Selective Inactivation of α-Ketoglutarate Dehydrogenase and Pyruvate Dehydrogenase: Reaction of Lipoic Acid with 4-Hydroxy-2-nonenal†

Kenneth M. Humphries and Luke I. Szweda*

Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4970

Received June 25, 1998; Revised Manuscript Received September 15, 1998

ABSTRACT: Previous research has established that 4-hydroxy-2-nonenal (HNE), a highly toxic product of lipid peroxidation, is a potent inhibitor of mitochondrial respiration. HNE exerts its effects on respiration by inhibiting α-ketoglutarate dehydrogenase (KGDH). Because of the central role of KGDH in metabolism and emerging evidence that free radicals contribute to mitochondrial dysfunction associated with numerous diseases, it is of great interest to further characterize the mechanism of inhibition. In the present study, treatment of rat heart mitochondria with HNE resulted in the selective inhibition of KGDH and pyruvate dehydrogenase (PDH), while other NADH-linked dehydrogenases and electron chain complexes were unaffected. KGDH and PDH are structurally and catalytically similar multienzyme complexes, suggesting a common mode of inhibition. To determine the mechanism of inhibition, the effects of HNE on purified KGDH and PDH were examined. These studies revealed that inactivation by HNE was greatly enhanced in the presence of substrates that reduce the sulfur atoms of lipoic acid covalently bound to the E2 subunits of KGDH and PDH. In addition, loss of enzyme activity induced by HNE correlated closely with a decrease in the availability of lipoic acid sulfhydryl groups. Use of anti-lipoic acid antibodies indicated that HNE modified lipoic acid in both purified enzyme preparations and mitochondria and that this modification was dependent upon the presence of substrates. These results therefore identify a potential mechanism whereby free radical production and subsequent lipid peroxidation lead to specific modification of KGDH and PDH and inhibition of NADH-linked mitochondrial respiration.

A large number of pathophysiological conditions, including cardiac reperfusion injury, are accompanied by an increase in free radical production and the subsequent peroxidation of membrane lipids (*1*−*16*). Mitochondria are a source of free radicals and are therefore a potential site of oxidative damage (*1*−*4*, *6*, *7*, *17*, *18*). Aldehydic products formed as a result of lipid peroxidation are believed to mediate, at least in part, free radical damage by virtue of their reactivity toward protein (*19*). Previous research in the laboratory has established that incubation of intact rat heart mitochondria with 4-hydroxy-2-nonenal (HNE[1]), a major product of lipid peroxidation, results in the inhibition of NADH-linked respiration (*20*). Inhibition occurred rapidly at concentrations of HNE expected under certain conditions of oxidative stress. HNE exerted its effects on mitochondrial respiration by decreasing steady-state concentrations of NADH. This was due to inactivation of α-ketoglutarate dehydrogenase (KGDH) by HNE (*20*).

KGDH, a Kreb's cycle enzyme which catalyzes the conversion of α-ketoglutarate to succinyl-CoA (*21*), is essential for mitochondrial function. Production of NADH by KGDH is necessary for mitochondrial respiration and oxidative phosphorylation (*22*, *23*). KGDH also plays a critical role in regulating the concentration of α-ketoglutarate, a metabolite produced by the oxidative metabolism of fats and carbohydrates, the malate−aspartate shuttle, and the oxidative deamination of amino acids (*24*). Because of the central role of KGDH in metabolism, inhibition of this enzyme by HNE would have profound effects on cellular homeostasis. It is therefore important to define the mechanism of HNE inactivation to accurately evaluate the role of this process during certain degenerative events associated with increased rates of mitochondrial free radical production and lipid peroxidation.

In the present study, we examined the effects of HNE on a number of mitochondrial proteins critical to respiration to determine the selectivity of HNE inactivation. Our results indicate that in intact cardiac mitochondria, HNE does not inhibit mitochondrial enzymatic functions unilaterally, but rather shows specificity toward the enzymes KGDH and pyruvate dehydrogenase (PDH). Mechanistic studies were performed, utilizing purified KGDH and PDH, to determine the molecular mechanism by which this highly susceptible nature is conferred. The results of this study indicate that the covalently bound cofactor lipoic acid, common to KGDH and PDH (*25*), is the site of HNE modification and inactivation. Thus, we have identified a likely target of free

† This work was supported by a Scientist Development Grant from the American Heart Association (9630025N) and with funds contributed in part by the AHA, Northeast Ohio Affiliate.

* Corresponding author: Department of Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106-4970. Telephone: (216)368-0035. Fax: (216)368-1693. E-mail: lxs54@po.cwru.edu.

[1] HNE, 4-hydroxy-2-nonenal; TFA, trifluoroacetic acid; MOPS, 4-morpholinepropanesulfonic acid; KGDH, α-ketoglutarate dehydrogenase; PDH, pyruvate dyhydrogenase; LA, lipoic acid; KLH, keyhole limpet hemocyanin; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride.

10.1021/bi981512h CCC: $15.00
Published on Web 10/22/1998

radical damage and a molecular mechanism whereby damage is mediated.

MATERIALS AND METHODS

4-Hydroxy-2-nonenal Synthesis and Preparation. 4-Hydroxy-2-nonenal dimethyl acetal was synthesized as previously described (*26*) by Dr. Lin Tsai (National Institutes of Health). Prior to use, HNE was generated by acid treatment of 4-hydroxy-2-nonenal dimethyl acetal (1.0 μL into 400 μL of 1:1 water/acetonitrile mixture containing 0.05% TFA). After 5.0 min, the solution was diluted with 400 μL 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 13750 M^{-1} cm^{-1}.

Isolation of Subsarcolemmal Mitochondria from Rat Heart. Sprague-Dawley rats (250−300 g) obtained from Zivic Miller laboratories were anesthetized with sodium pentobarbitol. An incision was made in the chest cavity, and animals euthanized by severing the diaphragm. 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 with a Polytron homogenizer (low setting, 2.0 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 concentration of approximately 25 mg/mL. Protein determinations were made using the bicinchoninic acid (BCA) method (Pierce), with BSA as a standard.

Incubation of Mitochondria with HNE. Mitochondria were diluted to a protein concentration of 0.5 mg/mL in a buffer containing 120 mM KCl, 5.0 mM KH_2PO_4, and 5.0 mM MOPS at pH 7.25 (buffer B). Respiration was initiated by the addition of glutamate (15 mM) or pyruvate (15 mM) and allowed to proceed in the presence or absence of 50 μM HNE for 5.0 min at 25 °C. After 5.0 min, remaining HNE was scavenged by the addition of 10 mM cysteine, and mitochondria were then diluted into appropriate assay buffers and disrupted as described below. Addition of cysteine at time = 0 min resulted in the rapid depletion of HNE and prevention of enzyme inactivation, indicating that cysteine acts as a potent scavenger of HNE.

Electron Transport Chain Assays. For analysis of complexes I−III, mitochondria were diluted into a buffer containing 35 mM KH_2PO_4, 5.0 mM $MgCl_2$, 2.0 mM NaCN, and 0.5 mM EDTA at pH 7.25 and sonicated (30 s, setting of 3.0, 100% pulse rate, VWR Scientific). Complex I was assayed by monitoring the consumption of NADH at 340 nm (ϵ = 6200 M^{-1} cm^{-1}) upon the addition of 100 μM NADH, 5.0 μM antimycin A, and 60 μM ubiquinone 1 (donated by Eisai, Japan) to 50 μg/mL of mitochondrial protein (*27*). Complex II was assayed by monitoring the reduction of DCPIP at 600 nm (ϵ = 21 000 M^{-1} cm^{-1}) upon addition of 90 μM DCPIP, 5.0 μM antimycin A, 60 μM ubiquinone 1, and 25 mM succinate to 25 μg/mL mitochondrial protein (*28*). Complex III was assayed by monitoring the reduction of cytochrome *c* at 550 nm (ϵ = 18 500 M^{-1} cm^{-1}) upon the addition of 50 μM cytochrome *c* and 60 μM reduced decylubiquinone to 10 μg/mL mitochondrial protein. Antimycin A sensitive complex III activity is presented (*28*). For analysis of complex IV, mitochondria were diluted into a buffer containing 35 mM KH_2PO_4, pH 7.2, and then sonicated for 10 s in a sonicator bath (Fisher Scientific). Complex IV was assayed polargraphically by the rate of oxygen consumption upon the addition of 50 mM ascorbate, 5.0 mM TMPD, and 1.0 mM cytochrome *c* to 30 μg/mL of mitochondrial protein (*28*). All assays were performed at room temperature.

Dehydrogenase Assays. Glutamate dehydrogenase was assayed by measuring the oxidation of NADH at 340 nm upon the addition of 50 μM NADH, 50 mM NH_4Cl, and 15 mM α-ketoglutarate to 50 μg/mL mitochondrial protein (*29*). Malate dehydrogenase was assayed by measuring the reduction of NAD^+ at 340 nm upon the addition of 0.5 mM NAD^+ and 15 mM malate to 50 μg/mL mitochondrial protein. α-Ketoglutarate and pyruvate dehydrogenases were assayed by measuring the reduction of NAD^+ at 340 nm upon the addition of 0.5 mM NAD^+, 200 μM TPP, 40 μM CoASH, and either 2.0 mM α-ketoglutarate or 4.0 mM pyruvate to 50 μg/mL mitochondrial protein (*30*). All NAD^+/NADH-linked assays were done in the presence of 2.5 μM rotenone to prevent NADH consumption by complex I. Assays were performed at room temperature with sonicated (30 s, setting of 3.0, 100% pulse rate, VWR Scientific) mitochondria in a buffer containing 35 mM KH_2PO_4, 5.0 mM $MgCl_2$, 2.0 mM NaCN, and 0.5 mM EDTA at pH 7.25.

Purified Enzyme Assays. KGDH and PDH, purified from porcine heart, were obtained from Sigma suspended in a solution containing 50% glycerol, 10 mg/mL bovine serum albumin (KGDH only), 30% sucrose, 2.5 mM EDTA, 2.5 mM β-mercaptoethanol, 0.5% Triton X-100, 0.005% sodium azide, and 25 mM potassium phosphate, pH 6.8. To measure the effects of HNE on KGDH and PDH, 19 mU enzyme were incubated at 37 °C in buffer B with given amounts of substrate and HNE for times indicated. Enzyme activity was assayed by measuring the production of NADH at 340 nm upon addition of 50 μM CoASH, 0.2 mM TPP, 0.5 mM NAD^+, 10 μM $CaCl_2$, and 0.5 mM α-ketoglutarate or 0.5 mM pyruvate. The E3 portion of KGDH was assayed, following incubation of the purified enzyme with HNE as described above, by the addition of 1.2 mU enzyme to buffer A containing 1.0 mM DTT, 3.0 mM lipoamide, and 2.5 mM NAD^+ (*31*).

Dithio-(bis)nitrobenzoic Acid (DTNB) Assay. To measure the ability of DTNB to bind to reduced lipoic acid, it was first necessary to remove β-mercaptoethanol from the KGDH and PDH preparations. It was found that KGDH and PDH, large enzyme complexes, were pelleted upon centrifugation and thus allowed the removal of packaging solution with ∼30% recovery of enzyme units. Remaining protein was composed of approximately 40% BSA and 60% KGDH. After centrifugation of 0.952 U of enzyme (5.0 min at 2000*g*, Eppendorf 5415C) the resulting pellet was tapped dry, and then resuspended in 250 μL buffer B to a concentration of approximately 1.0 mg/mL (BCA). Enzyme (35 μg) was then added to 1.0 mL buffer B containing 100 μM NADH and incubated for 2.0 min at 37 °C. Indicated amounts of HNE were then added and the mixture was allowed to incubate another 3.0 min at 37 °C. At 5.0 min, 100 μM NADH and 0.5 mM DTNB (Molecular Probes) were added to the

reaction mixtures, and the rate of DTNB reduction was measured at 37 °C by the increase at 412 nm (*30*, *32*). A blank containing no protein was incubated under identical conditions. Samples prepared in the same way were assayed for enzyme activity.

Preparation of Polyclonal Antibody to Lipoic Acid (LA). Lipoic acid antigen was prepared by EDC linking LA to keyhole limpet hemocyanin (KLH). Briefly, 3.1 mg LA (15 μmol), dissolved in dimethyl sulfoxide (DMSO), was reacted with 3.0 mg KLH and 1.4 mg EDC (7.0 μmol). The conjugation was performed at 25 °C in 12% DMSO, 0.1 M MES, 0.9 M NaCl, at pH 4.7. After 2.0 h, KLH was separated from reactants by gel filtration over a PD-10 column (Pharmacia) with 0.083 M NaH_2PO_4, 0.9 M NaCl, and stabilizers at pH 7.2 (Pierce) as eluant. Polyclonal antibody was then generated in New Zealand White female rabbits by Covance (Denver, PA) according to a standard immunization protocol, using Freund's incomplete adjuvant (FIA) throughout immunization. Antibody titer was assessed by ELISA, using lipoic acid linked BSA as the antigen.

Western Blot Analysis. Samples were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electophoresis (PAGE) and Western blot analysis. Briefly, following treatment of purified KGDH (containing approximately 40% BSA) or mitochondria with HNE, as described above, 1.5 μg/lane KGDH/BSA or 10.0 μg/lane of mitochondrial protein was applied to a series of two 10% polyacrylamide gels. One gel was stained with Coomassie blue to visualize the protein distribution. Protein present on the second gel was electroblotted onto a nitrocellulose membrane (0.45 μm). Membrane-immobilized proteins were incubated with anti-LA diluted 1:100 000 into blocking buffer (0.2% I-block from Pierce in PBS containing 0.1% Tween-20) for 1 h at 25 °C. After 3 × 5.0 min washes with PBS containing 0.1% Tween-20 (wash buffer), the membrane was incubated with goat anti-rabbit IgG conjugated to alkaline phosphatase (Tropix, diluted 1:10 000 in blocking buffer) for 1.0 h at 25 °C. The membrane was then rinsed 3 × 5.0 min with wash buffer and 2 × 5.0 min with assay buffer (20 mM Tris Base, pH 9.8, containing 1 mM $MgCl_2$). Following incubation with a chemiluminescent alkaline phophatase substrate (1.25 mM CSPD and 1.0 mg/mL Nitro-Block from Tropix in assay buffer) for 5.0 min, antibody binding was visualized by autoradiography.

Reaction of HNE and Lipoic Acid. Reduced LA (8.0 mM, Sigma) was reacted with HNE (6.0 mM) for 30.0 min at 25 °C in 20 mM KH_2PO_4, pH 8.0. At 30.0 min, the reaction was quenched by addition of 15 mM $NaBH_4$ in 0.1 mM NaOH. Reduction was allowed to proceed for 10.0 min at 25 °C. In addition to quenching the reaction, reduction also serves to stabilize LA-HNE adducts. Excess $NaBH_4$ was then removed by the addition of HCl, and the products were purified by reverse phase HPLC (Vydac C18 column; 0 to 50% acetonitrile/0.05% TFA versus H_2O/0.05% TFA in 20 min, flow rate of 1.0 mL/min). The products were collected in three discrete peaks (retention times = 22.25, 22.50, and 22.80 min), as monitored at 210 nm, and then analyzed with an electrospray triple quadropole mass spectrometer (Micromass Quattro-II Triple Quadropole Mass Spectrometer) by Dr. Yong Hong Chen, Cleveland State University. The mass spectra of the three product peaks were identical. Electrospray MS: observed M − 1, $m/z = 365.21$; calculated M −1 for $C_{17}H_{33}O_4S_2$, $m/z = 365.57$. MS/MS analysis was then performed by selecting the parent peaks at 365.21 and fragmenting them via a gas collision chamber. Daughter peaks (M − 1) are as follows: observed $m/z = 191.05$, calculated for $(M - HS{-}C_2H_4{-}CH{-}C_4H_8{-}CO_2 - H)^- = 191.31$; observed $m/z = 173.09$, calculated for $(M - HS{-}C_2H_4{-}CH{-}C_4H_8{-}CO_2 - H)^- = 173.14$. The fragmentation patterns are consistent with that of the Michael addition product (see Scheme 2 for schematic). It was not possible to distinguish between stereoisomers or a mixture of products, with HNE reacting at either the S6 or the S8 position.

Table 1: Effect of HNE on Mitochondrial Respiratory Enzyme Activities[a]

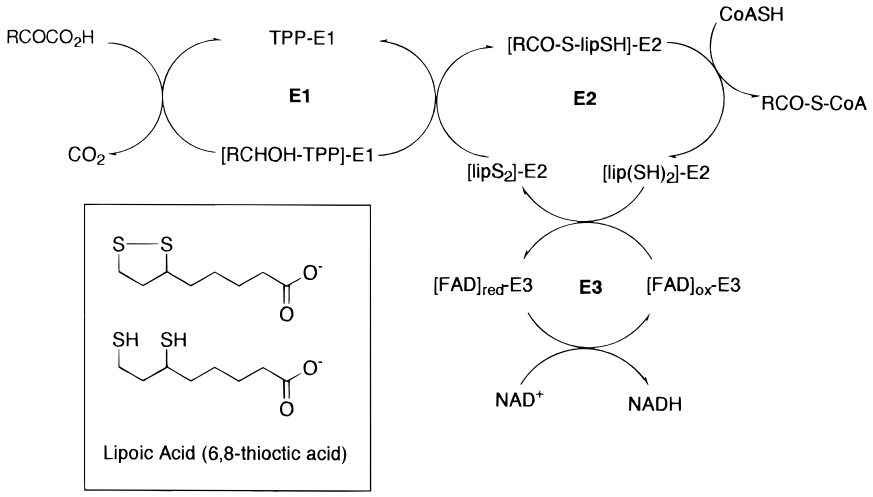
[HNE] enzyme	0 μM	50 μM
complex I	156.0 ± 7.6	162.0 ± 3.5
complex II	487.6 ± 13.3	445.2 ± 38.1
complex III	2409.9 ± 319.1	2415.5 ± 201.3
complex IV	509.1 ± 29.7	481.7 ± 21.3
glutamate dehydrogenase	30.2 ± 4.3	32.3 ± 0.7
malate dehydrogenase	78.1 ± 7.0	84.7 ± 5.9
pyruvate dehydrogenase	28.5 ± 1.3	22.5 ± 1.5*
α-ketoglutarate dehydrogenase	62.2 ± 1.5	35.7 ± 1.4†

[a] Intact mitochondria (0.5 mg/mL) were incubated in the absence and presence of 50 μM HNE for 5 min at 25 °C. Unreacted HNE was then scavenged by addition of 50 mM cysteine, mitochondria were disrupted, and enzyme activities determined as described in Materials and Methods. Values are expressed as nmol min^{-1} mg^{-1} mitochondrial protein and represent the mean ($n = 4$) ± standard deactivation. *P* values (determined from paired *t* test) indicate statistically significant decreases in activity: *, ≤ 0.002, †, ≤ 2×10^{-7}.

RESULTS

Effects of 4-Hydroxy-2-nonenal on Mitochondrial Enzyme Activities. Incubation of cardiac mitochondria with HNE results in the selective inactivation of KGDH and PDH. As shown in Table 1, exposure of mitochondria to 50 μM HNE for 5.0 min at 25 °C resulted in a 42.6% and a 21.0% decrease in KGDH and PDH activity, respectively. Inhibition required the presence of NADH-linked substrates (glutamate or pyruvate) during the incubation. In contrast, glutamate dehydrogenase and malate dehydrogenase activities were unaffected by HNE under the conditions of the experiments, indicating that the effects of HNE on KGDH and PDH are not general to all NADH-linked dehydrogenases. In addition, electron transport chain components (complexes I−IV) exhibited no loss in activity when mitochondria were treated with HNE. Thus, under the conditions of our experiments, observed declines in NADH-linked respiration is not the result of global disruption of respiratory enzyme activity but is due to the specific inactivation of KGDH and PDH by HNE. Structurally and catalytically similar, both dehydrogenases are comprised of multiple copies of three enzymes, α-ketoacid decarboxylase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoamide dehydrogenase (E3) (*21*, *25*). In addition, KGDH and PDH require the cofactors thiamine pyrophosphate bound to E1, lipoic acid covalently linked to the E2 subunit, and FAD^+ for E3 activity (Scheme 1). It is therefore likely that structural features common to KGDH and PDH render these enzymes susceptible to inactivation by HNE.

Scheme 1. Schematic Representation of the Reaction Mechanism of α-Ketoacid Dehydrogenases (*21*)[a]



[a] Abbreviations: α-ketoacid decarboxylase (E1); dihydrolipoyl transacetylase (E2); dihydrolipoamide dehydrogenase (E3); thiamine pyrophosphate (TPP); lipoic acid (lip). Inset: Oxidized and reduced lipoic acid.

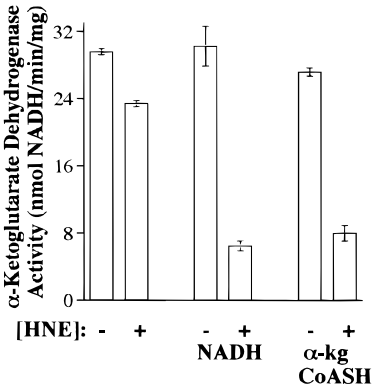


FIGURE 1: Effect of HNE on purified KGDH. Protein (4.0 mU) was incubated for 2.0 min at 37 °C in the absence or presence of substrate (10 μM NADH or 0.5 mM α-ketoglutarate and 2.5 μM CoASH) as indicated. After 2.0 min, HNE was added at a final concentration of 25 μM and allowed to incubate for 3.0 min at 37 °C. Unreacted HNE was then scavenged by addition of 10 mM cysteine and KGDH activity assayed as described in Materials and Methods.

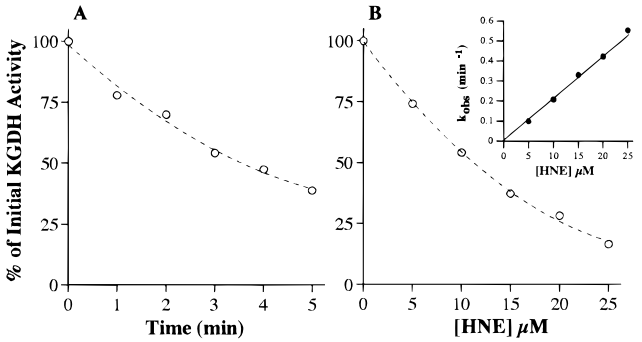


FIGURE 2: Fractional loss of KGDH activity as a function of time and HNE concentration. (A) Protein (4.0 mU) was incubated with 10 μM NADH for 5.0 min at 37 °C. During this incubation, HNE (10 μM) was added to expose protein to HNE for times indicated on the abscissa. (B) Protein (4.0 mU) was incubated with 10 μM NADH for 2.0 min at 37 °C followed by an additional 3.0 min exposure to concentrations of HNE as indicated on the abscissas. Following treatment, KGDH 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 KGDH.

Effects of HNE on Purified KGDH and PDH. The effects of HNE on purified KGDH and PDH were determined to elucidate the mechanism of inactivation. As shown in Figure 1, incubation of purified KGDH with 25 μM HNE for 3.0 min at 37 °C resulted in a 20.8% decrease in activity. The inhibitory effects of HNE were greatly enhanced in the presence of either NADH or α-ketoglutarate and CoASH (Figure 1), with a loss of 78.5% and 70.4% in activity, respectively. As shown in Figure 2A, KGDH activity decreased in a time-dependent manner. A semilogarithmic plot of remaining activity versus incubation time (not shown) was linear, indicating that inactivation occurred by a pseudo-first-order process. Exposure of KGDH to increasing concentrations of HNE (0−25 μM, 3.0 min) led to increased inactivation in a saturable fashion (Figure 2B). The linear relationship between HNE concentration and the apparent rate constant for inactivation (k_{obs}) (Figure 2B, inset) 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. This is in agreement with kinetic results obtained for HNE inactivation of KGDH in intact mitochondria (*20*). Inhibition of purified PDH by HNE was enhanced in the presence of substrate and followed pseudo-first-order kinetics (results not shown), suggesting a similar mechanism of inactivation.

Relationship between Loss of Lipoic Acid Sulfhydryls and Enzyme Activity. In the presence of NADH or CoASH and α-ketoglutarate/pyruvate, lipoic acid(s) on the E2 subunit of KGDH and PDH are maintained in the reduced state (Scheme 1). It is well established that HNE readily reacts with sulfhydryl groups (*19*). Therefore, due to conditions necessary for inhibition and the chemical nature of HNE, lipoic acid is a potential site of modification. It has previously been described that in the presence of NADH, DTNB binds to reduced lipoic acid on the E2 subunit and is then catalytically cleaved by the E3 subunit of KGDH or PDH (*30*, *32*). To test whether lipoic acid is a site of HNE modification, the ability of purified KGDH and PDH to reduce DTNB in the presence of NADH and HNE (0−25 μM) was evaluated. As shown in Figure 3, the rate of DTNB reduction decreased with increasing concentrations of HNE, correlating closely with a loss in KGDH activity. Further-

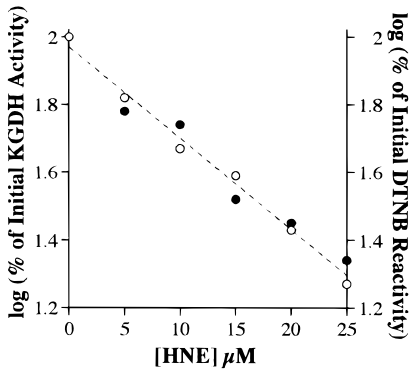
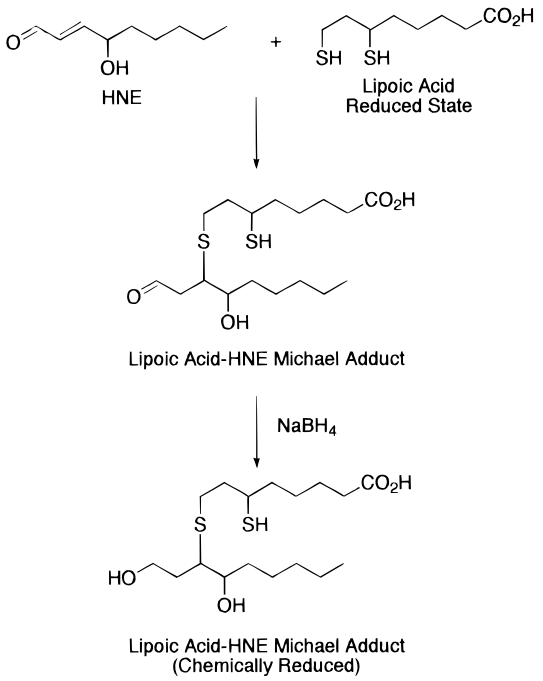


FIGURE 3: Semilogarithmic plot of KGDH activity and rate of DTNB reduction as a function of HNE concentration. Protein (12 mU) was incubated with 10 μM NADH for 2.0 min at 37 °C followed by an additional 3.0 min exposure to concentrations of HNE as indicated on the abscissa. Enzyme activity (○) and DTNB reduction (●) were then assayed as described in Materials and Methods.

Scheme 2. Reaction of Lipoic Acid with HNE Followed by Reductive Stabilization



more, under the same conditions, HNE had no effect on E3 activity, as measured by the rate of reduction of NAD^+ in the presence of dihydrolipoamide (results not shown). Identical results were obtained for PDH (results not shown). These observations indicate that, in the presence of substrate, lipoic acid is modified by HNE resulting in inactivation of KGDH and PDH.

Inspection of the chemical structures of HNE and reduced lipoic acid suggests that lipoic acid sulfhydryl group(s) would undergo 1,4 (Michael) addition to the double bond (C3) of HNE, thereby causing inactivation of KGDH and PDH. To determine the chemistry of lipoic acid−HNE interactions, model studies with HNE and reduced lipoic acid were performed. As depicted in Scheme 2, mass spectroscopic analyses (ES and MS/MS) of the products of this reaction were consistent with the formation of 1:1 lipoic acid−HNE Michael adduct(s) (Materials and Methods). Experiments

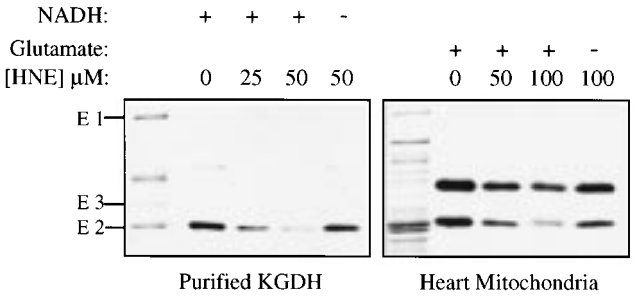


FIGURE 4: Western blot analysis of purified KGDH and mitochondria treated with HNE. Purified KGDH and mitochondria were incubated with HNE in the presence and absence of NADH (10 μM) or glutamate (15 mM) as indicated. Protein samples were separated on 10% polyacrylamide gels (1.5 μg of purified KGDH containing ~40% BSA/well or 10.0 μg of mitochondrial protein/well), and then electroblotted onto nitrocellulose membrane (0.45 μm) for Western blot analysis with anti-lipoic acid as primary antibody. Antibody binding was visualized after exposure of the blot to a chemiluminescent substrate (Materials and Methods). The first lane in each series is a representative protein profile obtained upon Coomassie staining. KGDH E1, E2, and E3 migrate at 95, 50, and 56 kDa, respectively.

to distinguish between HNE modification(s) at the S6 and S8 positions of lipoic acid are currently underway.

Immunochemical Evidence for HNE Modification of Lipoic Acid. Polyclonal antibodies were raised to KLH-linked lipoic acid (Materials and Methods). As judged by competitive ELISA, these antibodies are specific to lipoic acid and do not recognize HNE-modified lipoic acid (results not shown). This antibody preparation was therefore used to determine whether lipoic acid is modified when purified protein or mitochondria are treated with HNE. As shown in Figure 4 (purified KGDH), the antibody recognized one protein band assigned as the E2 subunit of KGDH based on molecular weight (~50 kDa). Upon treatment of purified enzyme with increasing amounts of HNE (0−50 μM), antibody recognition decreased. Loss in antibody binding was dependent on the presence of NADH during the incubation, thereby reflecting conditions required for inactivation.

Western blot analysis of mitochondrial protein using anti-LA antibody revealed the presence of two lipoic acid-containing proteins (Figure 4). The molecular masses of these proteins (~50 and ~65 kDa) approximate those of the E2 subunit of KGDH and PDH, respectively. Antibody binding was competed by LA (100 μM) confirming antibody specificity. In agreement with results obtained for purified KGDH, treatment of mitochondria with increasing concentrations of HNE (0−100 μM) resulted in a decline in the level of antibody binding (Figure 4). The decrease in recognition was dependent upon the presence of glutamate in the incubation mixture (Figure 4). Thus, loss of antibody recognition of lipoic acid for both HNE-treated KGDH and mitochondria occurs under conditions which promote enzyme inactivation, thereby providing further support for lipoic acid as the site of HNE modification and enzyme inactivation. Methods which combine immunochemical and mass spectral techniques, which will rigorously identify lipoic acid containing proteins and provide direct chemical evidence for lipoic acid−HNE adducts, are currently being developed in our laboratory.

DISCUSSION

Increased rates of free radical production have been associated with the progression of numerous degenerative diseases (*1*−*16*). Many of these conditions, in particular reperfusion injury, are accompanied by declines in mitochondrial function (*1*−*3*, *8*, *9*, *12*, *28*, *33*−*40*). Mechanisms by which free radicals mediate mitochondrial dysfunction have not, however, been established. Previously, we have demonstrated that 4-hydroxy-2-nonenal (HNE), a major product of lipid peroxidation, is a potent inhibitor of NADH-linked mitochondrial respiration (*20*). Loss of respiratory activity was due to inactivation of KGDH (*20*). In the present study, the effects of HNE on a variety of mitochondrial dehydrogenases and on electron transport chain complexes were examined to identify other potential sites of HNE-mediated inactivation. The results of this study demonstrate that incubation of cardiac mitochondria with micromolar concentrations of HNE results in the selective and rapid inactivation of KGDH and PDH. KGDH and PDH are structurally and catalytically similar (*21*, *25*), suggesting a common mode of inactivation. Mechanistic studies indicate that inhibition of both enzymes is due to covalent modification of lipoic acid residues bound to the respective E2 subunits.

The susceptibility of KGDH and PDH to HNE-induced inactivation is likely predicated by the chemical and structural properties of lipoic acid and HNE. It is well established that HNE, an α,β-unsaturated aldehyde, can react with the sulhydryl moiety of cysteine, the imidazole nitrogens of histidine, and the ϵ-amino group of lysine, via Michael addition (*19*, *41*−*48*). At neutral pH sulhydryls are the most reactive of these functional groups (*19*). On protein, reactivity is also dependent on the accessibility of the nucleophilic amino acid residues to HNE (*42*). Reduced lipoic acid is a strong nucleophile at physiological pH (*19*) and is located on the surface of the E2 subunit of KGDH and PDH (*21*, *25*). Model studies reveal that sulfhydryl group(s) on lipoic acid react with the double bond (C3) of HNE to form a Michael addition product (Scheme 2). Hydrophobic interactions are also likely to occur between the hydrocarbon chains of lipoic acid and HNE. Thus, lipoic acid represents a prime target for Michael addition to HNE. Furthermore, HNE is a hydrophobic compound which would be at greatest concentration in the lipid bilayer. KGDH, reported to be associated with the inner mitochondrial membrane (*49*, *50*), would therefore be particularly susceptible to modification (Table 1). Because many lipid peroxidation products are lipophilic electrophiles, this chemistry is not likely to be limited to HNE and may represent a common mechanism of cytotoxicity. Due to the sensitivity of KGDH and PDH to HNE inactivation, potential intracellular mechanisms for the removal of HNE and restoration of enzyme activity, perhaps glutathione-dependent, should be explored. In addition, it is interesting to note that lipoic acid can scavenge oxygen radicals (*51*, *52*). Due to the ability of KGDH and PDH to cycle between oxidized and reduced states of lipoic acid, these enzymes may function as antioxidants under certain conditions.

The highly susceptible nature of KGDH to HNE inactivation is of significance considering that a loss in activity would have profound effects on mitochondrial function. We have previously demonstrated that the degree of KGDH inactivation induced by treatment of intact mitochondria with HNE correlates closely with the loss of NADH-linked mitochondrial respiration (*20*). This observation suggests that production of NADH by KGDH is rate-limiting for mitochondrial respiration, a view consistent with previous findings (*22*, *23*). In contrast, it has been reported that electron-transport chain complexes must be significantly inhibited before mitochondrial respiration and ATP production are affected (*53*). The degree of inactivation required to induce a significant loss in respiration is dependent on the identity of the complex and on the source of mitochondria (*53*). Thus, while there have been numerous reports on disease-related declines in the activities of various electron-transport chain complexes (*9*, *33*, *35*, *39*), declines in KGDH and PDH activities may have relatively greater effects on mitochondrial respiration.

The cellular environment created during the progression of a number of degenerative conditions suggests that HNE inactivation of KGDH and PDH is likely to play a significant role in disease-related losses of mitochondrial function. Upon reperfusion, mitochondria exhibit declines in NADH-linked respiration (*34*−*37*, *39*, *40*) and increased rates of free radical production (*4*, *7*). This is accompanied by a rise in tissue and perfusate levels of HNE (*6*, *54*) and the appearance of HNE-modified protein (*40*). In addition, the preceding ischemic event induces a buildup of reducing equivalents (NADH) and a fall in the level of ADP (for review, see ref *55*), conditions which would favor the reduced state of lipoic acid on KGDH and PDH, priming these enzymes for modification by HNE during reperfusion. Furthermore, several neurodegenerative diseases, including Alzheimer's and Parkinson's, are characterized by an increase in free radical production (*8*−*10*, *12*), HNE modification(s) to protein (*11*−*14*, *16*), and declines in KGDH activity (*8*, *56*, *57*). While the results of our study strongly suggest that KGDH and PDH may be inactivated by HNE during the progression of certain degenerative events, a direct link between loss(es) of enzyme activity and the appearance of HNE-modified protein has not yet been established. Clearly, identification of likely targets and mechanisms of HNE inactivation is critical for direct assessment of the role of free radicals and lipid peroxidation in disease.

REFERENCES

1. Nohl, H., and Hegner, D. (1978) *Eur. J. Biochem. 82*, 563−567.
2. Nohl, H., Breuninger, V., and Hegner, D. (1978) *Eur. J. Biochem. 90*, 385−390.
3. Hansford, R. G. (1983) *Biochim. Biophys. Acta 726*, 41−80.
4. Das, D. K., George, A., Liu, X., and Rao, P. S. (1989) *Biochem. Biophys. Res. Commun. 165*, 1004−1009.
5. Dexter, D. T., Carter, C. J., Wells, F. R., Javoy-Agid, F., Agid, Y., Lees, A., Jenner, P., and Marsden, C. D. (1989) *J. Neurochem. 52*, 381−389.
6. Ambrosio, G., Flaherty, J. T., Duilio, C., Tritto, I., Santoro, G., Elia, P. P., Condorelli, M., and Chiariello, M. (1991) *J. Clin. Invest. 87*, 2056−20669.
7. Ambrosio, G., Zweier, J. L., Duilio, C., Kuppusamy, P., Santoro, G., Elia, P. P., Tritto, I., Cirillo, P., Condorelli, M., Chiariello, M., and Flaherty, J. T. (1993) *J. Biol. Chem. 268*, 18532−18541.
8. Beal, M. F. (1995) *Ann. Neurol. 38*, 357−366.
9. Mizuno, Y., Mori, H., and Kondo, T. (1995) *Intern. Med. 34*, 1045−1054.

10. Mizuno, Y., Ikebe, S.-I., Hattori, N., Nakagawa-Hattori, Y., Mochizuki, H., Tanaka, M., and Ozawa, T. (1995) *Biochim. Biophys. Acta 1271*, 265−274.
11. Yoritaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E. R., and Mizuno, Y. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2696−2701.
12. Markesbery, W. R. (1997) *Free Radical Biol. Med. 23*, 134−147.
13. Sayre, L. M., Zelasko, D. A., Harris, P. L., Perry, G., Salomon, R. G., and Smith, M. A. (1997) *J. Neurochem. 68*, 2092−2097.
14. Ando, Y., Brannstrom, T., Uchida, K., Nyhlin, N., Nasman, B., Suhr, O., Yamashita, T., Olsson, T., El Salhy, M., Uchino, M., and Ando, M. (1998) *J. Neurol. Sci. 156*, 172−176.
15. Beckman, K. B., and Ames, B. N. (1998) *Physiol. Rev. 78*, 547−581.
16. Montine, K. S., Reich, E., Neely, M. D., Sidell, K. R., Olson, S. J., Markesbery, W. R., and Montine, T. J. (1998) *J. Neuropathol. Exp. Neurol. 57*, 415−425.
17. Chance, B., Sies, H., and Boveris, A. (1979) *Physiol. Rev. 59*, 527−605.
18. Giulivi, C., Boveris, A., and Cadenas E. (1995) *Arch. Biochem. Biophys. 316*, 909−916.
19. Esterbauer, H., Shaur, R. J., and Zollner, H. (1991) *Free Radical Biol. Med. 11*, 81−128.
20. Humphries, K. M., Yoo, Y., and Szweda, L. I. (1998) *Biochemistry 37*, 552−557.
21. Reed, L. J. (1974) *Acc. Chem. Res. 7*, 40−46.
22. Cooney, G. J., Taegtmeyer, H., and Newsholme, E. A. (1981) *Biochem. J. 200*, 701−703.
23. Moreno-Sanchez, R., Hogue, B. A., and Hansford, R. A. (1990) *Biochem. J. 268*, 421−428.
24. Smith, C. M., Bryla, J., and Williamson, J. R. (1974) *J. Biol Chem. 249*, 1497−1505.
25. Perham, R. N. (1991) *Biochemistry 30*, 859−8512.
26. De Montarby, L., Tourbah, H., and Gree, R. (1989) *Bull. Soc. Chim. Fr.* (No. 3), 419−432.
27. Estornell, E., Fato, R., Pallotti, F., and Lenaz, G. (1993) *FEBS Lett. 332*, 127−131.
28. Veitch, K., Hombroeckx, A., Caucheteux, D., Pouleur, H., and Hue, L. (1992) *Biochem. J. 281*, 709−715.
29. Frieden, C. (1959) *J. Biol. Chem. 234*, 809−815.
30. Brown, J. P., and Perham, R. N. (1976) *Biochem. J. 155*, 419−427.
31. Linn, T. C. (1971) *Arch. Biochem. Biophys. 161*, 505−514.
32. Erfle, J. D., and Sauer, F. (1968) *Biochem. Biophys. Res. Commun. 32*, 562−567.
33. Scholte, H. R. (1988) *J. Bioenerg. Biomembr. 20*, 161−191.
34. Duan, J., and Karmazyn, M. (1989) *Can. J. Physiol. Pharmacol. 67*, 704−709.
35. Hardy, L., Clark, J. B., Darley-Usmar, V. M., Smith, D. R., and Stone, D. (1991) *Biochem. J. 274*, 133−137.
36. Boraso, A., Cargnoni, A., Comini, L., Gaia, G., Bernocchi, P., and Ferrari, R. (1993) *Mol. Cell. Biochem. 125*, 73−86.
37. Sciamanna, M. A., and Lee, C. P. (1993) *Arch. Biochem. Biophys. 305*, 215−224.
38. Luft, R. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 8731−8738.
39. van Jaarsveld, H., Kuyl, J. M., Alberts, D. W., and Wiid, M. N. (1994) *Res. Commun. Mol. Pathol. Pharmacol. 85*, 33−44.
40. Lucas, D. T., and Szweda, L. I. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 510−514.
41. Uchida, K., and Stadtman, E. R. (1993) *J. Biol. Chem. 268*, 6388−6393.
42. Szweda, L. I., Uchida, K., Tsai, L, and Stadtman, E. R. (1993) *J. Biol. Chem. 268*, 3342−3347.
43. Friguet, B., Stadtman, E. R., and Szweda, L. I. (1993) *J. Biol. Chem. 269*, 21639−21643.
44. Chen, J. J., Bertrand, H., and Yu, B. P. (1995) *Free Radical Biol. Med. 19*, 583−590.
45. Nadkarni, D. V., and Sayre, L. M. (1995) *Chem. Res. Toxicol. 8*, 284−291.
46. Tsai, L., and Sokoloski, E. A. (1995) *Free Radical Biol. Med. 19*, 39−44.
47. Cohn, J. A., Tsai, L., Friguet, B., and Szweda, L. I. (1996) *Arch. Biochem. Biophys. 328*, 158−164.
48. Siems, W. G., Hapner, S. J., and van Kuijk, F. J. G. M. (1996) *Free Radical Biol. Med. 20*, 215−223.
49. Porpaczy, Z., Sumegi, B., and Alkonyi, I. (1987) *J. Biol. Chem. 262*, 9509−9514.
50. Maas, E., and Bisswanger, H. (1990) *FEBS Lett. 277*, 189−190.
51. Packer, L., Tritschler, H. J., and Wessel, K. (1997) *Free Radical Biol. Med. 22*, 359−378.
52. Biewenga, G. P., Haenen, G. R. M. M., and Bast, A. (1997) *Gen. Pharmacol. 29*, 315−331.
53. Davey, G. P., Peuchen, S., and Clark, J. B. (1998) *J. Biol. Chem. 273*, 12753−12757.
54. Blasig, I. E., Grune, T., Schonheit, K., Rohde, E., Jakstadt, M., Haseloff, R. F., and Siems, W. G. (1995) *Am. J. Physiol. 269 (Heart Circ. Physiol. 38)*, H14−H22.
55. Reimer, K. A., and Jennings, R. B. (1992) in *The Heart and Cardiovascular System* (Fozzard, H. A., Haber, E., Jennings, R. B., Katz, A. M., and Morgan, H. E., Eds.) 2nd ed., pp 1875−1973, Raven, New York.
56. Mastrogiacomo, F., Bergeron, C., and Kish, S. J. (1993) *J. Neurochem. 61*, 2007−2014.
57. Kish, S. J. (1997) *Ann. N. Y. Acad. Sci. 826*, 218−228.

BI981512H