The Reactive Oxygen Species- and Michael Acceptor-inducible Human Aldo-Keto Reductase AKR1C1 Reduces the α,β-Unsaturated Aldehyde 4-Hydroxy-2-nonenal to 1,4-Dihydroxy-2-none*  

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The human aldo-keto reductase AKR1C1 (20α(Sα)-hydroxysteroid dehydrogenase) is induced by electrophilic Michael acceptors and reactive oxygen species (ROS) via a presumptive antioxidant response element (Burczynski, M. E., Lin, H. K., and Penning, T. M. (1999) Cancer Res. 59, 607–614). Physiologically, AKR1C1 regulates progesterone action by converting the hormone into its inactive metabolite 20α-hydroxyprogesterone, and pharmacologically this enzyme activates polycyclic aromatic hydrocarbon trans-dihydrodiols to redox-cycling α-quinones. However, the significance of its potent induction by Michael acceptors and oxidative stress is unknown. 4-Hydroxy-2-nonenal (HNE) and other α,β-unsaturated aldehydes produced during lipid peroxidation were reduced by AKR1C1 with high catalytic efficiency. Kinetic studies revealed that AKR1C1 reduced HNE (Km = 54 μM, kat = 8.8 min⁻¹) with a kat/Km similar to that for 20α-hydroxysteroids. Six other homologous recombinant AKRs were examined for their ability to reduce HNE. Of these, AKR1C1 possessed one of the highest specific activities and was the only isoform induced by oxidative stress and by agents that deplete glutathione (ethacrynic acid). Several hydroxysteroid dehydrogenases of the AKR1C subfamily catalyzed the reduction of HNE with higher activity than aldehyde reductase (AKR1A1). NMR spectroscopy identified the product of the NADPH-dependent reduction of HNE as 1,4-dihydroxy-2-none. The Km of recombinant AKR1C1 for nicotinamide cofactors (KmNADPH = 6 μM, KmNADH > 6 mM) suggested that it is primed for reductive metabolism of HNE. Isoform-specific reverse transcription-polymerase chain reaction showed that expression of HepG2 cells to HNE resulted in elevated levels of AKR1C1 mRNA. Thus, HNE induces its own metabolism via AKR1C1, and this enzyme may play a hitherto unrecognized role in a response mounted to counter oxidative stress. AKRs represent alternative GSH-independent/NADPH-dependent routes for the reductive elimination of HNE. Of these, AKR1C1 provides an inducible cytosolic barrier to HNE following ROS exposure.

Oxidative stress plays a detrimental role in a number of pathological conditions, including cancer, atherosclerosis, and several neurodegenerative disorders (1–5). One of the main classes of cytotoxic and genotoxic substances produced during oxidative stress is α,β-unsaturated aldehydes generated endogenously as a breakdown product of lipid peroxidation of ω-6 polyunsaturated fatty acids (6). Of these, HNE* is one of the major unsaturated aldehydes produced and may be more deleterious than the initial production of ROS. This metastable reactive metabolite possesses a longer half-life than ROS and can diffuse from its sites of origin in the cellular membranes. Since HNE is a Michael acceptor, it attacks nucleophilic targets intracellularly and can act as a potent cytotoxin (for a review, see Ref. 6). In vivo, HNE immunoreactive adducts have been detected in atherosclerotic plaques (7), in the degenerating neurons of the substantia nigra affected by Parkinson’s disease (8), and in the neurofibriillary tangles associated with Alzheimer’s disease (9). HNE is also genotoxic, since it will readily form propano-dGuo adducts, which can lead to promutagenic lesions (10, 11). Related etheno adducts may be derived from the more reactive bifunctional electrophile 4-oxo-2-nonenal (12, 13), and whether this can be metabolically derived from HNE is unknown.

Mammalian cells have developed multiple enzymatic pathways for the detoxification of these lipid peroxidation by-products. The best characterized of these enzymes include the GSTs, aldehyde dehydrogenase, and alcohol dehydrogenase (14, 15). GSTs catalyze conjugation of GSH to HNE via Michael addition at the C-3 carbon, thereby preventing further nucleophilic addition to this toxic compound. Aldehyde dehydrogenase catalyzes the oxidation of HNE to the innocuous 4-hydroxy-2-nonenonic acid, while alcohol dehydrogenase catalyzes reduction of the terminal aldehyde to its alcohol, yielding the unreactive metabolite DHN. Recently two members of the AKR2 superfamily, aldose reductase and FR-1, were also shown to catalyze HNE reduction (16–19), thereby expanding the repertoire of enzyme families that contribute to detoxification of HNE in hepatic and extrahepatic tissues.

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1 The abbreviations used are: HNE, 4-hydroxy-2-nonenal; AKR, aldo-keto reductase; ADH, alcohol dehydrogenase; DHA; dehydroascorbate; DHN, 1,4-dihydroxy-2-nonenone; GST, glutathione S-transferase; HSD, hydroxysteroid dehydrogenase; 3α-HSD, 3α-hydroxysteroid dehydrogenase (EC 1.1.1.213: A-face specific); 20α-hydroxysterrogenase, 20α-hydroxyprogren-4-en-3-one; PAH, polycyclic aromatic hydrocarbon(s); NQO1, NAD(P)H:quinone oxidoreductase 1; ROS, reactive oxygen species; PCR, polymerase chain reaction.

2 The nomenclature for the aldo-keto reductase superfamily was proposed by Jez et al. and adopted at the 5th International Symposium on Enzymology and Molecular Biology of Carbonyl Metabolism in Dead-wood, June 29 to July 3, 1996 (16). It is also described on the AKR superfamily home page on the World Wide Web.
In previous studies, a 20α(3α)-hydroxysteroid dehydrogenase (AKR1C1), a member of the AKR1C subfamily, was found to be potently induced by Michael acceptors and ROS in human colon and hepatoma cell lines through an apparent antioxidant response element-type mechanism (20–22). However, the physiological reason for the robust induction of this enzyme by electrophiles and ROS has remained unclear. The HSDs of this subfamily play important roles in steroid target tissues (for a review, see Ref. 23). Human AKR1C1–AKR1C4 are plastic enzymes and display 3α-, 17β-, and 20α-hydroxysteroid oxidase activity (24, 25). This plasticity indicates that these enzymes can interconvert potent androgens, estrogens, and progesterins with their inactive metabolites and have the potential to regulate occupancy of steroid hormone receptors based on their tissue distribution. In the case of AKR1C1, all of these reactions occurred, but the most catalytically efficient is the interconversion of progesterone to its inactive metabolite, 20α-hydroxyprogesterone, suggesting that this is its physiological role (25).

Several HSDs in the AKR1C subfamily also play roles in xenobiotic metabolism by oxidizing PAH trans-dihydriodiol proximate carcinogens to reactive and redox-active o-quinones (26–29). However, the roles of AKR1C1 in steroid and PAH metabolism fail to provide a rationale for its isoform-specific induction by electrophiles and ROS.

In the present studies, we sought to identify an activity that might explain the robust induction of AKR1C1 during oxidative insult. We found that, like its closely related rat homolog AKR1C9 (30), AKR1C1 weakly catalyzed reduction of DHA to regenerate ascorbic acid. However, the low specific activity observed suggested that this was not the ROS counter-response catalyzed by AKR1C1. Subsequent studies revealed that HNE and other α,β-unsaturated aldehydes produced during lipid peroxidation were substrates of the inducible AKR1C1 isoform and identified DHN as the product of the AKR1C1-catalyzed HNE reduction. HNE was also found to directly induce AKR1C1 expression. The induction of AKR1C1 by HNE and ROS suggests that it may belong to a battery of genes (GST, α-G-glutamylcysteine synthetase, etc.) that mount a counter-response to ROS during periods of electrophilic and oxidative stress.

**Experimental Procedures**

**Chemicals and Reagents—**Cell culture media and reagents were obtained from Life Technologies, Inc. Homogeneous recombinant AKR proteins, including aldolase reductase (AKR1A1), were overexpressed in *Escherichia coli* and purified as described previously (28, 31, 32). β-NADPH nucleotide cofactor was obtained from Roche Molecular Biochemicals. DHA, alkaliens, and 2,3-dimethoxynapthalene-1,4-dione were purchased from Sigma, and HNE was obtained from Calbiochem. Because HNE will self-polymerize on storage, only new lots of HNE were used in these experiments. All other chemicals used were of the highest grade available.

**Chemical Synthesis of 1,4-Dihydroxynonenone from 4-Hydroxy-2-nonenal—**DHN was synthesized by NaBH₄ reduction of HNE. Briefly, a 4-fold molar excess of NaBH₄ (25 μmol) was added dropwise to a solution of 1 mg HNE (6.4 μmol) in 4 ml of anhydrous methanol with continuous stirring over 10 min. Aliquots were removed over time and analyzed by TLC to monitor the progress of the reaction. Reactions were terminated by the addition of 1.0 ml of 0.1 N HCl to decompose unreacted NaBH₄. The solution was extracted with 3 × 20 ml of dichloromethane, and the extract was dried over anhydrous sodium sulfate and reduced under vacuum at room temperature. The chemically synthesized DHN was separated from the starting material by TLC, excised from silica, and resuspended in CDCl₃ for subsequent NMR analysis.

**Isolation and Characterization of the Product of HNE Reduction—**Large scale enzymatic incubations were conducted in 20 ml of 100 mM potassium phosphate, pH 6.0, containing 3.2 mM NADPH and 320 μM HNE solubilized in 2% methanol. Following the addition of the purified enzyme (100 μg) the reactions were incubated at 37 °C for 4 h and then terminated by the extraction of products with dichloromethane (three 20-ml aliquots). The organic solvent was dried with anhydrous sodium sulfate and then removed under reduced pressure. Following resuspension in dichloromethane, the resulting extract was subjected to TLC separation. DHN was separated from HNE by TLC in acetone/methanol (1:1). Detection was accomplished with methanol/formic acid (1:1) reagent followed by heating. TLC plates were analyzed by densitometry on a Unispec densitometer (Analytich, Newark, DE) to determine the chromatographic profile of HNE standard (R⁰ = 0.49), chemically synthesized DHN (R¹ = 0.20), and the enzymatic product. The enzymatic product was eluted from the silica with methanol, evaporated to dryness, and resuspended in CDCl₃ for NMR analysis.

**NMR Spectroscopy—** 1H NMR spectra were obtained on a Bruker AM-250 spectrometer operating at 250 MHz, using CDCl₃ as the solvent. All chemical shifts (δH) are in ppm downfield from tetramethylsilane. The spectra were referenced to residual protonated solvent residues as internal standards; for CDCl₃, δH = 7.3 ppm. Analysis of HNE gave the following spectral assignments: δH 8.09 (t, 3H, 9-CH₃); δH 1.25–1.67 (m, 4H and m, 4H, 5-CH₂, 6-CH₂, 7-CH₂, 8-CH₂); δH 3.63–3.78 (m, 1H, 1-CH₃); δH 5.83–6.05 (d, 1H, 2-CH J₃, 4, 3.0 Hz); δH 6.13–6.38 (t, 1H, 3-CH J₃, 4= 2–1 Hz and J₉, 1H = 2–1 Hz); δH 9.55–9.61 (d, 1H, 1-CHO). Analysis of chemically synthesized DHN and the AKR1C1 product gave the following spectral assignments, which were identical with the previously characterized spectral shifts of DHN (33): δH 6.09 (t, 3H, 9-CH₃); δH 1.25–1.67 (m, 4H and m, 4H, 5-CH₂, 6-CH₂, 7-CH₂, 8-CH₂); δH 4.21 (m, 3H, 1-CH₃, 4-CH); δH 5.69–5.90 (m, 2H, 2H, 2-CH, 3-CH; gave the expected 10-line hyperfine splitting pattern based on a J₉, 1H J₈, 1H and J₃, 1H, J₈, 1H coupling system). A residual CH₃Cl shift (δH = 5.29) was present in the chemically synthesized DHN, and a residual CH₃OH shift (δH = 3.44) was detected in the enzymatic product.

**Spectrophotometric Assays and Kinetic Characterization—**Kₐ and kₐ values were obtained by varying the substrate concentration at constant saturating cofactor concentration (180 μM NADPH or 2.3 mM NADPH⁺) in 1.0 ml systems containing 100 μM potassium phosphate buffer, pH 7.0, at 37 °C. Initial velocities at each substrate concentration were determined on a Beckman DU640 spectrophotometer by measuring the change in absorbance of pyridine nucleotide at 340 nm (ε = 6220 M⁻¹ cm⁻¹). Actual values of the kinetic constants were determined using ENSYFITTER to fit transformed data (substrate concentration versus initial rate) to a hyperbolic equation to provide estimates of kₐ and Vₐmax and their associated S.E. values (34). Kₛ(app) values for nicotinamide cofactors were obtained by varying NADH/NADPH concentration at a constant progesterone concentration (50 μM, limit of solubility) or by varying NAD⁺/NADPH concentration at a constant 20α-hydroxyprogesterone concentration (50 μM, limit of solubility).

**Cell Culture—** HepG2 hepatoma cells (passages 10–30) were maintained in Eagle’s minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and were passaged every 4 days at 1:10 dilution. For induction studies, 48 h prior to treatment, cells were seeded into 100-mm tissue culture dishes containing fresh medium. Two days later (~50–60% confluency), cells were exposed to HNE. Aliquots (10 μl) of 1000× stock solutions in acetone were added to 10 ml of fresh culture medium, and cells were incubated for the indicated times before harvesting.

**RNA Isolation and Northern Analysis of AKR1C1 mRNA—**Cellular RNA was isolated using the Trizol® reagent. Total RNA (10 μg) was separated by electrophoresis on 1.0% agarose/formaldehyde gels and transferred overnight to Duralon-UV membranes (Stratagene). Membranes were prehybridized in hybridization buffer (50% formamide, 10% dextran sulfate, 1 mM NaCl, and 1% SDS) with 100 μg/ml sheared salmon sperm DNA at 42 °C for 2 h. After prehybridization, membranes were hybridized to 10⁵ dpm of an [α-³²P]cATP probe corresponding to an 855-bp base pair EcoRI fragment of the human colon DD1 cDNA (pBluescript-hcDD kindly provided by Dr. Paul Ciaccio and Dr. Ken Tew). Signal intensities were measured using the PhosphorImager system (Molecular Dynamics, Inc., Sunnyvale, CA), and blots were exposed to x-ray film at ~80 °C overnight. For purposes of normalization, blots were stripped and reprobed with a 780-base pair PstI/BglI fragment of human GAPDH labeled by random priming. Quantitative Isoform-specific Reverse Transcription PCR of AKR1C1 mRNA—Total RNA (1 μg) from untreated and HNE-treated HepG2 cells was reverse transcribed into cDNA and subjected to 18 cycles of reverse transcription PCR amplification (94 °C for 45 s (denaturation), 60 °C for 45 s (annealing); 72 °C for 2 min (extension)) using a 1 μM concentration of the following isoform-specific primers: AKR1C1 (forward, 5’-dTATAGCGTGTAGGCGC-3’; reverse, 5’-cACCATG-
for HNE reduction, while the major 3 (alddehyde reductase) possessed intermediate specific activities (a 98% amino acid identity) AKR1C2 (type 3 to prostaglandins and HNE, respectively.

AKR1C1 and AKR1C3 Possess Low but Detectable Dehydroascorbate Reductase Activities—ROS can lead to uncontrolled GSH depletion, ascorbic acid oxidation, lipid peroxidation, and DNA damage unless cells mount an effective counter-response. A previous study reported that rat liver 3a-HSD (AKR1C9) functions as a dehydroascorbate reductase (30). Thus, human HSDs of the AKR1C subfamily were initially screened for their ability to catalyze the reduction of 1.5 mM DHA as a potential antioxidant activity. Interestingly, only two human isoforms that are induced by ROS and Michael acceptors (AKR1C1 and, to a lesser extent, AKR1C3) catalyzed the reduction of DHA to ascorbic acid. The low specific activities observed for the reduction of DHA (less than 8 nmol/min/mg) at millimolar substrate concentrations suggested that DHA was not the physiological substrate for the inducible AKR1C isoforms during periods of oxidative stress.

Reduction of 4-Hydroxy-2-nonenal by Multiple AKR Family Members—One of the most common cytotoxic products of lipid peroxidation is HNE. Like prostaglandins, HNE is derived from arachidonic acid and possesses several structural characteristics in common with prostaglandins (Fig. 1). HNE (200 m) was thus screened as a substrate for several human AKR1 subfamily members that share high sequence identity with bovine prostaglandin F synthase, which is involved in prostaglandin metabolism (Fig. 2). The ROS- and Michael acceptor-inducible AKR1C1 (20α3α-HSD) and its closely related (>98% amino acid identity) AKR1C2 (type 3 3α-HSD, bile acid-binding protein) homolog possessed the highest specific activities for HNE reduction of the six homogeneous recombinant AKRs studied. AKR1C3 (type 2 3α-HSD) and AKR1A1 (aldehyde reductase) possessed intermediate specific activities for HNE reduction, while the major 3α-HSDs in human and rat liver, AKR1C4 (type 1 3α-HSD) and AKR1C9 (rat 3α-HSD), respectively, possessed low activity toward HNE. Of these enzymes, AKR1C3 has recently been shown to be identical to human prostaglandin F synthase (35). In this comparison, AKR1C1 had a specific activity that was 3 times greater than that seen with AKR1A1. In previous studies, AKR1C1 was shown to have a specific activity 300 times less than AKR1A1 (36), raising the issue as to why this discrepancy exists. In the earlier studies, assays were conducted with non-physiologic amounts of HNE (4 mM). This led to a comparison of the catalytic efficiencies of AKR1C1 and AKR1A1 for HNE and an examination of whether HNE could inactivate either enzyme by Michael addition.

**RESULTS**

**Reduction of 4-Hydroxy-2-nonenal by Multiple AKR Family Members**—One of the most common cytotoxic products of lipid peroxidation is HNE. Like prostaglandins, HNE is derived from arachidonic acid and possesses several structural characteristics in common with prostaglandins (Fig. 1). HNE (200 m) was thus screened as a substrate for several human AKR1 subfamily members that share high sequence identity with bovine prostaglandin F synthase, which is involved in prostaglandin metabolism (Fig. 2). The ROS- and Michael acceptor-inducible AKR1C1 (20α3α-HSD) and its closely related (>98% amino acid identity) AKR1C2 (type 3 3α-HSD, bile acid-binding protein) homolog possessed the highest specific activities for HNE reduction of the six homogeneous recombinant AKRs studied. AKR1C3 (type 2 3α-HSD) and AKR1A1 (aldehyde reductase) possessed intermediate specific activities for HNE reduction, while the major 3α-HSDs in human and rat liver, AKR1C4 (type 1 3α-HSD) and AKR1C9 (rat 3α-HSD), respectively, possessed low activity toward HNE. Of these enzymes, AKR1C3 has recently been shown to be identical to human prostaglandin F synthase (35). In this comparison, AKR1C1 had a specific activity that was 3 times greater than that seen with AKR1A1. In previous studies, AKR1C1 was shown to have a specific activity 300 times less than AKR1A1 (36), raising the issue as to why this discrepancy exists. In the earlier studies, assays were conducted with non-physiologic amounts of HNE (4 mM). This led to a comparison of the catalytic efficiencies of AKR1C1 and AKR1A1 for HNE and an examination of whether HNE could inactivate either enzyme by Michael addition.

**Catalytic Efficiency of AKR1C1 for HNE**—Determination of kinetic constants for AKR1C1 at pH 7.0 and 37 °C revealed a $K_m$ of 34 μM and a $k_{cat}/K_m$ of 8.8 min$^{-1}$ for HNE. The catalytic efficiency for HNE ($k_{cat}/K_m = 0.27 \times 10^6$ mol$^{-1}$ min$^{-1}$) observed with AKR1C1 was considerably higher than that anticipated with AKR1A1. AKR1A1 was not saturable at 1 mM HNE, and in fact $v$ versus $[S]$ plots were entirely linear, indicating that under the conditions of the highest substrate concentrations employed the velocity was still pseudo-first order. Thus, the catalytic efficiency is not measurable and must be considerably lower than that observed with AKR1C1. Preincubation of AKR1C1 and AKR1A1 with 4 mM HNE (under assay conditions reported in Ref. 36) led to a time-dependent inactivation of AKR1C1 only (data not shown). Thus, an explanation for the inadvertently high specific activity reported for AKR1A1 can be explained by its lack of saturation and lack of time-dependent inactivation, both of which are observed with AKR1C1 at 4 mM HNE. Importantly, we find that AKR1C1 is not inactivated in the presence of NAD$^+$ Since cofactor protects AKR1C1 against this event, no inactivation occurs over the time course of the enzyme assay used to generate the steady-state kinetic parameters. When the catalytic efficiency of AKR1C1 for HNE is compared with its values for the NADP(H)-dependent oxidation/reduction of various physiological (20α-hydroxysteroids, prostaglandins) and xenobiotic (PAH trans-dihydriodiolis) substrates (Table I), HNE is reduced with a catalytic efficiency...
similar to that of 20α-hydroxysteroids, which are the presumed substrates of AKR1C1 in vivo.

Substrate Specificity of AKR1C1 for α,β-Unsaturated Aldehydes of Increasing Chain Length—To investigate the specificity of the AKR1C1-catalyzed reduction of HNE, a series of unsaturated aldehydes (alkenals) of increasing chain length were also screened as substrates of AKR1C1. Calculation of catalytic efficiencies revealed that \( k_{cat}/K_m \) increased over 20-fold as the length of the carbon chain increased from 5 to 9 carbons (Table II). The predominant effects were on \( K_m \) and were reflected in \( k_{cat}/K_m \). Interestingly, the presence of the 4'-hydroxyl group on 2-nonenal raises the \( K_m \) ~5-fold, while \( k_{cat} \) remained unchanged.

\( K_m \) of Recombinant Human AKR1C1 for NADP\(^+\) and NADPH Cofactors—To determine the nucleotide cofactor specificity of AKR1C1-catalyzed reduction of HNE, the ability of the enzyme to catalyze NADH- versus NADPH-dependent reduction of HNE was assessed (Fig. 3). AKR1C1 catalyzed HNE reduction with a 100-fold increase in activity using NADPH as cofactor compared with NADH. Kinetic constants of AKR1C1 for NAD(P)H were generated using 20α-hydroxyprogesterone and progesterone as a reversible oxidation/reduction substrate pair (Table III). AKR1C1 appears to be specific for the triphospho-containing nicotinamide cofactors, with \( K_m \) constants for NADP\(^+\) and NADPH in the low micromolar range. Since AKR1C1 fails to oxidize DHN using NADP\(^+\) as cofactor (data not shown), AKR1C1 appears to be a unidirectional NADPH-specific α,β-unsaturated aldehyde reductase. This suggests that AKR1C1 is primed to catalyze the NADPH-dependent reduction of α,β-unsaturated aldehydes.

Identification of 1,4-Dihydroxy-2-nonene as the Product of the AKR1C1-dependent Reduction of 4-Hydroxy-2-nonenal—Since members of the AKR1 family can possess either double bond reductase or aldehyde reductase activities (37), it was unclear whether AKR1C1 was catalyzing the reduction of the \( \Delta^2 \)-double bond or the terminal aldehyde in HNE or both. To distinguish between these possibilities, DHN was chemically synthesized, and its chromatographic profile was compared with that of the enzymatic product of the AKR1C1-catalyzed reaction. Thin layer chromatographic separation revealed that the AKR1C1-derived product (Fig. 4C) possessed an \( R_1 \) value (0.20) identical to that of chemically synthesized DHN (Fig. 4B), and the products of both reactions coeluted when the products were mixed (Fig. 4D).

To establish the structure of the AKR1C1-derived metabolite, the enzymatic product was extracted, isolated by TLC, and subjected to NMR spectroscopy (Fig. 5). The product of the enzymatic reaction gave identical chemical shifts to those assigned to the synthetic standard (Fig. 5, compare A and C). Importantly, there was loss of the aldehydic proton (\( \delta \sim 9.5 \) ppm) and retention of the vinylic protons of the \( \Delta^2 \)-double bond (\( \delta \sim 5.8 \) ppm), yielding a 10-line hyperfine splitting pattern characteristic of a \( J_{AB} \), \( J_{ABD} \), \( J_{ABD} \), and \( J_{BB} \), \( J_{BX} \), \( J_{BX} \) coupling system (Fig. 5, B and C), establishing the identity of the enzymatic product as DHN.

**TABLE I**

| Substrate | \( k_{cat}/K_m \) | min\(^{-1}\)M\(^{-1}\)
|-----------|------------------|
| Benzenedicarbonyl | 2.1 \times 10\(^3\) |
| Benzo[|chrysene-1,2-diol | 6.2 \times 10\(^4\) |
| Benzo[a]pyrene-7,8-diol | 1.2 \times 10\(^2\) |
| 7,12-Dimethylbenzanthracene-3,4-diol | 2.3 \times 10\(^3\) |
| 20α-Hydroxy-4-pregnen-3-one | 4.3 \times 10\(^3\) |
| 9α,11β-Prostaglandin E\(_2\) | 5.1 \times 10\(^5\) |
| Naphthalenedicarboxylic acid | 6.5 \times 10\(^3\) |
| 20β-Hydroxy-5α-pregn-3-one | 3.2 \times 10\(^3\) |

**TABLE II**

| Substrate | \( K_m \) | min\(^{-1}\)M\(^{-1}\) | \( k_{cat} \) | min\(^{-1}\)M\(^{-1}\) | \( k_{cat}/K_m \) | min\(^{-1}\)M\(^{-1}\) |
|-----------|----------|----------|----------|----------|----------|----------|
| 2-Pentenal | 660 \times 80 | 8.7 | 1.3 \times 10\(^4\) |
| 2-Hexenal | 195 \times 30 | 3.7 | 1.9 \times 10\(^4\) |
| 2-Heptenal | 62 \times 17 | 3.7 | 6.0 \times 10\(^4\) |
| 2-Octenal | 37 \times 9 | 3.4 | 9.2 \times 10\(^4\) |
| 2-Nonenal | 6.5 \times 0.8 | 2.3 | 3.5 \times 10\(^5\) |
| 4-Hydroxy-2-nonanal | 32 \times 11 | 2.7 | 8.4 \times 10\(^4\) |

**TABLE III**

| Cofactor | \( K_m \) | min\(^{-1}\)M\(^{-1}\) |
|----------|----------|----------|
| NADP\(^+\) | 1600 \times 280 | 4.1 \times 1.2 |
| NADPH | 6100 \times 2700 | 6.2 \times 0.9 |
was verified by reprobing the blot for the expression of GAPDH. A concurrent time-dependent induction of AKR1C1 enzyme activity was observed in HepG2 cells lysates following HNE treatment as measured by 1-acenaphthenol oxidation (Fig. 6B).

To verify that the ROS- and Michael acceptor-inducible AKR1C1 was responsible for the global increases in AKR1C mRNA caused by HNE, first strand cDNA libraries from untreated and HNE-treated HepG2 mRNA were PCR-amplified with isoform-specific primers for AKR1C1 and β-actin (Fig. 7). Isoform-specific semiquantitative reverse transcription PCR demonstrated that AKR1C1 was the AKR isoform induced following a 6-h exposure of HepG2 cells to 10 μM HNE. By contrast, primer pairs for AKR1C3, an isoform that shows modest induction by ROS, failed to detect induction of this isoform by HNE (data not shown). In previous experiments, we have shown that AKR1A1 is not induced by ROS or Michael acceptors (22).

**DISCUSSION**

Oxidative stress induces the expression of a battery of human enzymes by signaling to antioxidant response elements/electrophilic response elements present in the 5'-flanking region of their respective genes. Regulated genes include GSTs (38, 39), γ-glutamylcysteine synthetase (40, 41), metallothionein (42, 43), and NQO1 (44, 45). The proteins encoded by these inducible genes often represent either phase II (de)toxification enzymes (NQO1, GST) or enzymes involved in antioxidant defense (γ-glutamylcysteine synthetase, metallothionein). The pattern of induction of AKR1C1 by monofunctional and bifunctional inducers and oxidative insult strongly implies that AKR1C1 is another member of the antioxidant response element/electrophilic response element-inducible battery of genes (22). However, the reason for the robust induction of AKR1C1 by GSH-depleting electrophiles (e.g. ethacrynic acid) and/or ROS was unclear. Based on these observations, we determined whether AKR1C1 and/or other closely related human AKRs might either 1) regenerate endogenous antioxidants in the cell or 2) detoxify endogenous toxic metabolites generated during oxidative stress.

Several antioxidant vitamins are protective during oxidative stress, including α-tocopherol (46) and ascorbic acid (47, 48). The closely related 3α-HSD from rat liver (AKR1C9) was previously shown to catalyze the reduction of DHA to ascorbic acid (30). Since such an activity could contribute to the regeneration of an endogenous antioxidant in cells, we determined whether any human AKRs might also catalyze the reduction of DHA to ascorbic acid. Interestingly, DHA reduction was identified as an enzymatic activity of the inducible AKR1C1 and AKR1C3 isoforms. However, the low specific activity (~8 nmol/min/mg) observed at high (1.5 mM) concentrations of DHA made it unlikely that DHA represents a physiologically relevant substrate of AKR1C1 in vivo.

By contrast, HNE appears to be a physiological substrate of AKR1C1 with a catalytic efficiency similar to that of the 20α-hydroxysteroids. The various AKR1C isoforms displayed significant variability in their ability to catalyze HNE reduction, illustrating a notable dissociation of this function within the AKR1C subfamily. It is not surprising that HNE is also a substrate of AKR1C2, since AKR1C1 and AKR1C2 are 98% identical, with only a single conservative amino acid substitution within the active site. AKR1C1 may be preferentially induced by ROS and Michael acceptors over AKR1C2, which has a slightly higher specific activity for two reasons. First, in liver, AKR1C2 (bile acid-binding protein) will be saturated with bile acids, which act as potent inhibitors of AKR1C2 but are far weaker inhibitors of AKR1C1 (49, 50). Thus, AKR1C1 can continue to eliminate HNE in the presence of normal bile acid concentrations. As an aside, under conditions of cholestatic liver damage and/or bile acid duct ligation (51, 52), hepatic HNE metabolism is impaired, and this may result from inhibition of AKR1C1 by elevated bile acids. Second, AKR1C1 undergoes time-dependent inactivation by HNE and may serve a sacrificial role to eliminate HNE at high concentrations. Induction of AKR1C1 may be necessary to replenish AKR1C1 under these conditions.

AKR1C1 also demonstrated specificity for other α,β-unsaturated aldehydes. The catalytic efficiency of AKR1C1 increased over 20-fold as the length of the hydrocarbon backbone increased from five to nine carbons. This is almost entirely due to a decrease in $K_m$ and possibly reflects increased interaction of the hydrophobic substrate with hydrophobic residues predicted to line the substrate binding site (53).

The $K_m$ value of AKR1C1 for HNE (34 μM) is substantially lower than those of isozymes I and II of alcohol dehydrogenase, 250–1430 and 100 μM, respectively (54), which have long been considered to be the sole source of reductive HNE metabolism in many tissues. These $K_m$ values are important when it is considered that under normal conditions the HNE concentration is <1.0 μM but may rise significantly and approach concentrations of 100 μM under conditions of oxidative stress. These arguments would suggest that the $K_m$ constant for AKR1C1 is more favorable to handle physiological concentrations of HNE than that observed with isozymes I and II of alcohol dehydrogenase. The $K_m$ of AKR1C1 for HNE is, however, similar to that reported for the HNE-specific GST isoform GSTA4-4 (15) and the NADH-specific human class IV alcohol dehydrogenase (55). This warrants a comparison of the catalytic efficiencies of these enzymes to determine which is most important in HNE elimination.

Catalytic efficiencies of GSTA4-4 are 500-fold greater than that observed with AKR1C1. Although GSTA4-4 has been characterized in human fetal brain, it has been argued that this isozyme is ubiquitously expressed in a manner similar to its rodent counterparts (15). In addition, GST-catalyzed conjugation of HNE has been described in human liver, heart, cornea, and retina (56). Thus, GST-catalyzed conjugation of GSH to HNE is undoubtedly an important elimination pathway. How-
ever, under conditions of oxidative stress, there is a concomitant decrease in GSH leading to a change in redox state (increased GSSG:GSH) making non-GSH-dependent pathways an important second barrier to the toxic effects of HNE. Importantly, under conditions of oxidative stress (H_2O_2) and drugs that deplete GSH (e.g. ethacrynic acid), AKR1C1 can be induced up to 10-fold, and this induction may be a component of an effective counter-response to this stress.

The recently described human class IV alcohol dehydrogenase possesses a \( k_{\text{cat}} \) which is 2 orders of magnitude higher than that of the AKR1C1 isoform, suggesting that this enzyme is the better catalyst (57). However, the cytosolic concentrations of nicotinamide cofactors favor NAD\(^+\) over NADH and NADPH over NADP\(^+\), suggesting that NADPH-dependent reductases (i.e. AKRs) may be "primed" with the appropriate cofactor for HNE reduction. Calculation of the \( K_m \) constant of AKR1C1 for NADPH (\( K_m^{\text{NADPH}} \)) demonstrates that AKR1C1 will be saturated with NADPH under physiological conditions and will probably possess sufficient affinity for HNE to contribute to its overall reductive elimination. Together, these observations suggest an important role of AKR1C1 in the elimination of the toxic HNE.

To date, the only other enzymes with a similar low \( K_m \) constant for HNE are other AKR1 family members, aldose reductase and FR-1, which gave \( K_m \) values of 22 and 9 \( \mu M \), respectively (17–19). By contrast, AKR1A1 is not saturatable with these high concentrations of HNE (4 mM) and is unlikely to be a significant contributor to the elimination of HNE.

**FIG. 5.** Confirmation that DHN is the enzymatic product of AKR1C reduction of HNE by NMR spectroscopy. The product of the AKR1C1-dependent reaction was extracted, subjected to TLC purification, and then analyzed by high field proton NMR. In the NMR spectrum of both chemically and enzymatically synthesized DHN, note especially the loss of the aldehydic proton at \( \sim 10 \) ppm and the retention of the vinylic protons at \( \sim 5.8–6.2 \) ppm with new resonances, which gave the predicted 10-line splitting pattern for a \( J_{\text{AB}}, J_{\text{ANY}}, J_{\text{ABZ}} \) and \( J_{\text{BA}}, J_{\text{BC}} \) coupling system. A, HNE starting material. B, chemically synthesized DHN standard. C, enzymatically synthesized product. (The inset in A shows the structure of HNE and the splitting patterns for its methine protons; the inset in B shows the structure of DHN and the splitting patterns for its vinylic protons.)

**FIG. 6.** Induction of AKR1C mRNA and enzymatic activity by HNE. A, Northern blot analysis of AKR1C mRNA. HepG2 cells (3 \( \times \) 10^6) were seeded into 10-cm dishes, and 1 day later they were replaced with serum-free medium. Following serum withdrawal for 24 h, HNE (0–100 \( \mu M \)) was added in a final concentration of 0.1% ethanol. At various time points, total RNA was harvested. RNA (5 \( \mu g \)) from the various treatments was electrophoresed, transferred to membranes, and sequentially analyzed for AKR1C and GAPDH expression as described under "Experimental Procedures." Only the levels of the AKR1C transcript are shown. B, induction of AKR1C1 enzymatic activity by HNE. HepG2 cells were exposed to 10 \( \mu M \) HNE, and lysates were prepared over time and assayed for 1-acenaphthenol oxidation (22). Protein content for purposes of normalization was determined by the Bradford method. Data are expressed as -fold induction of AKR1C1-specific activity relative to the time 0 control.

**FIG. 7.** Isoform-specific induction of the ROS- and Michael acceptor-inducible AKR1C1 isoform by HNE. HepG2 cells were treated with Me_2SO (–) or 10 \( \mu M \) HNE (+), and total RNA was harvested after 6 h. RNA (1 \( \mu g \)) from each sample was reverse-transcribed into cDNA and then subjected to linear PCR amplification (12–24 cycles) using specific primer pairs for AKR1C1 and \( \beta \)-actin. PCRs were electrophoresed and visualized with ethidium bromide under UV light.
Reduction of 4-Hydroxy-2-nonanal by the ROS-inducible AKR1C1

AKR1C1 in the reductive elimination of HNE is shown.

Genestein, but not equimolar concentrations of the inactive isomer, depleted GSH. AKR1C1 is expressed at relatively high levels in multiple tissues associated with xenobiotic metabolism/oxidative stress, including lung, liver, and mammary gland (25). Importantly, AKR1C1 is the only known AKR with a low $K_{m}$ for HNE that is robustly induced by ROS and by agents that deplete GSH.

Given the multiplicity of potential HNE-derived metabolites, it was important to determine the identity of the product of the AKR1C1-dependent reduction of HNE. Certain AKR family members function as double bond reductases, and a single point mutation (H117E) is sufficient to introduce a double bond reductase activity into AKR1C9 (37). Whether AKR1C1 can function as a double bond reductase is unknown. Chromatographic separation and subsequent NMR spectroscopy confirmed the structure of the enzymatic product to be that of DHN. These findings verified that human AKR1C1 catalyzes reduction of the terminal aldehyde in HNE. Other experiments demonstrated that AKR1C1 does not function as an oxidoreductase with respect to HNE; it fails to oxidize DHN to DHN. These findings verified that human AKR1C1 catalyzes reduction of the terminal aldehyde in HNE. Other experiments demonstrated that AKR1C1 does not function as an oxidoreductase with respect to HNE; it fails to oxidize DHN to DHN.

DHN is a well characterized metabolite of HNE in multiple tissues in vivo. DHN appears to represent between 10 and 20% of the total metabolic fate of H2O2 resistance (66). The studies presented here as well as by others (17–19) imply that members of the AKR superfamily act as a cytosolic barrier to the products of lipid peroxidation in the absence of GSH. Of these, AKR1C1 is the isomerase that is induced by ROS and HNE as the counter-response to oxidative stress is mounted.

Fig. 8. AKR1C1 catalyzed reduction of HNE to DHN. The role of AKR1C1 in the reductive elimination of HNE is shown.

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The Reactive Oxygen Species- and Michael Acceptor-inducible Human Aldo-Keto Reductase AKR1C1 Reduces the \( \alpha,\beta \)-Unsaturated Aldehyde 4-Hydroxy-2-nonenal to 1,4-Dihydroxy-2-nonene

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