Metabolism of the Lipid Peroxidation Product, 4-Hydroxy-trans-2-nonenal, in Isolated Perfused Rat Heart*

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The metabolism of 4-hydroxy-trans-2-nonenal (HNE), an a,b-unsaturated aldehyde generated during lipid peroxidation, was studied in isolated perfused rat hearts. High performance liquid chromatography separation of radioactive metabolites recovered from [3H]HNE-treated hearts revealed four major peaks. Based on the retention times of synthesized standards, peak I, which accounted for 20% radioactivity administered to the heart, was identified to be due to glutathione conjugates of HNE. Peaks II and III, containing 2 and 37% radioactivity, respectively. Peak IV was due to unmetabolized HNE. The electrospray ionization mass spectrum of peak I revealed two prominent metabolites with m/z values corresponding to [M + H]⁺ of HNE and DHN conjugates with glutathione. The presence of 4-hydroxy-2-nonenal in peak III was substantiated using gas chromatography-chemical ionization mass spectroscopy. When exposed to sorbinil, an inhibitor of aldose reductase, no GS-DHN was recovered in the coronary effluent, and treatment with cyanamide, an inhibitor of aldehyde dehydrogenase, attenuated 4-hydroxy-2-nonenal-acid formation. These results show that the major metabolic transformations of HNE in rat heart involve conjugation with glutathione and oxidation to 4-hydroxy-2-nonenal acid. Further metabolism of the GS-HNE conjugate involves aldose reductase-mediated reduction, a reaction catalyzed in vitro by homogenous cardiac aldose reductase.

Incomplete detoxification of free radicals has been suggested to incite tissue injury and dysfunction associated with several disease states, drug toxicities and the degenerative processes of senescence (1). Recent evidence suggests that in addition to their well recognized cytotoxic effects, free radicals also participate in cell signaling and growth, and mediate apoptosis or programmed cell death (2–4). However, the mechanisms by which free radicals affect cellular metabolism and function are poorly understood. In general, free radicals are highly reactive and short lived. Their high reactivity prevents long range diffusion, which paradoxically limits their toxic potential (1). The biological effects of free radicals are, however, extensive, per-haps in part because they engender other reactive and stable oxidants. Several metastable products of radical reactions have been identified that can cause spatial and temporal extension of the initially local radical events. Of particular significance are a,b-unsaturated aldehydes, which are produced during b-scission of alkoxyl radicals derived from a-6 polyunsaturated fatty acids (5). These aldehydes are highly reactive and generated in high concentrations by peroxidative reactions of lipids and lipoproteins, and it has been suggested that they mediate and amplify the cellular effects of their radical precursors (5).

In addition to lipid peroxidation, high concentrations of a,b-unsaturated aldehydes are also generated by other biochemical processes, for instance, myeloperoxidase-catalyzed oxidation of amino acids (6), and oxidative modification of nucleosides (7). Furthermore, these aldehydes are present in many food substances and industrial and environmental pollutants (8), or they are produced in vivo during metabolism of several drugs, e.g. cyclophosphamide (9) and felbamate (10), and toxins such as benzene (11) and could thus represent a significant component of several acute or chronic toxicological states.

Accumulation of unsaturated aldehydes or their products has been observed under several pathological conditions, e.g. proteins modified by these aldehydes have been immunologically localized to atherosclerotic plaques of humans and animals (5, 12), and to lesions and neurons of patients with Alzheimer’s (13) and Parkinson diseases (14). In addition, these aldehydes may also be precursors or constituents of the age pigment lipofuscin (15, 16). Furthermore, high concentrations of aldehyde-derived DNA adducts have been found not only in animals exposed to carcinogens, but also in humans and animals even without carcinogen treatment (17), suggesting that these adducts may represent common background DNA lesions related to spontaneous carcinogenesis and aging.

Metabolic and physiological alterations and structural modifications due to a,b-unsaturated aldehydes may be an important component of tissue injury and dysfunction due to several cardiovascular pathological states associated with increased formation of reactive oxygen species, e.g. atherosclerosis, ischemia-reperfusion, or toxicity of anticancer drugs. While high concentrations of a,b-unsaturated aldehydes or their adducts have been detected under these states (5, 12, 18, 19), a causal relationship between aldehyde toxicity and oxidative stress remains to be established. However, to assess their contribution to specific pathological states and redox signaling, it is essential to identify the metabolism of these aldehydes. Nevertheless, the biochemical mechanisms for the metabolism of a,b-unsaturated aldehydes are not well understood (5).

In this communication we report on the metabolism of the
HNE-modified proteins have been detected in atherosclerotic cysteine, histidine and lysine residues of proteins, and displays dation. It combines spontaneously with glutathione, and with mal peroxidation (20). During oxidation of arachidonic acid for dride reduction of HNE. To a solution of HNE in methanol, 4 molar synthesis of GS-DHN, 0.15 GS-HNE was calculated on the basis of amino acid analysis. For the the decrease in absorbance at 340 nm. In 30 min times with dichloromethane and purified on HPLC as described below.

Experimental Procedures

Bovine hearts were obtained from the local abattoir, rat hearts were isolated from male Sprague-Dawley rats weighing 200–260 g. Human hearts were obtained within 6–8 h postmortem and kept frozen until use. NADPH, NAD, NADH, and aldehyde dehydrogenase were purchased from Sigma. Sorbinil (CP-45643; (+)-4S)-6-fluoropirrolo[4,4-3'H]-imidazolidine-2,5'-dione and tolrestat (Ay-27773; N-[6-methoxy-5-(trifluoromethyl)-1-naphthalenylthioxomethyl-N-thiomethyl-glycine) were gifts from Pfizer and Ayrest, respectively. All the other reagents were of the highest purity available.

Chemical Syntheses—HNE was synthesized as its dimethylacetal starting from dimethylacetal of fumaraldehyde as described previously (23). For the synthesis of [4-3H]HNE the dimethylacetal of HNE was oxidized to the 4-keto derivative using polymer-supported chromic acid as an oxidizing agent. The ketone was reduced to the dimethylacetal of HNE by using tritiated NaBH₄ and [4-3H]HNE obtained by acid hydrolysis was purified on HPLC. The [4-3H]HNE thus synthesized had a specific activity of 175 mCi/mmol.

1,4-Di-hydroxy-2-nonene (DHN) was synthesized by sodium borohydride reduction of HNE. To a solution of HNE in methanol, 4 molar excess of NaBH₄ was added, and the mixture was stirred for 60 min at room temperature. 0.1 m HCl was added dropwise to the reaction mixture to bring the pH to 2.0. The mixture was left in acidic conditions for 30 min (to decompose unreacted NaBH₄) and was extracted three times with dichloromethane and purified on HPLC as described below. Radiolabeled DHN was synthesized enzymatically by incubating 60 nmol of [4-3H]HNE with 300 milliliters of human placenta recombiant aldose reductase (AR) and 0.1 mCi NADPH in 0.05 m potassium phosphate, pH 6.0, containing 0.4 m Li₂SO₄. The reaction was monitored by following the decrease in absorbance at 224 nm. The enzyme was removed by ultrafiltration using an Amicon Centriprep-10, and DHN in the filtrate was purified on HPLC as described above. The identity and purity of DHN was established by NMR.

4-Hydroxy-trans-2-nonenic acid (HNA) was synthesized by incubating 100 nmol of HNE ([1H]HNE) with 1.0 unit of alddehyde dehydrogenase, and 1.5 mCi NAD in 0.1 m potassium phosphate, pH 7.4, at 25 °C. The reaction was monitored by following the increase in absorbance at 340 nm. The enzyme was removed by ultrafiltration and HNA in the filtrate was purified by HPLC.

The conjugate of GSH and HNE was synthesized by incubating 0.5 µmol of HNE ([1H]HNE) with 3-fold excess of GSH and 0.1 m potassium phosphate, pH 7.0, at 37 °C. The reaction was followed by monitoring decrease in absorbance at 224 nm. In 30 min approximately 90% of HNE was conjugated. After purification on HPLC, the conjugate was characterized by electrospray-ionization mass spectroscopy giving the pseudo molecular ion [M + H]⁺ at m/z of 464. The concentration of GS-HNE was calculated on the basis of amino acid analysis. For the synthesis of GS-DHN, 0.15 µmol of GS-HNE was incubated with 250 millimolar GSH, 0.1 m NADPH and 1.0 m NADP, 0.1 m potassium phosphate, pH 7.0, at 37 °C. The reaction was followed by monitoring the decrease in absorbance at 340 nm. In 30 min >85% of the conjugate was reduced. The reaction mixture was ultrafiltered and the filtrate was subjected to HPLC as described below.

HPLC Analysis—Synthesized standards and metabolites of HNE were separated by HPLC using a Rainin ODS C₈ column equilibrated with 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. The compounds were eluted using a gradient consisting of solvent A (0.1% aqueous trifluoroacetic acid) and solvent B (0.1% trifluoroacetic acid in 60% acetonitrile) at a flow rate of 1 ml/min. The gradient was established such that B reached 16.6% in 20 min, 41.5% in 35 min and held at 41.5% for 30 min. In an additional 10 min B reached 100%, and was held at this value for 20 min.

Measurement of HNE Metabolites—For measurement of HNE metabolites, isolated perfused rat heart preparations were used as described before (24). Briefly, Sprague-Dawley rats were anesthetized by an intramuscular injection of sodium pentobarbital (60 mg/kg of body weight). Following anesthesia, the thorax was opened and the hearts were removed. Retrograde perfusion was started via a cannula inserted into the aorta. A modified Krebs-Henseleit (KH) solution was equili- briated with a gas mixture of 95% O₂ + 5% CO₂, maintained at 37 °C, and perfused at a constant pressure of 100 cm water. The composition of the modified Krebs-Henseleit solution was (in mM): NaCl, 118; KCl, 4.7; MgCl₂, 1.25; CaCl₂, 3.0; KH₂PO₄, 1.25; EDTA, 0.5; NaHCO₃, 25, and glucose, 10; pH 7.4. In control experiments, the left ventricular pressure was measured at 5 mm Hg, using an intraventricular balloon. At the end of the experiment, the coronary perfusate was collected every 30 s for 3 min, after which perfusion was stopped, and the heart was removed, dissected into ventricles and aorta plus atria, and homogenized separately using a Polytron homogenizer in KH buffer diluted with an equal volume of 96% acetonitrile and 4% acetic acid at 4 °C. The final volume of the homogenate was 2 ml. The homo- genate was centrifuged at 10,000 x g for 30 min. The supernatant was extracted with dichloromethane, which was evaporated by bubbling nitrogen. The extract was reconstituted in a small amount of KH buffer.

EI Mass Spectrometry—EI-MS analyses were performed on a Finnigan MAT TSQ70 quadrupole instrument upgraded with TSQ 700 source (EI) and a 20-kV conversion dynode electron multiplier. The Vestec ES Source (PerSeptive Biosystems, Vestec Products, Cambridge, MA) was modified as described previously by Emmett and Caprioli (25). EI-MS operating parameters were as follows: needle voltage, 3.4 kV; nozzle voltage, 270 V; repeller voltage, 4 V, and source block temperature, 227 °C. To enhance source-induced fragmentation the repeller voltage was raised to 10 V, and bath nitrogen was introduced into the source at 25 cm/min. The spectra were acquired at a rate of 0.82 µl/min. Spectra were acquired at the rate of 275 atomic mass units/s over the mass range of 100–650 atomic mass units.

Analysis of DHN and HNA by GC/CIMS—All samples were derivatized with 10 µl of diethyl ether and 50 µl of N-methyl-N-(t-butyldimethylsilyl)-trifluoroacetamide for 40 min at room temperature. An aliquot (1 µl) of the mixture was utilized for GC/CIMS analysis. GC/CIMS analysis was performed using a Finnigan Incon 50 GS/MS system (Finnigan-MAT, San Jose, CA) fitted with a chemical ionization source held at a temperature of 150 °C using ammonia as the reagent gas. Gas chromatographic separation was achieved using a DB-5 fused silica capillary column (30 m x 0.25 mm inside diameter, 0.25-µm film thickness, J & W Scientific, Folsom, CA), which was held at 115 °C for 5 min, programmed at a 4 °C/min rate at 165 °C and then to 250 °C at a rate of 5 °C/min. The column was held at the final temperature for 5 min. Other parameters were: injection, 270 °C, and transfer line, 300 °C. To avoid a large peak due to the

1 The abbreviations used are: HNE, 4-hydroxy-trans-2-nonenal; HNA, 4-hydroxy-trans-2-nonenic acid; DHN, 1,4-dihydroxy-2-nonen; AR, aldose reductase; AR, inhibitor; KH, Krebs-Henseleit; EIS, electrospray-ionization; MS, mass spectroscopy; GC, gas chromatography; CIMS, chemical ionization mass spectroscopy; HPLC, high performance liquid chromatography.
homogenate and centrifuged at 10,000 \( \times g \) for 1 h at 4 °C. Prior to enzyme activity determinations, the homogenate was reduced by incubating with 0.1 m dithiothreitol for 60 min at 37 °C. Excess dithiothreitol was removed by a Sephadex G-25 (PD-10) column equilibrated with nitrogen saturated 0.1 m potassium phosphate, pH 7.0. Aldose reductase was purified from bovine heart essentially as described previously (26). The enzyme activity was monitored using 10 mm D-t-glyceraldehyde and 100 \( \mu \)m NADPH in 0.05 m potassium phosphate, pH 6.0, containing 0.4 m lithium sulfate on a Gilford response spectrophotometer at 340 nm. One unit of enzyme activity is defined as 1 \( \mu \)mol of NADPH oxidized per min. Homogeneity of the enzyme was established by the presence of a single protein band upon \( \beta \)-mercaptoethanol-SDS-polyacrylamide gel electrophoresis. For Western blot analysis, antibodies raised against homogenous human placental AR were used.

RESULTS

**Metabolism of \[^{3}H\]HNE in Perfused Rat Heart**—In our experimental set-up, isolated rat hearts perfused at a constant pressure of 100 cm water, developed a left ventricular pressure of 136 ± 3, and a systolic pressure of 141 ± 3 mm Hg. The mean heart rate was 339 ± 8 beats/min, and the coronary flow rate was 16 ± 0.7 ml/min (n = 29). The isolated hearts were stable, and 60 min of perfusion with KH buffer led to <10% loss in developed pressure. For studying HNE metabolism, however, no pressure recordings were made. An intraventricular balloon was not used because of possible interference with endocardial flow (27). Moreover, oxygen consumption in the perfused heart (without a balloon) is directly proportional to the aortic perfusion pressure (28), which might change in the presence of the balloon and could potentially alter HNE metabolism.

As described under “Experimental Procedures,” 1.0 ml of \[^{3}H\]HNE (25–200 nmol in KH buffer) was injected into the aorta. This concentration of HNE is too small to affect myocardial contractility or coronary flow (29). In seven similar experiments, 64 ± 5% of the radioactivity injected into the aorta was collected in the 22 ± 3 ml of the perfusate. Approximately 8 to 10% of the radioactivity was recovered in the supernatant of the homogenates of the heart tissues. Upon HPLC separation of the metabolites present in the coronary effluent of rat hearts perfused with \[^{3}H\]HNE, four peaks were evident (Fig. 1, I–IV). Total recovery in peaks I–IV averaged 74 ± 5% of radioactivity injected into the column. Peak IV (retention time, \( t_R = 65 \) min), displayed a high absorbance at 224 nm, and co-eluted with externally added reagent HNE, indicating that it is due to unmetabolized HNE present in the sample. The amount of radioactivity recovered in peak IV averaged 29 ± 6% (n = 6; Table I). Peak II accounted for 1.7 ± 0.6% of the radioactivity and appears to be due to DHN, since the \( t_R \) of this peak corresponded to that of enzymatically synthesized DHN, and this peak was co-injected with radiolabeled DHN.

Peak III accounted for 37 ± 6% of the radioactivity (Table I) and co-eluted with HNA synthesized by aldehyde dehydrogenase-catalyzed oxidation of HNE. For further characterization, fractions corresponding to peak III (\( t_R = 58 \) min) were collected, silylated, and subjected to GC/CIMS as described under “Experimental Procedures.” Fig. 2A shows the elution profile of peak III on GC. The peak was further identified by GC/CIMS analysis. The fragmentation pattern of the metabolite in peak III, as shown in Fig. 2B, corresponds to HNA. To ensure that no DHN was present in this peak, chemically synthesized DHN was also silylated and subjected to GC/CIMS. Under the conditions used, DHN eluted on GC with a \( t_R \) of 18 min (Fig. 2C). As evident from Fig. 2A no metabolite with a similar \( t_R \) was observed in the collected peak III.

Peak I contained the most polar metabolites and represented 19 ± 2% of the radioactivity (n = 6). The \( t_R \) of these metabolites corresponded to the glutathione conjugates of HNE and DHN. To characterize the metabolites present in this peak further,
ions with respect to the m/z 464 ion (data not shown).

For synthesis of reagent GS-DHN, GS-HNE was incubated with recombinant AR and 0.1 mM NADPH. After reduction, the enzyme was separated from the reaction mixture by ultrafiltration, and the filtrate was further purified on HPLC using the gradient described above. The fractions eluting with a tR of 45 min were collected, dried in a SpeedVac, and used for ESI-MS. The only major peak observed in the ESI-MS spectrum of the product (Fig. 3B) appeared at m/z of 466.3, which was ascribed to the [M + H]+ of GS-DHN (molecular mass 465). To ascertain whether GS-DHN and GS-HNE could be individually identified in a mixture, approximately 80% of GS-HNE was reduced by AR (as assessed from the decrease in NADPH absorbance). The product and the untransformed GS-HNE were purified as before and used for ESI-MS. The ESI-MS spectrum of the mixture showed two clearly separated peaks appearing at m/z 466 (GS-DHN) and 464 (GS-HNE). In addition, a peak due to GSH generated from the breakdown of the conjugates was also evident (at m/z 307). The ratio of GS-DHN and GS-HNE recovered as such (calculated from the m/z 466 and 464 peaks) was 1:0.2, which is comparable to the ratio expected on the basis of the percent conversion calculated from NADPH oxidation (data not shown). These measurements show that even though the difference in the m/z values between GS-HNE and GS-DHN is only 2, the two metabolites are clearly resolved by this technique, and that the ratio of 464 and the 466 peaks approximately reflects the relative proportion of GS-HNE and GS-DHN present in the sample.

Fig. 4 shows the ESI-MS spectrum of peak I obtained from HPLC separation of the metabolites present in the coronary effluent of rat hearts treated with [3H]HNE. Peaks corresponding to GS-HNE and GS-DHN having m/z values of 464.0 and 466.1, respectively, are clearly evident and well resolved. The peak heights of the two conjugates are comparable, suggesting that roughly equal amounts of GS-HNE and GS-DHN are present in the effluent. A prominent peak was observed at m/z 446.3, which corresponds to the loss of a single water molecule from GS-HNE (also observed with reagent GS-HNE; Fig. 3A).

The peak at m/z 308.2 was also observed and was attributed to GSH, which presumably arises from the in-source fragmentation of GS-HNE. Other prominent peaks, observed at m/z 424.9, 430.9, and 466.2 were not identified. Notably, no significant peaks corresponding to GS-HNA (expected molecular mass of 479 Da), or GS-HNA lactone (molecular mass, 461 Da) were observed.

Separation of the metabolites in the supernatant of the [3H]HNE perfused hearts also revealed four major peaks (Fig. 1B). The total radioactivity recovered in the ventricles was 5 ± 0.6% (n = 3). An additional 4–5% radioactivity was associated with the aorta and the associated blood vessels. In addition, approximately 4% of radioactivity was associated with the ace-
tic acid-acetonitrile-insoluble precipitate of the heart homogenate, presumably due to protein-bound HNE. Thus the total radioactivity remaining in the tissue was 12–16% of the administered dose. As in case of the perfusate, peak I contained a mixture of GS-HNE and GS-DHN, peak II displayed a $t_R$ identical to DHN, and peak III was attributed to HNA and peak IV to unmetabolized HNE. The percent radioactivity present in each of these peaks is listed in Table I. A similar pattern of metabolites was observed when the heart was exposed to 24–200 nmol of HNE.

**Identification of the Metabolic Pathways—**Our previous investigations (30) show that HNE and GS-HNE are excellent substrates of the polyol pathway enzyme AR. Since the heart is particularly rich in AR, we examined the effect of the AR inhibitor-sorbinil on HNE metabolism in the heart. In addition we also studied the effects of cyanamide, which inhibits aldehyde dehydrogenase (31), a potential catalyst for the conversion of HNE to HNA. For these experiments, the hearts were perfused with the KH buffer as before, and after 10 min of equilibration, 0.2 mM sorbinil or 2 mM cyanamide was added to the KH buffer. After 20 min of perfusion with the KH buffer containing the drug, 55 nmol of \[^{3}H\]HNE were injected into the heart, and the metabolites were separated as before.

In the hearts perfused with \[^{3}H\]HNE in the presence of sorbinil, 62% of the radioactivity was recovered in the coronary effluent. When separated on HPLC, only two major peaks were evident (Fig. 5A), and the total radioactivity recovered on HPLC was 83%. Peak I, which contains the glutathione conjugates accounted for 21%, whereas peak III, which corresponds to HNA, accounted for 65% of the total radioactivity recovered, indicating that upon inhibition of AR, oxidation of HNE to HNA is increased. The ESI-MS spectrum of peak I revealed the presence of GS-HNE (at m/z 464), but no peak corresponding to GS-DHN (at m/z 466) was observed (Fig. 6A), indicating that sorbinil completely inhibited reduction of GS-HNE to GS-DHN.

The metabolism of HNE was also profoundly altered in the presence of cyanamide, an inhibitor of aldehyde dehydrogenase. In the presence of 2 mM cyanamide, peak III corresponding to HNA accounted for 20% of the radioactivity, whereas the radioactivity present in peak I, which contained the glutathione conjugates was increased to 69% from 19% (Fig. 5B). The electrospray mass spectrum (Fig. 6B) shows both GS-DHN and GS-HNE, and the ratio of the two conjugates was 1:0.4, indicating that in the presence of cyanamide the reduction of the conjugate is facilitated, and as compared with GS-HNE more of GS-DHN is formed.

In the HPLC eluates of hearts perfused with both sorbinil and cyanamide, the conjugates represented the major HNE metabolites and accounted for 42% of the radioactivity (Fig. 5C). A significant (17%) proportion of HNE was recovered unmetabolized, whereas HNA accounted for 30% of the metabolites. Little or no radioactivity was recovered in the peak corresponding to DHN. Upon ESI-MS, the ratio of GS-DHN to GS-HNE was 1:2.25 (Fig. 6C), indicating again that inhibition of AR, attenuates the formation of the reduced conjugate.

To further identify the enzymatic processes catalyzing the formation of GS-DHN, pyridine coenzyme linked transformation of GS-HNE was examined in cardiac homogenates using 0.1 mM NADPH, NADP, NADH, and NAD. As shown in Table II, the major coenzyme-linked oxidation/reduction of GS-HNE in all the homogenates examined was associated with the oxidation of NADPH, with little or no activity due to NAD, NADP, or NADH. The NADPH-catalyzed reduction of GS-HNE was at least 2–3-fold higher than that associated with other coenzymes both at pH 7.0 and more so in pH 6.0 buffer containing...
7.0, or 0.05 M K-phosphate, containing 0.4 M lithium sulfate, pH 6.0. The concentration of all the coenzymes used was 0.1 mM, and the GS-HNE adduct for catalysis was recognized by antibodies raised against human AR. When tested with GS-HNE as substrate, the catalytic rate of the enzyme \( k_{\text{cat}} \) was 29 min\(^{-1} \), with a \( K_m \) of GS-HNE of 29 ± 8 \( \mu \)M, suggesting that homogenous AR is capable of catalyzing the reduction of GS-HNE.

**DISCUSSION**

The results of this study show that several enzymatic pathways contribute to the metabolism of HNE in the heart. A distinctive feature of HNE metabolism in isolated perfused hearts was that most of the HNE administered was rapidly transformed and extruded into the effluent. Only a small percent of the radioactivity was retained by the tissue. These data suggest that endogenous aldehydes generated by lipid peroxidation or other metabolic processes also may be metabolized similarly and extruded from the heart. In the absence of comparable studies with other tissues, it remains to be determined whether such rapid extrusion is a general feature of aldehyde metabolism or whether this phenomenon is characteristic of the heart. Nonetheless, efficient metabolism and extrusion of HNE metabolites indicates that estimates of lipid peroxidation based on measurements of free aldehydes or their protein adducts will grossly underestimate the extent of peroxidative activity under specific pathological conditions. For complete assessment, therefore, it may be essential not only to measure free aldehydes but also their metabolites in the intracellular as well as extracellular space.

One of the major pathways of HNE metabolism in the heart appears to be oxidation to HNA. In the liver, HNA is generated by aldehyde dehydrogenase-catalyzed oxidation of HNE (5), and a similar enzymatic pathway may be responsible for HNA formation in the heart. The heart contains several aldehyde dehydrogenases (32), including the mitochondrial aldehyde dehydrogenase (33), which may be responsible for catalyzing HNE oxidation. This view is strengthened by the observation that in the presence of the mitochondrial aldehyde dehydrogenase inhibitor cyanamide, the formation of HNA was significantly attenuated. Similar to other metabolites, HNA was also rapidly extruded from the heart. Although additional investigations will be required to identify the specific transporter(s) involved in this process, it is likely that the efflux of HNA (a long chain fatty acid analog) may be mediated in part by the fatty acid transporter, which is expressed in the rat heart (34) and, interestingly, is up-regulated during oxidative stress (35).

In addition to oxidation, conjugation with glutathione (GSH) also appears to be a major route of HNE metabolism in the heart. We find that in the perfused rat heart 30% of the metabolized HNE in the effluent was in the form of GSH conjugates, while 50% of the metabolized intracellular HNE was present in the form of GSH conjugates. The extent to which HNE is conjugated with GSH in the heart is thus comparable to that observed in liver (5, 36). While HNE spontaneously reacts with GSH to form a Michael adduct (5), it has been proposed that intracellular formation of GS-HNE is due to glutathione S-transferase-mediated catalysis, which enhances adduct formation 600 times over the spontaneous rate (37). Several glutathione S-transferases have been localized to the heart, of which glutathione S-transferase 6.9 from rat heart has been shown to efficiently catalyze the formation of GS-HNE (38). In addition, the “HNE-specific” glutathione S-transferase (4-4 in rats and 5-8 in humans), suggested to have evolved specifically to metabolize lipid peroxidation products, is also present in cardiac tissues (39), although the localization and the kinetic

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**FIG. 6.** Electrospray-mass spectra of metabolites of \(^{3} \text{H}\)HNE collected in coronary effluents of hearts perfused with either 0.2 mM sorbinil (A), 2 mM cyanamide (B), or 0.2 mM sorbinil and 2 mM cyanamide (C). Coronary effluents were collected, pooled, and separated on HPLC, and fractions corresponding to peak 1 (see Fig. 5) were analyzed by ESI-MS. Peaks at \( m/z \) 464 and 466 were ascribed to GS-HNE and GS-DHN, respectively.

**TABLE II**

Pyridine coenzyme-linked transformation of the GS-HNE adduct in homogenates of bovine, rat, and human hearts

| Heart | Coenzyme and pH | 6.0 | 7.0 | 6.0 | 7.0 | 6.0 | 7.0 | 6.0 | 7.0 |
|-------|-----------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Bovine | NAD | ND  | ND  | ND  | ND  | 0.3 | 3.3 | 1.8 |
| Rat   | NADP | 1.9 | 1.9 | 7.7 | 3.8 | ND  | 34.6| 17.2|
| Human | NADH | 1.6 | 0.8 | ND  | 0.8 | 4.8 | 1.6 |
|       | NADPH| ND  | ND  | ND  | ND  | ND  | ND  | ND  |
|       | ND   | ND  | ND  | ND  | ND  | ND  | ND  | ND  |

* ND, not detectable.

0.4 M lithium sulfate, indicating that AR-catalyzed reduction may be the predominant route of GS-HNE reduction.

To examine whether the homogenous cardiac AR was in fact capable of catalyzing the reduction of GS-HNE, the enzyme was purified to homogeneity as described under “Experimental Procedures.” The purified enzyme migrated as a single band on SDS-polyacrylamide gel electrophoresis, and on Western blot analysis was recognized by antibodies raised against human placenta AR (results not shown). The homogenous enzyme was activated 3-fold by lithium sulfate and inhibited (>80%) by 0.2 mM sorbinil; features consistent with AR isolated from other tissues.

The enzyme displayed a \( K_m \) of glyceraldehyde of 37 ± 7 \( \mu \)M and a \( k_{\text{cat}} \) of 27 min\(^{-1} \), which is consistent with the glyceraldehyde reducing activity of AR isolated from other tissues.

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properties of the rat heart enzyme have not been studied.

The metabolic fate of GSH-xenobiotic adducts in most tissues involves either active extrusion via the GS-X transporter or conversion into mercapturates. The formation of mercapturic acid derivatives involves serial and catalytic conversion of GS-X to X-Cys-Gly, Cys-X, and finally to N-acetyl cysteine-X (X-mercapturic acid) (40). These compounds could be further reduced or oxidized resulting in several N-acetylcysteine derivatives which are then excreted. However, under the present experimental conditions, we found no evidence for the formation of HNE-derived mercapturic acids. Because high concentrations of the GSH conjugates were recovered in the perfusate, we conclude that these do not undergo further metabolism in the heart. The major biochemical pathways of HNE metabolism in heart are outlined in Scheme 1.

In addition to GS-HNE, significant amounts of the reduced form of the conjugate-GS-DHN, were also recovered in perfusates of hearts exposed to HNE. Reduced and oxidized forms of GSH conjugates have not been distinguished previously, since HPLC analysis, used in most studies, often fails to separate GS conjugates with very similar properties. The problem is further aggravated by aldehyde-specific pre-column derivitization, which excludes nonaldehydic forms of the conjugate. Although different forms of the conjugate could be resolved by thin layer chromatography (e.g. see Grune et al. (41)), in the absence of additional analysis, assignment of chemical structure based on RF values alone remains ambiguous. In this regard, ESI-MS appears to be the method of choice. The ability to resolve submicromolar concentrations of GSH derivatives that differ by only 2 m/z value attests to the general utility of the technique for identification of GS conjugates. However, in the absence of radioactive labeling, in-source fragmentation, and unequal ionization of the conjugates may confound quantitative measurements, and may require additional modifications to this technique.

The reduced conjugate, GS-DHN, could arise either from catalytic reduction of GS-HNE or conjugation of DHN with GSH. However, due to loss of the conjugated aldehyde following reduction, it is unlikely that DHN could spontaneously react with GSH, and for the same reason, enzymatic catalysis of the adduct formation between GSH and DHN may also be inefficient. Thus, it appears unlikely that GS-DHN arises from the formation of adduct between DHN and GSH. This view is further supported by our observation that perfusion of isolated hearts with [3H]DHN did not result in the formation of GS-DHN (data not shown). Thus, the most likely route of GS-DHN formation appears to be enzyme-catalyzed reduction of GS-HNE. This reductive transformation in the heart is catalyzed by the polyol pathway enzyme AR. This conclusion is based on the following observations: (a) the formation of GS-DHN is completely suppressed in heart perfused with HNE in the presence of the AR inhibitor sorbinil, (b) in homogenates of human, rat and bovine hearts, the major catalytic activity associated with GS-HNE was NADPH-mediated reduction, which could be attributed to AR, and (c) homogenous AR purified from heart efficiently catalyzed the reduction of GS-HNE with high affinity. Although AR also catalyzes the reduction of unconjugated HNE (to DHN) (30), it appears that in the presence of high GSH concentration (−3 mM in cardiac sarcoplasm) (38) and GST activity, conjugation with GSH is favored. Thus, in GSH-competent cells, reduction of GS-HNE (rather than free HNE) may be the primary activity of AR associated with HNE metabolism.

Although a large fraction of the conjugate was recovered in the reduced form, the metabolic significance of this reductive transformation remains to be established. In previous studies, both oxidized and reduced forms of mercapturic acid derivatives of HNE and acrolein have been shown (42, 43). However, it is not clear whether these redox transformations occur with the GS-X, X-Cys-Gly, Cys-X, or N-acetyl-Cys-X. In this regard, our report constitutes the first unequivocal demonstration of reduction of GSH conjugates, and identifies a specific enzymatic process associated with this transformation. No GS-HNA adducts were recovered from HNE-treated hearts. However, mercapturic acids of HNA and its lactone cysteine have been recovered from the urine of HNE-treated rats, but these could arise from oxidation of Cys-Gly-HNE and N-acetyl-Cys-HNE rather than GS-HNE. Our data do not distinguish between these possibilities, but nevertheless, indicate that, at least in the heart, oxidation of GS-HNE (in contrast to free HNE) is not catalyzed by an aldehyde dehydrogenase, and that the conjugation of HNA with GSH does not efficiently compete with HNA extrusion.

Studies with mercapturic acids of acrolein show that in contrast to the oxidized and untransformed mercapturates, the reduced mercapturic acid derivative is not toxic (43), suggesting that reduction may represent a metabolic transformation to abrogate the toxicity of GSH conjugates. While the GSH conjugates are covalent adducts, several of these can spontaneously dissociate. The GS-HNE adduct, for instance, readily dissociates at neutral pH into GSH and HNE (44). Such dissociation, which may lead to the generation of HNE at GSH-
deficient sites, has been suggested to be responsible for their persistent toxicity and trans-organ effects (45). Reduction, however, prevents dissociation and may thus prevent long term and/or distal toxicity. That reduction is an important determinant of HNE toxicity is consistent with the reports that inhibition of AR exacerbates HNE toxicity to vascular smooth muscle cells (46, 47), and our own observations that superfusion with sorbinil accelerates HNE-induced hypercontracture of isolated cardiac myocytes. In addition, we have also observed that HNE-induced opacification of the ocular lens is exacerbated by inhibitors of AR (48). Together, these data suggest that AR-mediated metabolism is an important component of HNE detoxification in several tissues, under normal and certain pathological conditions.

The pathophysiological role of AR in mediating hyperglycemic injury has been extensively studied (49). Although the specific mechanisms by which hyperglycemic activation of AR causes tissue injury continues to be a matter of debate, several studies have shown that pharmacological inhibitors of AR (ARIs) prevent, delay, or in some cases even reverse, tissue injury-associated with several pleiotrophic complications of long term diabetes such as cataractogenesis, neuropathy, nephropathy and retinopathy (49). Nevertheless, some trials with these drugs have yielded uncertain results, and even in in vitro early protection seen with ARIs decreases upon long term use (49, 50). While many reasons may underlie the ambiguous results obtained with ARIs, our observation that AR contributes to the detoxification of lipid-derived aldehyde, and that ARIs exacerbate the cytotoxic effects of these aldehydes suggest that these drugs may under some circumstances exacerbate oxidative injury, and should, therefore, be used with caution (perhaps with additional antioxidative intervention).

Our results also suggest a new role for AR. Based on its ability to catalyze sorbitol formation, it is believed that the chief physiological role of AR is glucose metabolism. However, glucose is a poor substrate for AR ($K_m \approx 50$ to $100$ mM) (49), and the reduction of other long chain hydrophobic and aromatic aldehydes is catalyzed more efficiently ($K_m$ GS-HNE $\approx 0.03$ mM). In addition, the unusual tight binding of AR to NADPH, primes the catalytic cycle, and permits wide substrate specificity by minimizing the contribution of aldehyde binding steps to stabilization of the transition state (51). Thus structural and thermodynamic features of AR are optimized to catalyze essentially irreversible reduction of a range of aldehydes rather than a specific substrate-product pair; characteristics ideal for the detoxification of multiple aldehydes and their glutathione conjugates generated during lipid peroxidation. While catalytic reduction of several aldehydes has been demonstrated with the isolated enzyme, our results provide the first reported evidence for in vivo reduction of a substrate other than glucose. Based on these results, and those showing exacerbation of aldehyde injury by ARIs (46–48), we speculate that AR may represent an integral component of secondary antioxidative defenses.

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