitochondria were stored at 4 °C and exhibited no change in state 3 or state 4 respiratory rates for up to 5.0 h.

Incubation of Mitochondria with HNE and Assay for O_2 Utilization. Mitochondria were diluted to a protein concentration of 0.5 mg/mL in assay buffer (120 mM KCl, 5.0 mM KH_2PO_4, 5.0 mM MOPS, and 1.0 mM EGTA at pH 7.25). State 2 respiration was initiated by the addition of glutamate (15 mM) or α-ketoglutarate (15 mM) and allowed to proceed for 5.0 min at room temperature. At appropriate times during this 5.0 min period, HNE (0−100 μM) was added. After 5.0 min of state 2 respiration, state 3 respiration was initiated by addition of ADP (0.33 mM). Upon depletion of ADP, state 4 respiration was monitored. For uncoupled respiration, 60 μM 2,4-dinitrophenol was added instead of ADP. Oxygen consumption was measured using a Clark-style oxygen electrode (Instech). The substrate concentrations and assay buffer used were optimized to yield maximal rates of respiration. Respiratory rates did not vary by greater than 10% between mitochondrial preparations. Acetonitrile and TFA present in the HNE solution had no effect on mitochondrial respiration at concentrations present during incubation of mitochondria with HNE.

Assays for Complex I Activity. Complex I was assayed according to the method of Estornell (*30*). Briefly, following treatment of mitochondria with 100 μM HNE for 0 and 5.0 min, mitochondria (0.5 mg/mL) were diluted into 50 mM KCl, 10 mM Tris-HCl, 1.0 mM EDTA, and 2.0 mM KCN to a final concentration of 25 μg of protein/mL, and then sonicated (30 s, setting of 3.0, 50% pulse rate, VWR Scientific). Upon the addition of 5.0 μM antimycin A, 50 μM ubiquinone-1 (donated by Eisai, Japan), and 75 μM NADH, consumption of NADH was monitored at 340 nm. Assays were performed at room temperature, and rates represent rotenone-sensitive activity.

The NADH dehydrogenase domain of complex I was assayed by monitoring the reduction of potassium ferricyanide (*31*). Mitochondria (0.5 mg/mL) were diluted to 50 μg/mL in assay buffer, then sonicated as above. The reduction of ferricyanide (500 μM) was monitored at 420 nm after the addition of 100 μM NADH.

The NADH oxidase assay was employed to determine the ability of mitochondria to utilize exogenously added NADH (*31*). Mitochondria (0.5 mg/mL) were diluted to a protein concentration of 0.1 mg/mL with a hypotonic solution (20 mM K_2HPO_4, 0.1 mM EDTA, pH 7.25) to induce swelling and provide access of NADH and cytochrome *c* to the electron transport chain. Oxygen consumption was then monitored, via a Clark-style electrode, after the addition of 7.0 μM cytochrome *c* and 0.2 mM NADH.

Assay for Mitochondrial NADH Levels. A dual excitation wavelength fluorimeter (Johnson Research Center, excitation at 365 nm with a reference excitation at 430 nm and emission measured at 467 nm) was used to measure production and consumption of NADH in intact mitochondria (0.5 mg/mL) (*32*) at 25 °C. Known quantities of NADH were added to 0.5 mg/mL mitochondria for calibration.

Dehydrogenase Assays. Following incubation with HNE, mitochondria were diluted 1:10 to a concentration of 50 μg of protein/mL in assay buffer (120 mM KCl, 5.0 mM KH_2PO_4, 5.0 mM MOPS, and 1.0 mM EGTA at pH 7.25) containing 5.0 mM *N*-acetyl-L-cysteine. *N*-Acetyl-L-cysteine was included in the buffer to react with remaining HNE and prevent further inactivation. Mitochondria were then sonicated as described above. Glutamate dehydrogenase activity was calculated as the rate of disappearance of NADH monitored at 340 nm upon addition of 20 mM α-ketoglutarate, 50 mM NH_4Cl, 3.5 μM rotenone, and 50 μM NADH (*33*). The inclusion of rotenone was found to block NADH consumption not related to glutamate dehydrogenase activity. α-Ketoglutarate dehydrogenase activity was measured as the rate of NADH formation upon addition of 2.5 mM NAD^+, 200 μM thiamine pyrophosphate, 130 μM CoASH, and 2.0 mM α-ketoglutarate (*34*). NAD^+, present in the assay mixture, was found to prevent consumption of NADH.

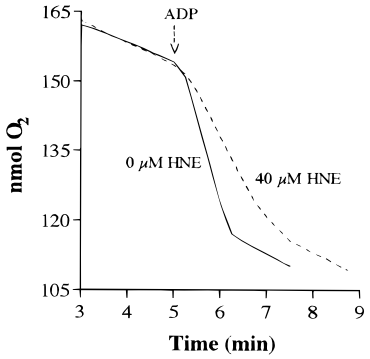

FIGURE 1: Effect of 4-hydroxy-2-nonenal on mitochondrial respiration. Mitochondria at a concentration of 0.5 mg/mL were incubated for 5.0 min with 15 mM glutamate at 25 °C in the presence or absence of 40 μM HNE. Oxygen consumption was monitored beginning at 3.0 min. State 3 respiration was initiated with the addition of 0.33 mM ADP at 5.0 min. State 4 respiration was evaluated as the rate of oxygen consumption following depletion of ADP.

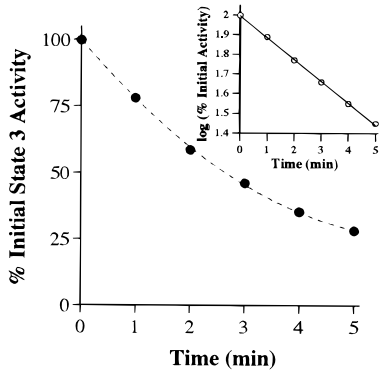

FIGURE 2: Fractional loss of mitochondrial respiration as a function of time in the presence of 4-hydroxy-2-nonenal. Mitochondria at a concentration of 0.5 mg/mL were incubated with 15 mM glutamate for 5.0 min at 25 °C. The abscissa indicates the time of exposure of mitochondria to 80 μM HNE. State 3 respiration was measured following addition of 0.33 mM ADP. The inset presents the data expressed as the log of fractional loss of mitochondrial state 3 respiration with respect to time.

Assays were performed at room temperature. The molar absorptivity used to determine NADH concentration was 6200 M^{-1} at 340 nm.

RESULTS

Effects of 4-Hydroxy-2-nonenal on Mitochondrial Respiration. Incubation of cardiac mitochondria with 4-hydroxy-2-nonenal (HNE) results in a rapid decline in the rate of ADP-dependent (state 3) respiration. Control mitochondria exhibited state 3 and state 4 respiratory rates of 72.9 and 10.5 nmol O_2 min^{-1} mg^{-1}, respectively, with glutamate as a substrate (Figure 1). These values correspond to a respiratory control ratio (state 3/state 4) of 6.9 and an ADP/O of 2.8. Approximately 50% of the original state 3 respiratory activity was lost when mitochondria (0.5 mg/mL) were treated with 40 μM HNE for 5.0 min at 25 °C (Figure 1). State 4 respiration and the ADP/O ratio were not significantly affected by HNE (Figure 1). As shown in Figure 2, the rate of state 3 respiration declined in a time-dependent manner in the presence of HNE. A semilogarithmic plot of remaining activity versus incubation time (Figure 2 inset) is linear over the experimental interval examined, demonstrating that

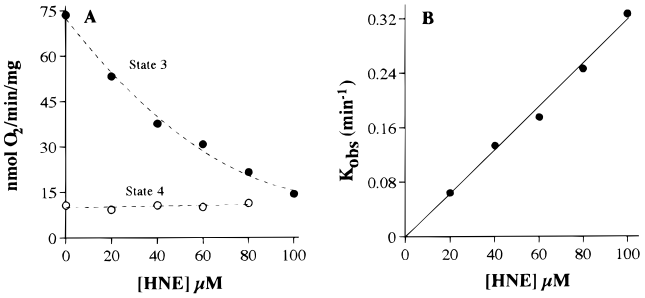

FIGURE 3: Rate of mitochondrial respiration as a function of 4-hydroxy-2-nonenal concentration. (A) Mitochondria at a concentration of 0.5 mg/mL were incubated for 5.0 min at 25 °C with 15 mM glutamate and at concentrations of HNE indicated on the abscissas. State 3 respiration was initiated with addition of 0.33 mM ADP at 5.0 min. State 4 respiration was evaluated as the rate of oxygen consumption following depletion of ADP. (B) Mitochondria were incubated with HNE for times consistent with initial rate conditions. The observed rate constant for inhibition (k_{obs}) is equal to $0.693/t_{1/2}$, where $t_{1/2}$ is defined as the time in minutes required for HNE to induce a 50% decline in the rate of state 3 respiration.

inactivation occurred by a pseudo first-order process. This was true at all HNE concentrations (0−100 μM) tested.

Exposure of mitochondria to increasing concentrations of HNE (0−100 μM, 5.0 min) led to increased inactivation of state 3 respiration in a saturable fashion (Figure 3A). The concentration of HNE required for half-maximal inhibition (IC_{50}) was approximately 40 μM under the conditions of these experiments. The linear relationship between HNE concentration and the apparent rate constant for inactivation (k_{obs}) (Figure 3B) indicates that the rate of inactivation is first-order with respect to HNE concentration. The fact that this line intersects at the ordinate indicates that inactivation is essentially irreversible. Consistent with these results, dilution of mitochondria following treatment with a given concentration of HNE did not alter the degree of inactivation. No significant change in state 4 respiration (Figure 3A) or ADP/O ratio was observed in mitochondria treated with 0−100 μM HNE for 5.0 min. However, prolonged exposure of mitochondria to 100 μM HNE resulted in loss of state 4 respiration (results not shown).

Effect of HNE on Uncoupled Mitochondrial Respiration. To examine whether inhibition of ADP-dependent (state 3) respiration was due to inactivation of adenine nucleotide translocase (ANT) and/or ATPase, the effects of HNE on uncoupled respiration were investigated. Use of an uncoupler results in maximum rates of electron transport and O_2 consumption independent of ADP transport or ATP synthesis. HNE-induced declines in the rate of uncoupled respiration would therefore suggest damage to electron transport components and/or a drop in the supply of reducing equivalents (NADH). We observed that HNE inhibited uncoupled respiration in a concentration-dependent manner (results not shown). The IC_{50} was identical to that observed for respiration coupled to ATP synthesis (Figure 3), indicating that observed inhibition by HNE is not due to inactivation of ANT or ATPase.

Effects of HNE on NADH- and Succinate-Linked Respiration. HNE inhibited both ADP-dependent and uncoupled respiration when α-ketoglutarate was used as substrate. The time- and concentration-dependent degree of inactivation were nearly identical to those observed with glutamate as

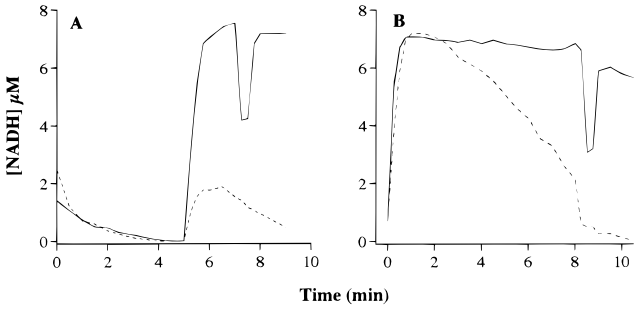

FIGURE 4: Effect of 4-hydroxy-2-nonenal on NADH production and on steady-state NADH levels. (A) Mitochondria at a concentration of 0.5 mg/mL were incubated for 5.0 min at 25 °C in the presence (- - -) or absence (—) of 100 μM HNE. NADH production was then initiated by the addition of 15 mM α-ketoglutarate at 5.0 min. The ability of the mitochondria to consume NADH was measured upon addition of 0.2 mM ADP at 7.0 min. (B) Mitochondria at a concentration of 0.5 mg/mL were incubated at 25 °C in the presence of 15 mM α-ketoglutarate followed by the addition of 0 μM (—) and 100 μM (- - -) HNE at 2.0 min. The ability of the mitochondria to consume ADP was then measured upon addition of 0.2 mM ADP at 8.0 min.

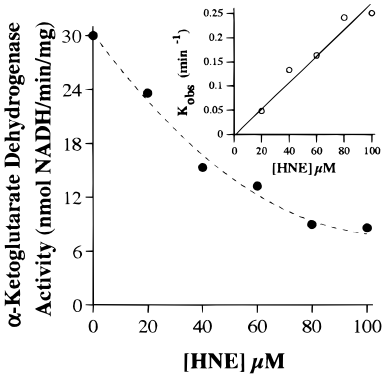

FIGURE 5: Effect of 4-hydroxy-2-nonenal on α-ketoglutarate dehydrogenase activity. Mitochondria (0.5 mg/mL) were incubated for 5.0 min at 25 °C with HNE at concentrations indicated on the abscissas. Following treatment with HNE, α-ketoglutarate dehydrogenase activity was assayed as described in Materials and Methods. (Inset) The observed rate constant for inhibition (k_{obs}) is equal to $0.693/t_{1/2}$, where $t_{1/2}$ is defined as the time in minutes required for HNE to induce a 50% decline in the rate of NAD^+ reduction by α-ketoglutarate dehydrogenase.

substrate (data not shown). In contrast, succinate-linked respiration was unaffected by HNE (data not shown) at all HNE concentrations tested (0−100 μM). Glutamate and α-ketoglutarate are NADH-linked substrates which result in electron flow through complexes I, III, and IV. In contrast, electrons flow through complexes II, III, and IV when succinate is used as substrate. HNE therefore appears to inhibit NADH-linked state 3 respiration by inactivation of complex I and/or by decreasing the availability of NADH to complex I.

Effect of HNE on Complex I Activity. To test whether HNE exerts its inhibitory effects on complex I, ubiquinone-1 was employed as an electron acceptor upon addition of NADH to sonicated mitochondria. Antimycin A (5.0 μM) and CN^- (2.0 mM), inhibitors of complex III and IV, respectively, were included in the assay mixture ensuring assay of complex I. Under these conditions, NADH was consumed at a rate of 86.2 ± 11.2 nmol NADH min^{-1} mg^{-1}. Mitochondria treated with 100 μM HNE for 5.0 min exhibited no significant change in complex I activity (90.2 ± 6.4 nmol NADH min^{-1} mg^{-1}). When complex I activity was measured using either the NADH oxidase or NADH dehydrogenase assay, no HNE-mediated decline in activity was observed. Thus, under the conditions of the experiments, it appears that complex I and its components are unaffected by HNE.

Effects of HNE on Mitochondrial NADH Synthesis and Utilization. The effects of HNE on NADH production and consumption can be conveniently monitored in intact mitochondria using fluorescence spectroscopy (*32*). As shown in Figure 4A, preincubation of mitochondria (0.5 mg/mL) with 100 μM HNE for 5.0 min resulted in a significant reduction in the level of NADH upon addition of α-ketoglutarate. When NADH was allowed to reach steady-state levels with α-ketoglutarate as substrate, addition of HNE resulted in a time-dependent decrease in NADH concentration (Figure 4B). Declines in NADH levels during state 2 respiration (Figure 4A,B) would result from decreased rates of NADH production relative to NADH consumption. With glutamate as substrate, HNE (100 μM) exerted less of an effect on the level of NADH (70% of the original level after 5.0 min). Upon addition of ADP, however, NADH levels fell rapidly and failed to recover, reflecting active complex I but an inability to deal with increased demand (data not shown). Thus it appears that HNE inhibits state 3 respiration by decreasing the availability of NADH to complex I.

There are a number of mechanisms by which HNE may affect the level of NADH. It is unlikely that HNE causes leakage of NAD^+/NADH from the mitochondria. Mitochondria treated with HNE were not permeable to NADH and did not exhibit increases in state 4 respiration typical of permeabilized mitochondria. Alternatively, NADH may be consumed by the enzymatic reduction of HNE, thereby limiting the levels of NADH available for respiration. However, inhibition of aldehyde dehydrogenase (3.0 mM cyanamide), an enzyme believed to be critical for the reduction of HNE (*35*), had no effect on HNE-mediated declines in respiration. It is therefore likely that HNE exerts its effects on respiration by inhibiting the synthesis of NADH.

Effect of HNE on the Activities of Glutamate Dehydrogenase and α-Ketoglutarate Dehydrogenase. Studies were performed to determine whether HNE limits NADH synthesis by direct inactivation of glutamate dehydrogenase and/or α-ketoglutarate dehydrogenase. Following treatment of mitochondria with (0−100 μM) HNE for 5.0 min at 25 °C, *N*-acetylcysteine was added to scavenge unreacted HNE, mitochondria were disrupted by sonication, and the respective dehydrogenase activities were assayed. While HNE had relatively little effect on glutamate dehydrogenase activity (21.8 ± 0.2 and 20.7 ± 0.3 nmol NADH min^{-1} mg^{-1} at 0 and 100 μM HNE respectively), α-ketoglutarate dehydrogenase was highly susceptible to HNE inactivation. As shown in Figure 5, HNE inhibited α-ketoglutarate dehydrogenase in a concentration-dependent manner. The IC_{50} (~40 μM) for HNE inactivation was nearly identical to that observed for state 3 respiration when glutamate (Figure 3) or α-ketoglutarate was used as a substrate (data not shown). Oxidation of glutamate by glutamate dehydrogenase would result in the accumulation of α-ketoglutarate due to inactivation of α-ketoglutarate dehydrogenase. The conversion of glutamate to α-ketoglutarate by glutamate dehydrogenase is thermodynamically unfavorable ($K_{eq} = 1.8 \times 10^{-13}$), and a

buildup of α-ketoglutarate would be expected to reduce the rate of glutamate utilization. These results are therefore consistent with observed declines in state 3 respiration with glutamate or α-ketoglutarate as substrate. Thus, inactivation of α-ketoglutarate dehydrogenase appears to be the primary mechanism by which HNE inhibits mitochondrial respiration under the conditions of our experiments. Because the rate of NADH consumption is low during state 4 respiration (Figure 1), state 3 respiration would be expected to be more sensitive to HNE inactivation (Figure 3).

DISCUSSION

Mitochondria are a major site of free-radical production during normal and pathophysiological conditions (*1*, *2*, *4*−*9*) and are therefore likely targets of oxidative damage. To understand the role free radicals play in certain degenerative events, it is critical to define mechanisms by which free radicals impair mitochondrial function. Previous research has established that polyunsaturated fatty acids of membrane lipids are highly susceptible to oxidative damage (*3*, *18*). 4-Hydroxy-2-nonenal (HNE) is a major product of lipid peroxidation and is perhaps the most reactive and cytotoxic (*18*). While it has been shown that HNE can inhibit respiration (*36*, *37*) in mitochondria isolated from rat liver and kidney cortex, the kinetics and mode of inactivation have not been established. We provide evidence that, in intact cardiac mitochondria, HNE specifically inhibits NADH-linked respiration by reducing the steady-state level of NADH. This was due to inactivation of α-ketoglutarate dehydrogenase. α-Ketoglutarate dehydrogenase was inactivated at micromolar concentrations of HNE and over relatively short periods of time. Therefore, HNE-induced declines in α-ketoglutarate dehydrogenase activity may represent an important mechanism by which free radicals and lipid peroxidation mediate mitochondrial dysfunction.

HNE has been shown to reach micromolar concentrations in the perfusate during reperfusion of ischemic rat heart (*28*) and in certain tissues under conditions of oxidative stress (for review, see ref *18*). Due to the amphipathic nature of HNE, much higher concentrations are likely to occur in lipophilic environments, particularly in regions were it is formed, such as the inner mitochondrial membrane. Thus, the concentrations of HNE and time required for inhibition of respiration suggest that HNE-mediated mitochondrial dysfunction would take place during degenerative events associated with increased free-radical generation by mitochondria. In addition, HNE appears to be a key mediator of lipid peroxidation damage. Malonaldehyde (MDA), an abundant lipid peroxidation product (*3*, *18*), had no effect on mitochondrial respiration at concentrations up to 1.0 mM. Furthermore, no decline in respiration was observed when mitochondria were exposed to *t*-2-nonenal at concentrations where HNE induced maximal levels of inactivation. The hydroxyl group at the C4 position of HNE enhances the electrophilic nature of HNE relative to *t*-2-nonenal. The decrease in state 3 respiration caused by HNE is likely the result of specific chemistry between nucleophilic residue(s) on α-ketoglutarate dehydrogenase and the highly electrophilic double bond (C3) of HNE. Thus, while numerous forms of oxidative damage may occur *in vivo*, HNE represents an important mediator of this damage.

While aberrations in adenine nucleotide transporter and components of the electron transport chain have been observed during reperfusion (*10*−*12*), a decline in α-ketoglutarate dehydrogenase activity has not been investigated as a contributing factor to decreased NADH-linked respiration. Metabolic flux through α-ketoglutarate dehydrogenase appears to be a critical determinant of the rate of respiration (Figure 4) (*38*, *39*). HNE-mediated declines in α-ketoglutarate dehydrogenase activity may therefore have profound effects on the rate of respiration. This enzyme may be particularly susceptible to HNE modification because of its apparent association with the inner mitochondrial membrane (*40*) and because HNE is produced and may be localized in the membrane (*18*). In addition, α-ketoglutarate dehydrogenase contains covalently-linked lipoic acid, essential cofactors for activity. Sulfhydryl moieties are highly reactive toward HNE (*18*), making lipoic acid a likely target of HNE modification. The susceptibility of this enzyme to HNE inactivation is consistent with previous research demonstrating that α-ketoglutarate dehydrogenase is inactivated under conditions of increased lipid peroxidation *in vitro* (*41*). Future research will therefore address the molecular mechanism(s) by which HNE inactivates α-ketoglutarate dehydrogenase and the relative contribution of this process to the loss of mitochondrial function(s) associated with cardiac reperfusion injury and other degenerative events. It is important to note that HNE may affect other components of mitochondrial respiration which are not rate limiting under the conditions of our experiments. The relative levels and importance of such modifications may vary depending on the physiological and/or pathophysiological state of the mitochondria. We will therefore continue to identify mitochondrial proteins susceptible to HNE damage and conditions under which these modifications occur.

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