ANTIOXIDANTS & REDOX SIGNALING
Volume 5, Number 5, 2003

Forum Original Research Communication

Age-Related Increase in 4-Hydroxynonenal Adduction to Rat Heart α-Ketoglutarate Dehydrogenase Does Not Cause Loss of Its Catalytic Activity

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ABSTRACT

4-Hydroxynonenal (HNE), a product of ω-6 polyunsaturated fatty acid peroxidation, impairs mitochondrial respiration *in vitro* by adducting the α-ketoglutarate dehydrogenase complex (KGDC) and inhibiting its activity. The present study seeks to define whether aging increases HNE adduction to rat heart KGDC, and whether such adduction impacts KGDC activity. We found that hearts from old rats exhibit significantly ($p \leq 0.01$) higher HNE-modified mitochondrial proteins when compared with those from young rats. Among these proteins, dihydrolipoamide succinyltransferase, the E2k component of KGDC, was most markedly modified ($p \leq 0.01$) by HNE with age. As opposed to that seen *in vitro,* no significant change in electrophoretic mobility or impairment in enzyme activity was observed. On the contrary, KGDC activity increased onefold ($p \leq 0.01$) in old rats, suggesting that the aging myocardium is not affected by HNE adduction or compensates for such damage. Further analysis revealed that heightened KGDC activity was not due to increased protein content or gene expression, but correlates with a lower K_m for α-ketoglutarate. Thus, contrary to that observed *in vitro,* the measurement of HNE-KGDC adduct in rat heart is more relevant as a marker of age-related protein oxidation than a factor of mitochondrial dysfunction. *Antioxid. Redox Signal.* 5, 517–527.

INTRODUCTION

THE CYTOTOXICITY OF LIPID PEROXIDATION is attributed, in part, to the formation of reactive aldehydes that modify proteins. Among these aldehydes, 4-hydroxynonenal (HNE) is one of the most reactive and abundant end products generated by the controlled oxidation of ω-6 polyunsaturated fatty acids (6). Increased HNE levels are associated with a variety of pathological conditions, including ischemia/reperfusion injury, neuronal death, and nephropathies (1, 15, 19, 21). Under basal conditions, cellular HNE steady-state levels amount to ~1 μ*M,* and increase to 10 μ*M* under pathophysiological conditions (6). It is not currently known whether the rate of formation or steady-state levels of HNE increase in tissues during the aging process, nor the significance of HNE adduction to cell function in aging (17, 22, 38). HNE is highly reactive toward proteins. It reacts with free sulfhydryl groups producing thioether adducts that further undergo cyclization to form hemiacetals (36). HNE also reacts with histidine and lysine residues to form stable Michael addition-type adducts referred to as advanced lipoxidation end products (ALEs). *In vitro,* HNE modifies a large array of model polypeptides (4, 34–36). Previous work on oxidative damage showed that heart mitochondrial proteins are susceptible to oxidative modification by HNE

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in vitro (11, 12). In particular, HNE, at supraphysiological concentrations (50 μ*M*), significantly inhibited the activity of the α-ketoglutarate dehydrogenase complex (KGDC) and pyruvate dehydrogenase complex, and also markedly diminished mitochondrial respiration. However, there is no direct evidence that HNE forms adducts with mitochondrial proteins *in vivo*.

KGDC is an ideal target protein to compare the *in vitro* with the *in vivo* situation because of its aforementioned susceptibility to HNE adduction *in vitro* and because of its role as a governor of the citric acid cycle. KGDC produces NADH via the oxidative decarboxylation of α-ketoglutarate and also forms succinyl-CoA, a major intermediate not only for the citric acid cycle, but also a precursor for heme biosynthesis. Additionally, there is no clear understanding how aging affects citric acid cycle enzyme function and, consequently, the supply of NADH for the electron transport chain and ATP synthesis.

Thus, the primary goal of the present study was to determine the extent of HNE-KGDC adduction in the aging rat heart and the consequences to KGDC activity. Furthermore, if a significant age-related increase in HNE adduction was found, a subsequent goal was to determine whether steady-state levels of HNE-KGDC adduct could be modulated by dietary supplementation with *R*-α-lipoic acid (LA). LA is a natural dithiol compound that could lower HNE adduction either through direct scavenging of reactive aldehydes or by inducing reduced glutatione (GSH) synthesis and glutathione *S*-transferase-mediated detoxification of HNE (3, 7).

MATERIALS AND METHODS

Modification of KGDC by HNE in vitro

KGDC, from porcine heart (Sigma, St. Louis, MO, U.S.A.), supplied in 50% glycerol containing 10 mg/ml bovine serum albumin (BSA), 30% sucrose, 2.5 m*M* EDTA, 2.5 m*M* EGTA, 2.5 m*M* β-mercaptoethanol, 0.5% Triton X-100, 0.005% sodium azide, and 25 m*M* potassium phosphate, pH 6.8, was used in all *in vitro* studies. KGDC (100 μg of protein) was incubated with up to 2 m*M* HNE (Oxis International, Inc., Portland, OR, U.S.A.) in 50 m*M* sodium phosphate buffer, pH 7.2, for 2 h at 37°C. The reaction was stopped by scavenging excess HNE with 10 m*M* cysteine, followed by the addition of sample buffer [312 m*M* Tris-HCl, pH 6.8, 10% (wt/vol) sodium dodecyl sulfate (SDS), 50% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, 0.05% (wt/vol) bromophenol blue], prior to SDS–polyacrylamide gel electrophoresis (PAGE) and western blotting as further described below.

Porcine heart KGDC activity was measured in the presence of 0–0.6 m*M* HNE by following the increase in absorbance at 340 nm. KGDC (5 mU) was incubated with HNE at 30°C for 30 min in 50 m*M* Tris-HCl, pH 7.6, and the reaction initiated by adding 2 m*M* NAD^+, 0.6 m*M* thiamine pyrophosphate (TPP), 0.24 m*M* CoASH, 0.1 m*M* dithiothreitol (DTT), 1 m*M* $MgCl_2$, 20 μ*M* $CaCl_2$, and 5 m*M* α-ketoglutarate.

Nano-LC ESI-MS/MS

Polypeptides contained in the porcine heart KGDC preparation were identified using a nanoscale liquid chromatography system coupled to electrospray ionization tandem mass spectrometry (nano-LC ESI-MS/MS). Proteins were separated by 7.5% SDS-PAGE under reducing conditions and stained with Coomassie Blue R-250. Five prominent bands were removed from the gel, destained in 5% methanol/7% acetic acid, washed with 100 m*M* NH_4HCO_3, dehydrated with acetonitrile, and dried under vacuum before digestion with modified porcine trypsin (Promega Corp., Madison, WI, U.S.A.) in 50 m*M* NH_4HCO_3, 5 m*M* $CaCl_2$, overnight at 37°C. Five microliters of the peptide digest was injected onto a 25-cm long PicoFrit column (New Objective, Cambridge, MA, U.S.A.) packed with 5-μm particle Luna C_{18} silica (Phenomenex, Torrance, CA, U.S.A.), and separated using a gradient of acetonitrile supplemented with 0.1% acetic acid and 0.01% trifluoroacetic acid delivered at a flow rate of 0.3 μl/min (5). Nano-LC ESI-MS/MS analyses were performed on an LC-Quadrupole ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, U.S.A.). The LC-Quadrupole was operated in the MS mode with the spraying potential set at 2.5 kV, the inlet capillary heated to 180°C, the capillary potential set at 46 V, and a maximum injection time of 50 ms. The instrument was set to acquire a full MS scan between 400 and 2,000 *m/z* followed by a MS/MS scan. For operation in the MS/MS mode, the maximum injection time was increased to 500 ms, the isolation width set to 1 Da, and the collision energy set to 30% with a 30-ms activation time. Using a data-dependent algorithm, the first most intense ion (>2×10^4 counts) was selected for MS/MS. To identify peptide fragments, MS/MS spectra were compared with the National Center for Biotechnology Information (NCBI) protein database using the Sequest software (Thermo Finnigan, San Jose, CA, U.S.A.).

Animals and diets

Rats (Fischer 344, male), both young (4 months) and old (24–28 months), from the National Institute on Aging were housed in cages in a controlled environment (temperature 22 ± 2°C, 12-h light/12-h dark cycle) and fed AIN-93M diet *ad libitum* supplemented or not with LA (2 g/kg of diet) for up to 6 weeks. LA was a gift from Dr. Hans Tritschler (Asta Medica, Germany). LA is the "natural" stereoisomer, preferentially used by α-ketoacid dehydrogenases (27). Animals had free access to food and water until they were killed. Food consumption data indicated an average intake of 114 and 152 mg of LA/kg/day in old and young F344 rats, respectively.

Isolation of mitochondria

Rat heart mitochondria were isolated using the method of Palmer *et al.* (24). In brief, rats were anesthetized with diethyl ether, a midlateral incision was made in the chest, and the heart was removed and minced in cold 0.3 *M* mannitol containing 5 m*M* MOPS, 5 m*M* KH_2PO_4, 1 m*M* $CaCl_2$, and 0.1% BSA (fatty acid-free, fraction V; Calbiochem–Novabiochem Corp., La Jolla, CA, U.S.A.), pH 7.4. Minced heart was subjected to collagenase treatment (type IV, Sigma) at 4°C for 40 min, whereupon digestion was stopped with EGTA and the buffer decanted. The heart digest was homogenized using a Potter–Elvehjem homogenizer in 8 volumes of cold 0.3 *M* mannitol, 5 m*M* MOPS, 5 m*M* KH_2PO_4, 1 m*M* EGTA, 0.1 m*M* *n*-tosyl-L-phenylalaninechloromethyl ketone, aprotinin (8 TIU/L),

0.5 μ*M* leupeptin, 0.5 μ*M* pepstatin A, and 0.1% BSA (fatty acid-free), pH 7.4. The homogenate was then centrifuged at 1,500 *g* for 10 min at 4°C. The supernatant was further centrifuged at 10,000 *g* for 10 min at 4°C. The resulting pellet was washed twice by centrifugation at 10,000 *g* for 10 min at 4°C and resuspended to a final concentration of 15 mg of protein/ml. This crude preparation is enriched in subsarcolemmal mitochondria, but also contains interfibrillary mitochondria as well. Protein content was determined using the bicinchoninic acid method (Pierce, Rockford, IL, U.S.A.) and BSA as a standard.

Western blotting

Crude mitochondria were solubilized in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 mixed with sample buffer [125 m*M* Tris-HCl, pH 6.8, 4% (wt/vol) SDS, 20% (vol/vol) glycerol, 2% (vol/vol) β-mercaptoethanol, 0.02% (wt/vol) bromophenol blue] and then heat-denatured. Proteins (70 μg) were subjected to reducing SDS-PAGE, electroblotted onto nitrocellulose membranes, and then incubated with monoclonal antibodies to HNE modified by keyhole limpet hemocyanin (HNE-KLH; Oxis International), antiserum to bovine KGDC, or IgG anti-porcine lipoamide dehydrogenase (Rockland, Gilbertsville, PA, U.S.A.) in blocking buffer (Tris-buffered saline with Tween-20 containing 5% nonfat dry milk and 0.5% BSA). Membranes were rinsed three times for 10 min and incubated with horseradish peroxidase-conjugated secondary antibodies. Antibody binding was visualized using an ECL™ Western Blotting System (Amersham Pharmacia Biotech Inc., Pistacaway, NJ, U.S.A.) and band density quantified using an AlphaImager™ imaging system (Alpha Innotech Corp., San Leandro, CA, U.S.A.). The level of HNE adduct to a specific protein was obtained by sequential blotting of the membrane for HNE-reactive proteins, stripping in 62.5 m*M* Tris, 2% (wt/vol) SDS, 100 m*M* β-mercaptoethanol for 30 min at 50°C, and then blotting with antibodies to the specific protein. Nonspecific binding of HNE-KLH antibodies with mitochondrial proteins was determined by incubating the antibodies with 5 mg of HNE-BSA prior to western blotting. HNE-BSA was produced by incubating BSA with 1 m*M* HNE, 1 m*M* EDTA in PBS overnight at room temperature followed by extensive dialysis against PBS.

KGDC activity in F344 rat heart

KGDC activity was measured in freshly isolated mitochondria by following the reduction of NAD^+ at 340 nm (at 30°C) upon the addition of 2 m*M* NAD^+, 0.3 m*M* TPP, 0.12 m*M* CoASH, 2.6 m*M* cysteine HCl, 25 m*M* oxamate, and 6 m*M* α-ketoglutarate to 50 μg/ml mitochondrial protein. KGDC was assayed in 50 m*M* Tris-HCl, pH 7.6, 1 m*M* $MgCl_2$, and 10 μM $CaCl_2$ from mitochondria solubilized with 50 m*M* Tris-HCl, pH 7.6, containing 0.15% *n*-octyl β-D-glucopyranoside.

KGDC kinetic characteristics

Kinetics were determined spectrophotometrically using a 96-well spectrophometric plate reader (SpectraMax, Molecular Dynamics, Sunnyvale, CA, U.S.A.). NADH appearance was monitored at 340 nm in 200 μl final volume for 10 min at 30°C. Under these conditions, molar absorptivity ϵ was 3,350 M^{-1} cm^{-1}. The assay mixture was composed of 2 m*M* NAD^+, 0.6 m*M* TPP, 0.24 m*M* CoASH, 0.1 m*M* DTT, 25 m*M* oxamate, 1 m*M* $MgCl_2$, 20 μ*M* $CaCl_2$, 0.2–5 m*M* α-ketoglutarate in 50 m*M* Tris-HCl, pH 7.6, and 15 μg of mitochondrial proteins solubilized with 0.15% (wt/vol) *n*-octyl β-D-glucopyranoside in 50 m*M* Tris-HCl, pH 7.6. Given the hyperbolic profile of the initial velocity as a function of substrate α-ketoglutarate concentration, the KGDC-catalyzed reaction was considered to follow Michaelis–Menten kinetics. Velocity was determined from the linear portion of the time curve and plotted according to Lineweaver–Burk double-reciprocal representation. Kinetic characteristics (K_m, K_m/V_{max}) were obtained graphically from computer-generated least-squares fit.

RT-PCR

Total RNA was extracted from myocardium using the SV Total RNA Isolation System (Promega Corp.). The first DNA strand was synthesized using a RETROscript™ kit (Ambion Inc., Austin, TX, U.S.A.). Target DNA was amplified by PCR using the synthetic oligonucleotide primers (forward 5′GTTCCATCACCAGCAAATGGCATC, reverse 5′TTCTGAGCGCAGGCCTTTAGCAG) that amplify a 324-bp fragment of dihydrolipoamide succinyltransferase (GenBank D90401) (23). The amplified fragment was purified by agarose electrophoresis and sequenced using an ABI 377 Prism DNA sequencer. Automated DNA sequencing confirmed that the PCR product was identical to the intended template. Relative quantitative PCR was carried out using a QuantumRNA™ 18S Internal Standards kit (Ambion Inc.) and 3 μCi as [α-^{32}P]dCTP (Amersham Pharmacia Biotech Inc.) per reaction. Quantification was performed by the scanning densitometry of the autoradiographs.

Statistical analysis

Data were analyzed by unpaired two-tailed Student's *t*-test or single-factor ANOVA followed by Student–Newman–Keuls multiple comparison test using Statview package (SAS Institute Inc., Cary, NC, U.S.A.). Proportions were arc sine transformed prior to analysis. All statistical tests were performed to the 95% level of confidence.

RESULTS

In vitro modification of KGDC by HNE

To determine the extent that HNE modifies KGDC, porcine heart KGDC was incubated with increasing concentrations of HNE, separated by reducing SDS-PAGE, and stained with Coomassie Blue R-250 or immunoblotted with antibodies against KGDC and HNE-protein. In the absence of added HNE, protein separation and Coomassie Blue staining revealed five major polypeptides, identified using a nano-LC system coupled to ESI-MS/MS as the three enzyme components of KGDC: α-ketoglutarate dehydrogenase (E1k), dihydrolipoamide succinyltransferase (E2k), and dihydrolipoamide dehydrogenase (E3). In addition to the KGDC components, BSA and mitochondrial creatine kinase (mtCK) were also identified as con-

taminants of the commercial enzyme preparation (Table 1 and Fig. 1A). Incubating KGDC with increasing concentrations of HNE (0–2 m*M*) caused a noticeable shift in the electrophoretic mobility of both E1k and E2k polypeptides (Fig. 1A and B) when subjected to SDS-PAGE and immunoblotting. The mobility of E1k was slowed down, implying the dose-dependent

TABLE 1. IDENTIFICATION OF PROTEINS CONTAINED IN PORCINE HEART KGDC PREPARATION USING AMINO ACID SEQUENCE TAGS OBTAINED FROM NANO-LC ESI-MS/MS ANALYSES*

Identified protein	*Matching species*	*NCBI number*	*Molecular mass (Da)†*	*Sequence coverage (%)*	*Peptide sequence*	*Position*
α-Ketoglutarate dehydrogenase (E1k component of KGDC)	Human	2160381	113,404	31	SWDIFFR	75–81
					LVEDHLAVQSLIR	123–135
					GHHVAQLDPLGILDADLDSSVPADIISSTDK	141–171
					LGFYGLDESDLDK	172–184
					VFHLPTTTFIGGQESALPLR	185–204
					FEEFLQR	264–270
					KWSSEK	271–276
					LNVLANVIR	315–323
					LEAADEGSGDVK	337–348
					SSPYPTDVAR	457–466
					QKPVLQK	533–539
					YAELLVSQGVVNQPEYEEEISKYDK	540–564
					ICEEAFAR	565–572
					TVDWALAEYMAFGSLLK	650–666
					EGIHIR	667–672
					LSGQDVER	673–680
					GTFSHR	681–686
					HHVLHDQNVDK	687–697
					QILLPFR	845–851
					KPLIIFTPK	852–860
					SFDEMLPGTHFQR	871–883
					VIPEDGPAAQNPENVK	884–899
					IEQLSPFPFDLLLKEVQK	930–947
					AKPVWYAGR	980–988
					NPAAAPATGNKK	989–1,000
Dihydrolipoamide dehydrogenase (E3 component of KGDC)	Porcine	118675	54,151	10	GIEMSEVR	110–117
					ADGSTEVINTK	167–177
					SEEQLKEEGIEYK	405–417
					TNADTGMVK	431–440
					EANLASFGK	496–505
Dihydrolipoamide succinyltransferase (E2k component of KGDC)	Porcine	18203301	48,946	26	DDVITVK	69–75
					TPAFAESVTEGDVR	76–89
					VEGGTPLFTLR	135–145
					AKPAEAPAAAAPK	154–166
					LGFMSAFVKAS	280–288
					AFALQEQPVVNAVIDDTTK	289–309
					GLVVPVIR	328–335
					NVETMNYADIER	336–347
					TISELGEK	348–355
Mitochondrial creatine kinase	Mouse	20908987	47,443	21	LFPPSADYPDLR	47–58
					VMKHPTDLDASK	138–149
					YVLSSR	159–164
					LSEMTEQDQQR	210–220
					DWPDAR	244–249
					GIWHNYDK	250–257
					VISMEK	271–276
					RVFER	282–286
					GLKEVER	290–296
					GWEFMWNER	302–310
					ILENLR	345–350

*MS/MS ion search was performed using the following parameters: peptide tolerance ± 1 Da, MS/MS tolerance ± 0.5 Da, monoisotopic mass, one missed cleavage.

†Value from the NCBI protein database.

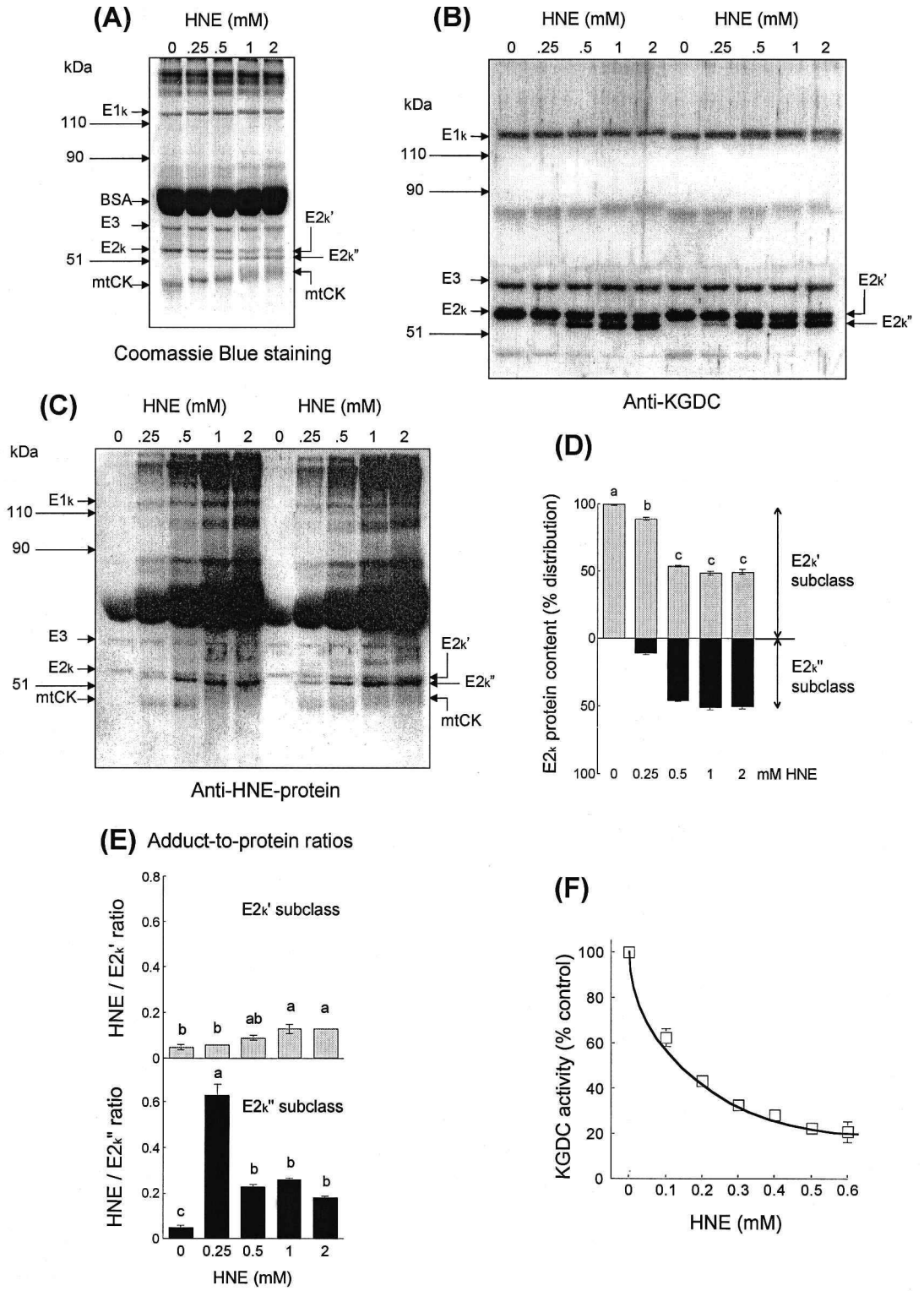

FIG. 1. ***In vitro*** **modification of KGDC by HNE.** Polypeptide constituents of commercial KGDC (containing BSA and mtCK) were incubated with HNE (0–2 m*M*) for 2 h at 37°C, and separated by SDS-PAGE, then stained with Coomassie Blue (**A**) or transferred onto nitrocellulose membranes and immunoblotted with antibodies to KGDC (**B**) or HNE-protein (**C**). Coomassie Blue staining showed that the electrophoretic mobility of the E2k component of KGDC (dihydrolipoamide succinyltransferase) and mtCK was markedly altered. E2k formed a doublet, labeled E2k' and E2k", where E2k" showed greater relative mobility. (**D**) Distribution of E2k into E2k' and E2k" subclasses upon treatment with HNE (means ± SD for two experiments). (**E**) Extent of HNE adduction of KGDC among E2k' and E2k" polypeptides (means ± SD for two experiments). Values not sharing a common letter are significantly different ($p \leq 0.05$). (**F**) HNE caused a loss of KGDC activity (IC_{50} = 0.138 m*M*). The purified enzyme (5 mU) was incubated with 0–0.6 m*M* HNE at 30°C for 30 min in 50 m*M* Tris-HCl, pH 7.6 (means ± SD for three experiments).

adduction of the protein by HNE (Fig. 1C). Interestingly, the electrophoretic mobility of the E3 component did not change appreciably, indicating no significant HNE binding irrespective of the concentration of HNE used (Fig. 1C). The addition of HNE also altered the electrophoretic mobilities of BSA and mtCK, the other identified polypeptides in the commercial KGDC preparation. MtCK mobility was markedly retarded by HNE, whereas the mobility of BSA was only slightly affected (Fig. 1A).

E2k was the enzyme most affected by HNE *in vitro*. E2k exhibited a pattern that suggests its segregation into two subclasses of polypeptides, which were subsequently designated E2k' and E2k" (Fig. 1A–C). E2k" showed a greater electrophoretic mobility caused perhaps by a partial refolding that could result from intramolecular HNE cross-links (36). E2k" levels increased with added HNE up to 0.5 m*M*. At higher HNE concentrations, the proportion of E2k' to E2k" remained unchanged and evenly distributed (Fig. 1D). E2k" was adducted by HNE to a greater extent than E2k' (Fig. 1C,E). The degree of HNE addition to E2k' increased gradually up to 1 m*M* added HNE, which then plateaued (Fig. 1E). In contrast, the extent of HNE modification of E2k" was the greatest at 0.25 m*M* HNE, and declined by 60% at higher HNE concentrations (Fig. 1E). This apparent biphasic kinetics could be due to the refolding of E2k", thus preventing its complete modification by HNE, and the lack of detectable reactivity of HNE-KLH antibodies with HNE cross-links. Thus, by mass action, the anti-HNE-protein signal does not increase proportionally to the anti-E2k" signal, thereby leading to a lower HNE/E2k" ratio.

Incubating KGDC with increasing concentrations of HNE (0–0.6 m*M*) resulted in a dose-dependent loss of activity (Fig. 1F). HNE-mediated inhibition of KGDC was not affected by the buffer (Tris versus sodium phosphate). IC_{50} was 0.138 m*M* with either buffer (data not shown). Thus, interference due to the binding of HNE to Tris is excluded under the present conditions.

These results indicate that HNE, at submillimolar concentrations, reacts with a variety of proteins *in vitro* by affecting their electrophoretic mobility as a result of addition and cross-link formation. Among the polypeptides identified, dihydrolipoamide succinyltransferase (the E2k component of KGDC) is particularly prone to HNE modification.

Detection of HNE adduct formation in heart mitochondrial proteins by western blotting

Because the aging process results in marked increases in lipid-derived aldehydes and oxidatively modified proteins, we hypothesized that KGDC might be increasingly modified with age. Moreover, given the propensity of HNE to bind to E2k *in vitro,* the enzyme component that bears lipoyllysine residues, we sought to determine whether dietary LA could modulate HNE adduction via thiol exchange.

Mitochondrial proteins isolated from the hearts of young and old rats were separated by SDS-PAGE under reducing conditions, transferred onto nitrocellulose membranes, and subjected to western blot analysis using antibodies to HNE-protein. Then the membranes were stripped of bound antibodies and reprobed with KGDC antiserum. Overall, HNE modification of mitochondrial proteins in the 25–120 kDa range was significantly ($p \leq 0.01$, $n = 5$) greater (by 35%) in old animals, as judged by the density of the bands (Fig. 2A). Among the polypeptides modified by HNE in old rat hearts, E2k (dihydrolipoamide succinyltransferase), identified based on its molecular mass (~54 kDa), reacted markedly with HNE-protein antibodies (Fig. 2A). The detection of HNE-E2k adduct was abolished by the incubation of HNE-KLH antibodies with 5 mg of HNE-BSA prior to western blotting (Fig. 2B). Density ratios of HNE-E2k and E2k bands (adduct-to-protein ratio) showed a significant ($p \leq 0.01$) onefold increase in the heart mitochondria of old animals fed the control diet when compared with their young counterparts (Fig. 2C). Thus, heart aging is associated with an increase in HNE modification of mitochondrial proteins, in general, and E2k, in particular. But unlike the *in vitro* situation, there is no evidence of a shift in the electrophoretic mobility of adducted proteins.

HNE levels *in vivo* are kept at low steady-state concentrations by enzymatic conjugation to GSH and subsequent removal from the cell (3, 7). Given that tissue GSH levels decline with age, therapeutic interventions to maintain GSH levels may lessen the extent of HNE-protein modification (23, 29, 37). Feeding LA, a natural compound with low toxicity (LD_{50} ~1,000 mg/kg body weight in rats) to old rats, restores GSH levels and, thus, may indirectly act to limit HNE formation (8). Also, as a thiol-containing molecule, dihydrolipoate (reduced LA) may directly conjugate lipid peroxidation products, thereby preventing reactive aldehydes from adducting proteins. However, under the conditions of the present study, feeding LA for 2 or 6 weeks did not significantly lower the age-related increase in HNE-E2k adduct formation in rat hearts (Fig. 2C).

KGDC activity, content, mRNA, and kinetic characteristics in F344 rat heart

Owing to the age-associated increase in HNE modification of E2k, we surmised that KGDC activity could be adversely affected, as previously shown *in vitro,* and thus compromise the production of NADH for the electron transport chain and ATP synthesis. To determine whether increased HNE adduction affected KGDC function, KGDC activity in mitochondrial extracts from young and old rat hearts was monitored. Contrary to an anticipated decline in enzyme activity, we observed a significant ($p \leq 0.01$) onefold increase in KGDC activity in old compared with young rats (Fig. 3A), suggesting there is no correlation between HNE binding and KGDC activity in the heart.

Because the lipoamide moiety may be a target of HNE adduction (34), we determined whether dietary LA supplementation for 2 weeks could affect KGDC activity. However, LA supplementation did not significantly affect heart KGDC activity within each age group (Fig. 3A), implying that the myocardial LA status of old F344 rats is adequate for bioenergetics.

To assess whether the enhanced KGDC activity observed in old rat hearts was linked to increased KGDC protein content or gene expression, we quantified the amounts of KGDC enzyme components by western blot and the mRNA levels of E2k by RT-PCR. E2k was chosen to represent the overall KGDC because E2k is specific to KGDC, particularly susceptible to ALE adduct formation (Figs. 1C and 2), and the most abundant component of the holoenzyme. As shown in Fig. 3B,

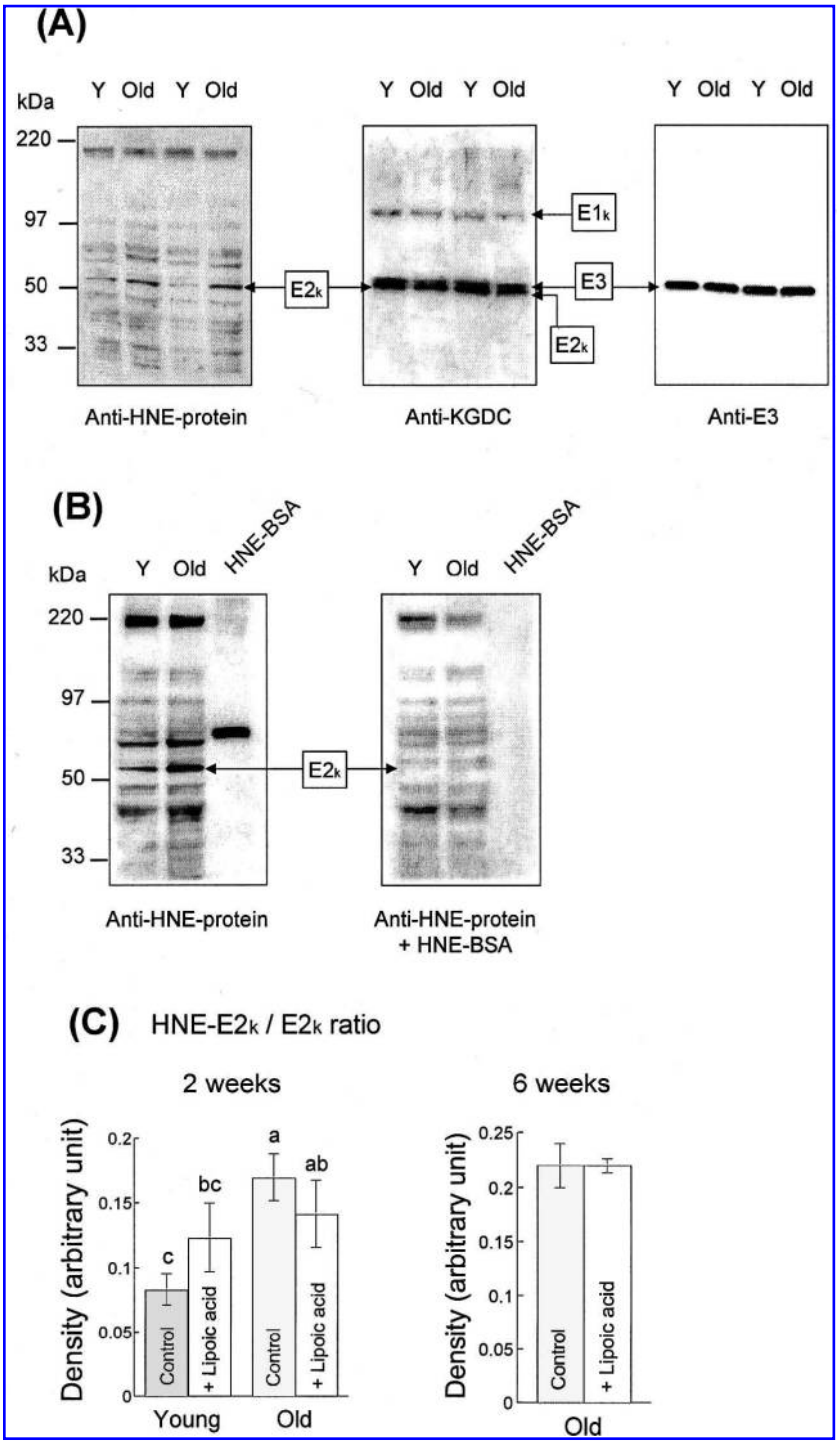

FIG. 2. HNE adduction of E2k increases markedly in the aging myocardium. (**A**) Immunoblot showing the extent of adduction to mitochondrial proteins isolated from young and old rat hearts. A ~54-kDa band identified as E2k (dihydrolipoamide succinyltransferase) is most markedly adducted by HNE in the aging myocardium. Proteins were separated by 7.5% SDS-PAGE under reducing conditions and transferred onto nitrocellulose membrane. The membrane was incubated successively with antibodies to HNE-protein, bovine KGDC, and porcine E3, and stripped of bound antibodies between incubations. (**B**) Immunoblot of mitochondrial proteins blotted with antibodies to HNE-protein in the presence or absence of competing antigen (HNE-BSA, 5 mg). Note the age-related increase in HNE-E2k modification is abolished by the presence of competing antigen. The blot is representative of three experiments. (**C**) Quantification of the level of modification of E2k by HNE in the hearts of young and old rats fed the LA supplement or the control diet for 2 or 6 weeks. Immunoblotting was performed as described above. The membrane was incubated with antibodies to HNE-protein, then stripped and incubated with KGDC antiserum to reveal E2k. Data are shown as means ± SD for five to seven animals for the 2-week trial and three animals for the 6-week trial. Values not sharing a common letter are significantly different ($p \leq 0.01$).

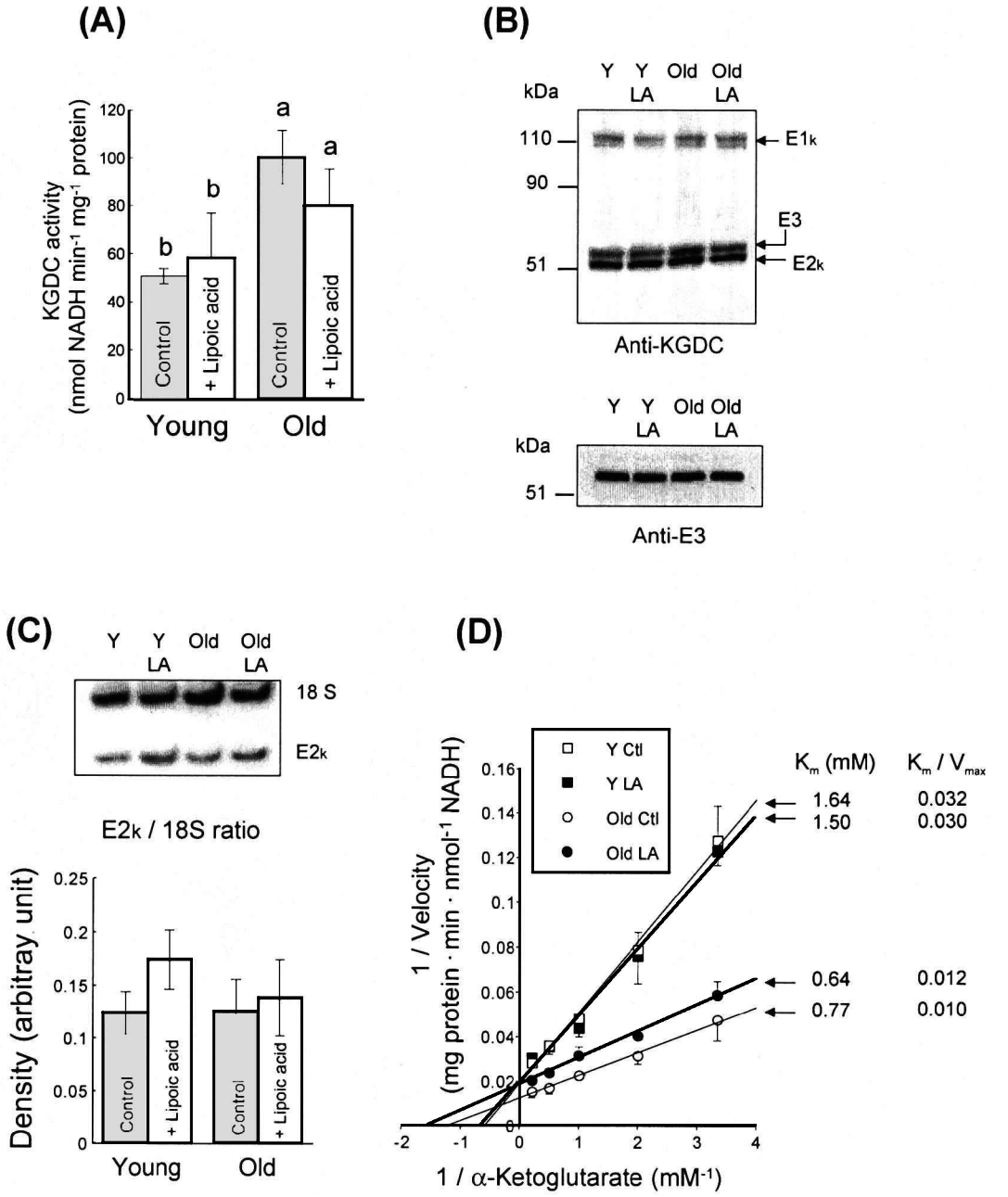

FIG. 3. Status of KDGC in the hearts of young and old rats fed or not fed the LA supplement for 2 weeks. Rat heart KGDC activity (**A**), protein content (**B**), mRNA levels of E2k (**C**), and kinetic characteristics of KGDC (**D**) were determined as described in Materials and Methods. Irrespective of dietary treatment, old rats exhibit higher KGDC activity without any increase in protein or mRNA levels. Old rat heart KGDC shows a greater affinity (lower K_m) for α-ketoglutarate. Data are shown as means ± SD of five to seven animals for KGDC activity and content, three animals for E2k mRNA levels, and four animals for the kinetics study. Values not sharing a common letter are significantly different ($p \leq 0.01$).

KGDC enzyme content did not change with age or diet, nor was there an effect of age or diet on E2k mRNA levels (Fig. 3C).

In the absence of any change in protein or message content, the rise of KGDC activity seen in old rat hearts may be explained by alteration to its kinetic characteristics, particularly catalytic efficiency. Analysis of KGDC kinetics revealed a change in the K_m for substrate α-ketoglutarate among age groups (Fig. 3D). Regardless of dietary treatment, the K_m was markedly lower (1.1–1.3-fold) in old compared with young rat hearts, indicating a greater affinity for the substrate and suggesting faster catalysis per enzyme complex. The slope (K_m/V_{max}) of the double-reciprocal plot was also affected by the age of the animal, but not by LA supplementation. In contrast with that of young rats, heart KGDC of old rats maintained its velocity near V_{max} at low substrate concentrations.

Taken together, the present study provides no evidence of deleterious functional consequences associated with the binding of HNE to KGDC in the aging rat heart, contrary to antic-

ipated results based on *in vitro* experiments. It rather suggests that the extent of HNE modification *in vivo* remains below the threshold where KGDC becomes inactivated.

DISCUSSION

HNE is one of the most reactive and abundant aldehydes produced during lipid peroxidation (6). Its broad range of reactivity with proteins is well documented *in vitro*. In general terms, HNE forms stable adducts with certain protein residues referred to as ALEs. In the present study, although HNE reacted with the major proteins of the porcine heart KGDC preparation (E1k, E2k, and E3 components, and also BSA and mtCK), E2k was most markedly modified. Upon HNE treatment, E2k separated into two distinct polypeptide classes that could be fully resolved by SDS-PAGE under reducing conditions. This phenomenon, also reported for glyceraldehyde-3-phosphate dehydrogenase (36), may be explained by the formation of intramolecular cross-links that give the polypeptide a more globular shape, allowing greater mobility on SDS-PAGE. HNE is known to form Michael addition-type adducts via conjugation with cysteine, lysine, and histidine residues. Also, the carbonyl group of HNE creates Schiff base derivatives with amino groups of proteins. In the case of KGDC, HNE also covalently binds to the lipoyllysyl moiety of E2k producing a thioether adduct (12). Cross-link formation within a polypeptide is a severe modification and leads to marked loss of function *in vitro*. These *in vitro* observations were the basis for evaluating the level and impact of HNE-associated mitochondrial protein modification in the aging rat heart.

Increased rates of reactive oxygen species have been associated with the progression of numerous degenerative diseases and also the aging process (21, 30, 31). There is good evidence that the aging heart is under heightened oxidative stress (33). This elevated oxidative stress may be due, in part, to increased lipid peroxidation and subsequent formation of stable unsaturated aldehydes that, in turn, react with proteins. Traditionally, oxidative protein modification has been observed in long-lived proteins, such as collagen and elastin, as opposed to proteins whose half-lives are measured in minutes or days. However, HNE may form at higher rates in organelles such as mitochondria where a high oxygen flux constantly occurs. It has been proposed that large quantities of lipid-derived aldehydes may be present locally in the cellular compartments leading to HNE concentrations in the high micromolar range (14). Thus, even relatively short-lived proteins, such as KGDC, may exhibit higher steady-state HNE adduction. Whether the rate of formation of lipid-derived adducts increases or whether their degradation slows with age remains to be tested.

Herein we report a significant ($p \leq 0.01$) 35% increase in the overall HNE modification of heart mitochondrial proteins in old rats compared with their young counterparts. This finding supports the earlier view that lipid peroxidation contributes to the heightened pro-oxidant state seen in the aging heart. We further show that dihydrolipoamide succinyltransferase (the E2k component of KGDC) was most markedly modified by HNE, corroborating previous *in vitro* observations of E2k susceptibility to HNE adduction. However, despite this heightened adduction, the activity of the KGDC was not decreased in the heart. On the contrary, old rats exhibited higher ($p \leq 0.01$) cardiac KGDC activity than young rats. These results suggest that possible detrimental effects of HNE on NADH steady-state concentrations are either negligible *in vivo* or offset by adaptive mechanisms that may involve substrate flux through the citric acid cycle. To determine whether gene and protein expression was associated with the rise in KGDC activity in old rat hearts, we measured mRNA levels of E2k and the content of KGDC constituents. These analyses indicate that neither message levels nor protein content could account for the change in activity, and suggest rather an increase of KGDC catalytic efficiency in the aging myocardium. This view is indeed supported by the onefold difference in K_m for α-ketoglutarate and twofold change in K_m/V_{max} between young and old rat heart KGDC. A lower K_m and K_m/V_{max}, such as the one seen in old rat hearts, would enhance the catalytic rate especially at low substrate concentrations.

The nature of the K_m alteration affecting KGDC in old rat myocardium is not clear at this point. Located in the mitochondrial matrix, KGDC is a key regulatory enzyme complex of the citric acid cycle and catalyzes the irreversible oxidative decarboxylation of α-ketoglutarate, yielding succinyl-CoA and NADH. The production of NADH is tightly associated with KGDC activity; thus, any functional decrement would have profound consequences on cellular bioenergetics. KGDC activity is modulated by various factors, including allosteric regulation by NAD^+/NADH and succinyl-CoA, and activation by Ca^{2+} and Mg^{2+} (25). Although aging may affect the steady-state levels of these organic and inorganic regulators, their effects on KGDC activity will not persist under the *in vitro* conditions used to assay KGDC. In the present study, the solubilization and dilution of crude mitochondria prior to assay would preclude any allosteric effects attributed to the loose binding of NAD^+, NADH, or succinyl-CoA. A selective activation by Ca^{2+} or Mg^{2+} can also be dismissed because the assay includes both of these salts in excess. To persist *in vitro,* the age-associated alteration to KGDC activity must result from a permanent conformational change of the enzyme complex itself, such as that induced by the alteration of the complex composition or, alternatively, the covalent binding of an activator. Crucial to KGDC activity is the concerted action of its three catalytic components (E1k, E2k, and E3), each present as multiple copies per complex. The absolute number of these components has not been determined for rat KGDC, but mammalian and *E. coli* KGDC alike are composed of six E1k and E3 dimers attached to the surfaces of a cubic (24-meric) E2k core (18). Thus far, there is no evidence to suggest that aging affects complex composition, nor can it be presently ascertained that modification of this composition, by addition or subtraction of enzymic components, would stimulate activity. Moreover, increased HNE binding to KGDC is certainly discernible in hearts from old rats, but should not be considered as activating factor for KGDC. To the contrary, all the reports dealing with HNE and isolated mitochondria or purified KGDC unambiguously show that HNE inhibits KGDC activity. Thus, we interpret that HNE adduction to KGDC is too low to adversely affect enzyme activity.

Deleterious consequences of HNE inactivation may be avoided by mechanisms that include the removal of HNE. There are three main HNE-metabolizing enzymes in the heart: glutathione *S*-transferase, aldehyde dehydrogenase, and aldose reductase (6, 32). Further study is required to evaluate the role played by these enzymes during myocardial aging. It should be

pointed out that, although mitochondria may display higher steady-state levels of HNE than the cytosol, the levels of HNE present in mitochondria are not well characterized. Also, more relevant than its overall steady state, HNE flux to the mitochondria should be considered given that HNE is quite stable and can traverse biological membranes. The general view is that cellular HNE concentration is ~1 μ*M* and can increase to 10 μ*M* under pathophysiological conditions (6). However, these concentrations are manyfold lower than those used to inhibit mitochondrial enzymes *in vitro* (50–100 μ*M*), suggesting that the extent of HNE-mediated protein inactivation may be quite small *in vivo*. Thus, the increased modification of KGDC seen in the aging rat heart should be viewed as a marker of aging rather than an indicator of mitochondrial dysfunction.

Supplementing the diet of old rats with LA significantly improves many indices of age-associated oxidative stress. LA supplementation lowers both steady-state levels of malondialdehyde (a marker of lipid peroxidation) and 8-oxodeoxyguanosine while reversing the age-related decline in GSH and ascorbate content (9, 16). In addition to improving general antioxidant status, LA enhances glucose uptake (2). Basal concentrations of unbound LA are very low *in vivo* (13, 28). However, following dietary supplementation, there is a brief increase in unbound LA inside all cells of the body, especially the heart, brain, and liver. LA is rapidly absorbed from the diet, transported to tissues, and taken up by cells (26), where it is reduced enzymatically to dihydrolipoate in mitochondria (10, 27). LA is also a coenzyme of mitochondrial α-ketoacid dehydrogenases, such as KGDC, and its supplementation in the diet of old animals may be beneficial to mitochondria-supported bioenergetics. Although the body can synthesize LA, there may be a greater need for LA during aging due to insufficient synthesis and/or increased utilization. Overall, dietary supplementation with LA (2 g/kg of diet) for 2 weeks had a modest effect on KGDC activity, implying that endogenous synthesis of LA is adequate for the function of cardiac KGDC. Feeding LA for up to 6 weeks did not lower the degree of HNE adduction to E2k. The transient postprandial accumulation of unbound LA may account for this observation.

In summary, there is an age-dependent increased adduction of mitochondrial proteins by HNE, including KGDC, a key enzyme of the citric acid cycle. However, the extent of HNE binding remains low and, thus, does not have deleterious functional consequences to KGDC. Thus, HNE-KGDC adduct formation should be viewed as a marker of aging rather than an indicator of mitochondrial dysfunction. The myocardium of old F344 rats may increase the catalytic efficiency of KGDC via K_m adjustment to maintain adequate levels of NADH for the electron transport chain.

ACKNOWLEDGMENTS

This work was supported by National Institute on Aging grant RIAG17141A.

ABBREVIATIONS

ALE, advanced lipoxidation end product; BSA, bovine serum albumin; ESI-MS/MS, electrospray ionization tandem mass spectrometry; GSH, reduced glutathione; HNE, 4-hydroxynonenal; KGDC, α-ketoglutarate dehydrogenase complex; KLH, keyhole limpet hemocyanin; LA, *R*-α-lipoic acid; LC, liquid chromatography; mtCK, mitochondrial creatine kinase; NCBI, National Center for Biotechnology Information; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TPP, thiamine pyrophosphate.

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Received for publication April 22, 2003; accepted July 14, 2003.

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