Antioxidative Function and Substrate Specificity of NAD(P)H-dependent Alkenal/one Oxidoreductase

A NEW ROLE FOR LEUKOTRIENE B4 12-HYDROXYDEHYDROGENASE/15-OXOPROSTAGLANDIN 13-REDUCTASE*

Received for publication, June 13, 2001, and in revised form, August 23, 2001
Published, JBC Papers in Press, August 23, 2001, DOI 10.1074/jbc.M105487200

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There are several known routes for the metabolic detoxication of α,β-unsaturated aldehydes and ketones, including conjugation to glutathione and reduction and oxidation of the aldehyde to an alcohol and a carboxylic acid, respectively. In this study, we describe a fourth class of detoxication that involves the reduction of the α,β-carbon=carbon double bond to a single bond. This reaction is catalyzed by NAD(P)H-dependent alkenal/one oxidoreductase (AO), an enzyme heretofore known as leukotriene B4 12-hydroxydehydrogenase, 15-oxoprostaglandin 13-reductase, and dithiolethione-inducible gene-1. AO is shown to effectively reduce cytotoxic lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) (k_{cat}/K_m = 4.0 × 10^9 min^{-1}; k_{cat}/K_m = 3.3 × 10^7 min^{-1} M^{-1}) and acrolein (k_{cat}/K_m = 2.2 × 10^6 min^{-1}; k_{cat}/K_m = 1.5 × 10^6 min^{-1} M^{-1}) and common industrial compounds such as ethyl vinyl ketone (k_{cat}/K_m = 9.6 × 10^5 min^{-1}; k_{cat}/K_m = 8.8 × 10^5 min^{-1} M^{-1}) and 15-oxoprostaglandin E2 (k_{cat}/K_m = 2.4 × 10^5 min^{-1}; k_{cat}/K_m = 2.4 × 10^5 min^{-1} M^{-1}). Furthermore, transfection of human embryonic kidney cells with a rat liver AO expression vector protected these cells from challenge with HNE. The concentration of HNE at which 50% of the cells were killed after 24 h increased from ~15 μM in control cells to ~70 μM in AO-transfected cells. Overexpression of AO also completely abolished protein alkylation by HNE at all concentrations tested (up to 30 μM). Thus, we describe a novel antioxidative activity of a previously characterized bioactive lipid-metabolizing enzyme that could prove to be therapeutically or prophylactically useful due to its high catalytic rate and inducibility.

Dithiolethiones have been shown to be potent cancer chemopreventive agents that up-regulate several classical carcinogen detoxication and antioxidative enzymes, including glutathione S-transferases (GSTs), epoxide hydrolase, UDP-glucuronosyl-

transferases, and NAD(P)H:quinone reductase (1–3). Additional protective enzymes were identified via a differential hybridization screen for 3H-1,2-dithiole-3-thione-inducible genes of rat liver (4). One such gene, DIG-1, was found to be homolog of an inactivator of leukotriene B4 (LTB4) in porcine kidney and act via an NADP+‐dependent dehydrogenation of the 12-hydroxy group of LTB4 (5). The human homolog of this leukotriene B4 12-hydroxydehydrogenase was later cloned and found to have similar substrate specificity (6). A contemporary study identified an enzyme from porcine lung that could reduce the 13,14-carbon=carbon double bond of 15-oxoprostaglandins via an NAD(P)H-dependent mechanism (7). Following the determination of the sequence of this enzyme, a BLAST search revealed its virtual identity to porcine kidney and human leukotriene B4 12-hydroxydehydrogenase. Recently, the porcine homolog has been shown to also inactivate the autacoid lipoxin A4 via an NAD(P)H-dependent reduction (8).

Treatment of LTB4 with DIG-1 purified from rat liver was shown to abolish the chemoattractant activity of LTB4 as well as its ability to induce the respiratory burst of neutrophils (9). Nevertheless, because of a significant sequence homology to quinone reductase (4) and a more vigorous NAD(P)H-dependent activity, we postulated a more prominent role in chemoprotection that extended beyond the metabolism of bioactive lipid messengers. Thus, in this study, the substrate specificity of DIG-1 was probed using various α,β-unsaturated carbonyl compounds.

The process of lipid peroxidation involves the free radical-mediated conversion of polyunsaturated fatty acids to smaller aliphatic, straight chain α,β-unsaturated aldehydes and saturated ketones and aldehydes (10). Of the α,β-unsaturated aldehydes detected, 2-alkenals, 4-hydroxy-2-alkenals, or 2,4-alkadienals are the most common (11). 4-Hydroxy-2-nonenal (HNE) is an abundant and extensively studied product of lipid peroxidation. At physiological concentrations, HNE has been shown to inhibit DNA and protein synthesis, induce apoptosis, alter cell signaling pathways, covalently bind to proteins, and form exocyclic DNA adducts (12). It is mutagenic and considered carcinogenic. HNE is a major constituent of oxidized low density lipoproteins, and HNE–protein adducts are abundant in atherosclerotic plaques and senile plaques associated with Alzheimer’s disease (12–14). Thus, HNE has been implicated in the etiology of cancer, heart disease, and Alzheimer’s disease (15–17).

DIG-1 was found to be very effective in catalyzing the hydrogenation and thus detoxication of a wide variety of α,β-unsaturated thione-inducible gene-1; HNA, 4-hydroxynonanal; ROS, reactive oxygen species.
urated aldehydes and ketones, including lipid peroxidation products, and cytotoxic and mutagenic ketones that are environmentally pollutants (18, 19). For this reason, we propose to rename DIG-1 as NADPH-dependent alkenal/one oxidoreductase (AO).

We previously demonstrated that AO is inducible in rat liver by dithiolethione administration and is thus regulated similarly to phase 2 and antioxidative enzymes (4). In this study, we demonstrate that cells transfected with an AO expression plasmid are well protected from cytotoxic actions of HNE. Thus, we conclude that AO may play a prominent role in protecting cells from the cytotoxic and mutagenic products of lipid peroxidation. Coupled with its ability to inactivate the pro-inflammatory LTB_4, AO appears to be an important attenuator of both the initiation and propagation of the detrimental effects of inflammation.

MATERIALS AND METHODS

Chemicals and Reagents—Most α,β-unsaturated carbonyl compounds were purchased from Sigma, HNE, 4-hydroxy-2,hexenal, and 15-oxoprostaglandin E_2 were purchased from Cayman Chemical Co., Inc. (Ann Arbor, MI). Cell culture media and reagents were purchased from Life Technologies, Inc. EBNA-293 cells and the pCEP4 plasmid were obtained from Invitrogen (Carlsbad, CA).

Expression of AO in Escherichia coli and Purification—AO cDNA from rat liver was isolated previously (4); cloned into the pTrcHisA vector (Invitrogen), which inserts a 6-His tag into the N terminus of the protein, and sequenced. This plasmid, pTrcHis/AO, was then used to transform chemically competent E. coli DH5α. AO was expressed as described previously (20). Recombinant AO was purified using nickel-nitrilotriacetic acid Superflow resin (Qiagen Inc.) as previously described (21). Eluted fractions were analyzed by SDS-polyacrylamide gel electrophoresis with Coomasie Blue staining. Those fractions with >95% purity were pooled and dialyzed against 1 liter of dialysis buffer (10 mM potassium Pi (pH 7.3), 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% Tween 20) overnight at 4 °C. Protein concentration was determined using the BCA reagent (Pierce), and the mixture was incubated with shaking at 37 °C for 24 h and then in serum-free medium containing 0.5% FCS and 0.1% Pen-Sigma (Life Technologies, Inc.) according to the manufacturer’s protocol.

Chromatographic Identification of Carboxyls and Not α,β-Unsaturated Alcohols as the AO Metabolites of α,β-Unsaturated Carbonyls—15-nmol of benzylidene acetone were metabolized by AO using a reaction mixture identical to that used for the chalcone assay. Reactions were incubated at 30 °C for 35 min and then stopped by the addition of 300 μl of ethyl acetate. The samples were mixed and centrifuged, and the organic layer was removed and evaporated. Products were then analyzed by HPLC in the same manner as described for chalcone with the following changes: A 50:50 mixture of acetonitrile/water was used as a mobile phase, and detection occurred at 220, 250, and 285 nm. trans-4-Phenyl-3-buten-2-ol was synthesized according to a protocol used by Burczynski et al. (55) to synthesize 1,4-dihydronoxone. The product was verified by 1H NMR.

4-Hydroxynonenal was synthesized as described previously (38) and verified by 1H NMR. HNE TLC studies were carried out with a 30% ethyl acetate and 70% hexane solvent system and stained with a phosphomolybdic acid reagent. Altecite Reagents (Newark, DE) thin-layer chromatography mass spectrometric studies were performed on a PE-Sciex API 150EX mass spectrometer (PE Biosystems) using positive ion detection.

Synthesis of trans-2-Nonenal- and Nonanal-2,4-Dinitrophenylhydrazine—2,4-Dinitrophenylhydrazine Conjugates—trans-2-Nonenal- and nonanal-2,4-dinitrophenylhydrazine (DNPH) conjugates were synthesized by adding 200 μl of each chemical to 100 ml of DNPH-saturated 2 M HCl (23). The mixtures were stirred and incubated in the dark at room temperature overnight. Precipitates were then filtered and washed with 2 N HCl to remove unreacted DNPH. Dilutions were dissolved in 100 ml of ethyl acetate and sequentially washed three times with 100 ml of 2 N HCl, once with 100 ml of H₂O, and once with 100 ml of brine. The organic layer was dried with anhydrous sodium sulfate and evaporated. The trans-2-nonenal conjugate was recrystallized from ethanol and water, whereas the nonanal conjugate was recrystallized from N,N-dimethylformamide and water. The nonanal–DNPH conjugate was further purified using preparative TLC plates and a 9% ethyl acetate and 91% hexane solvent system.

Identification of Nonanal as the AO Metabolite of trans-2-Nonenal—The concentration of trans-2-nonenal was 10 μg/ml. A 50:50 mixture of acetonitrile/water was added dropwise to 200 ml of 0.5× phosphate-buffered saline. DNPH (115 mg) and 600 μg of AO were then added. The mixture was incubated with shaking at 37 °C for 16 h. The solution was cooled to 4 °C, and 50 ml of concentrated HCl were added. DNPH was then added to saturation, and the mixture was incubated with shaking at 37 °C for 3 h. This aqueous mixture was extracted three times with 150 ml of ethyl acetate. The organic extracts were pooled and washed six times with 170 ml of 2 N HCl, twice with 400 ml of distilled water, and once with 400 ml of brine. The organic extract was dried with anhydrous sodium sulfate and evaporated. The product was then purified using preparative TLC plates and a 9% ethyl acetate and 91% hexane solvent system. The product was eluted from the silica slabs with dichloromethane, evaporated to dryness, and resuspended in Me₂SO-d₆. A Varian UNITY Plus 500-MHz NMR spectrometer was used to obtain the 1H NMR spectra of the standards and metabolite. Chemical shifts (δ) are referenced to tetramethylsilane. Authentic trans-2-nonenal–, 2,4′-dinitrophenyldiazine gave the following spectral assignments: δ 0.87–0.89 (t, 3H, 9-CH₃), 1.26–1.46 (m, 2H, and m, 6H, 5-CH₂, 6-CH₂, 7-CH₂, and 8-CH₂), 2.21–2.26 (q, 2H, 4-CH₂), 6.25–6.36 (m, 2H, 2-CH₂ and 3-CH₂, J = 21, 7.84–7.87 (d, 1H, 6-CH₂), 8.32–8.37 (m, 2H, 1-CH and 5-CH), 8.85 (d, 1H, 3-CH), and 11.43 (s, 1H, NH). 1H NMR analysis of the 2′,4′-dinitrophenyldiazine conjugate of the AO metabolite nonanal–2,4′-dinitrophenyldiazine gave identical spectral assignments. Spectral assignments are as follow: δ 0.86–0.89 (t, 3H, 9-CH₃), 1.26–1.58 (m, 2H, and m, 10H, 3-CH₂, 4-CH₂, 5-CH₂, 6-CH₂, 7-CH₂, and 8-CH₂), 2.21–2.38 (m, 2H, 2-CH₂), 7.82–7.85 (d, 1H, 6-CH), 8.60–8.63 (t, 1H, 1-CH), 8.32–8.35 (dd, 1H, 5-CH), 8.85–8.84 (d, 1H, 3-CH), and 11.34 (s, 1H, NH).

Transfected Cell Activity Assay and 4-Hydroxy-2-nonenal Toxicity Studies—pCEP4/AO- and pCEP4-transfected cells were plated in 96-well culture plates at a cell density of 3000 cells/well. They were grown in complete medium supplemented with 200 μg/ml hygromycin B for 24 h and then in serum-free medium containing 0–500 μM NHE. Cell
viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (24). AO-expressing and control cells were grown in 100-mm culture dishes to 90% confluency for an AO activity assay. Cells were scraped from the dish, washed with phosphate-buffered saline, lysed in phosphate-buffered saline, and centrifuged at 10,000 \( \times g \) for 30 min at 4 °C. 5 \( \mu \)g of pCEP4/AO and 50 \( \mu \)g of pCEP4 supernatants were then used in a standard chalcone assay with the following modifications. The reaction was allowed to proceed for 20 min, and NADPH was added to a final concentration of 0.25 mm.

Slot Blot with Anti-4-hydroxy-2-nonenal Primary Antibody—pCEP4/ AO-transfected and control cells were grown to 90% confluency in 60-mm dishes in complete medium. The medium was then replaced by complete serum-free medium containing 0, 15, or 30 \( \mu \)M HNE. Cultures were incubated for 2 h and then harvested, lysed, and briefly sonicated. Whole cell sonicates were vortexed, and 20 \( \mu \)g of protein were loaded into each well of a slot-blot apparatus (Schleicher & Schuell). A 1:1000 dilution of anti-4-hydroxy-2-nonenal Michael adduct polyclonal rabbit serum (Calbiochem) was used as the primary antibody, and a 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody was used as the secondary antibody. Blots were developed using Eastman Kodak BIOMAX MS film and ECL reagents (Amersham Pharmacia Biotech).

RESULTS

Substrate Specificity of AO—Several \( \alpha,\beta \)-unsaturated aldehydes and ketones were found to be effective substrates for AO (Fig. 1A). These substrates included products of lipid peroxidation (trans-2-alkenals, trans-4-hydroxy-2-alkenals, and trans, trans-2,4-alkadienals); short chain aliphatic alkenes and ketones, which are commonly used industrial reagents (methyl and ethyl vinyl ketones); and aromatic compounds used in fragrances and flavorings (cinnamaldehyde and benzylidene acetone). A series of trans-2-alkenals (compounds 2-8) was used to investigate the effect of hydrophobicity on substrate kinetics. Increasing carbon chain length was inversely proportional to substrate \( K_m \), which saturated at 50 \( \mu \)M and a nine-carbon length (Fig. 1B). Thus, increased hydrophobicity was generally associated with better substrate binding. The catalytic rate constant (\( k_{cat} \)) for this series increased with hydrophobicity until the substrates reached a nine-carbon length and then began to decrease (Fig. 1C). This could be due to less than optimal alignment of catalytic residues of AO and the substrate \( \alpha,\beta \)-double bond caused by the increased bulk of additional methylene groups. trans-2-Nonenal proved to be the best substrate of the series. Interestingly, acrolein (compound 1) did not follow the \( K_m \) trends of the other trans-2-alkenals (Table I).

The kinetics of reduction of several other substrates defined further trends in the substrate specificity of AO. 4-Hydroxylation decreased the catalytic efficiency of the unsaturated nonenals (compounds 6 and 13), but increased the catalytic efficiency of the unsaturated hexenals (compounds 4 and 12). Methylation at the \( \alpha \)-carbon markedly increased the \( K_m \), but did not affect the \( k_{cat} \) significantly (compounds 16 and 17). Methylation at the \( \beta \)-carbon also had drastic negative effects, as mesityl oxide was not a substrate for AO (compounds 11 and 32). Ketones were generally better substrates than aldehydes (compounds 16 and 18, 2 and 11, and 5 and 14). Attachment of a methyl group to methyl vinyl ketone (compound 9) at C-1 to form ethyl vinyl ketone (compound 10) decreased its \( K_m \) 5-fold, but did not affect its extremely high \( k_{cat} \). If 15-oxoprostaglandin E\(_1\) (compound 20) could be considered an example of longer hydrophobic attachments to the carbonyl carbon, then increasing hydrophobicity at this position greatly augmented substrate binding. Unsaturation at the C-4→C-5 bond decreased both the catalytic rate and substrate binding (compounds 6 and 15). A phenyl group attached to the \( \beta \)-carbon imparted a low \( K_m \) to the molecule (compounds 16 and 18). This result could reflect \( \pi \)-bonding to an aromatic residue of the hydrophobic active site or simply increased substrate hydrophobicity. The increased affinity associated with this group was exploited to verify that \( \alpha,\beta \)-unsaturated amides, esters, and carboxylic acids (compounds 24–26) were not substrates for AO (Fig. 2B). Several additional molecules of these classes were screened and produced no detectable AO-mediated NADPH oxidation or inhibition of AO. Malondialdehyde (compound 28), glycyglycine (compound 27), the dithiolethiones 3H-1,2-dithiole-3-thione (compound 29) and oltipraz (compound 30), and 2-cyclohexenone (compound 31) also did not interact with AO.

On the other hand, a few molecules that were found to inhibit AO are shown in Fig. 2A. Progesterone (compound 21), like 2-cyclohexenone, is an \( \alpha,\beta \)-unsaturated ketone and is constrained to a trans-configuration about the carbonyl carbon−\( \alpha \)-carbon single bond. Adoption of the cis-configuration may be important for proper alignment of reactive moieties in the active site. Because progesterone was a good competitive inhibitor of AO, the additional hydrophobicity imparted to the molecule by its B-, C-, and D-rings and methyl groups must allow it to tightly bind in the active site of the enzyme. Cyclovalone (compound 22) has previously been identified as a potent inducer of phase 2 enzymes (25). However, in this study, it inhibited AO noncompetitively. Quercetin (compound 23) also inhibited AO noncompetitively. Quercetin has been reported to bind to both the nicotinamide dinucleotide- and aldehyde-binding sites of aldehyde dehydrogenase (26) and may do so with AO as well.

Chromatographic Identification of the Products of the Reduction of \( \alpha,\beta \)-Unsaturated Aldehydes and Ketones by AO—To verify that AO utilized NADPH to catalyze the reduction of the \( \alpha,\beta \)-double bond and not the carbonyl double bond, benzylidene acetone (compound 18) was metabolized by AO to completion, extracted, and subjected to analysis by HPLC. The metabolite (Fig. 3D) and benzyl acetone standard (Fig. 3B) both eluted at 11.8 min. A NADPH-minus control was performed, and the resulting compound (Fig. 3E) was found to have a retention time (10.3 min) identical to that of benzylidene acetone (Fig. 3F)
Monitored at 285 nm; benzyl acetone chromatographs (Fig. 3, and similar compounds that do not interact with AO. A NADPH-mediated reduction of the buten-2-ol chromatograph (Fig. 3, benzylidene acetone chromatographs (Fig. 3, A-trans-position about the carbonyl carbon 3-trans–4-phenyl-3-buten-2-ol standard was synthesized and analyzed on a mass spectrometer. Large peaks at m/z 181.0 and 213.0 were apparent when the metabolite was dissolved in methanol and analyzed by electrospray ionization mass spectrometry using positive ion detection. These peaks undoubtedly correspond to the Na+ adducts of HNA and HNA-methanol hemiacetal, respectively. Second, to verify that 4-hydroxynonal (HNA) is the metabolic product of AO reduction, HNA was synthesized and compared with the HNE metabolite by TLC. HNA and the metabolite were found to have identical Rf values, not to stain with potassium permanganate, and not to be detectable by UV light. On the other hand, HNE had a slightly lower Rf value than the metabolite, stained with potassium permanganate, and was detectable by UV light. 1,4-Dihydroxy-2-nonenal has been reported to have a much lower Rf value under similar TLC conditions (55) and was not detected in any of our studies.

\begin{table}
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\hline
\textbf{Compound} & \textbf{Km} & \textbf{kcat} & \textbf{kcat/Km} \\
& \textbf{min} & \textbf{min}^{-1} & \textbf{min}^{-1} \textbf{m}^{-1} \\
\hline
1. Acrolein & 0.15 & 223 & 1.5 \times 10^6 \\
2. Crotonaldehyde & 3.9 & 483 & 1.2 \times 10^6 \\
3. trans-2-Pentenal & 2.4 & 1010 & 4.2 \times 10^5 \\
4. trans-2-Hexenal & 1.0 & 1730 & 1.7 \times 10^5 \\
5. trans-2-Octenal & 0.36 & 5380 & 1.5 \times 10^5 \\
6. trans-2-Nonenal & 0.046 & 5670 & 1.2 \times 10^6 \\
7. trans-2-Decenal & 0.04 & 4250 & 1.1 \times 10^6 \\
8. trans-2-Dodecenal & 0.06 & 3610 & 6.0 \times 10^5 \\
9. Methyl vinyl ketone & 0.58 & 9370 & 1.6 \times 10^6 \\
10. Ethyl vinyl ketone & 0.11 & 9600 & 8.8 \times 10^5 \\
11. 3-Penten-2-one & 0.49 & 1110 & 2.3 \times 10^5 \\
12. 4-Hydroxy-2-hexenal & 0.21 & 803 & 3.8 \times 10^3 \\
13. 4-Hydroxy-2-nonenal & 0.12 & 4040 & 3.3 \times 10^3 \\
14. 3-Nonen-2-one & 0.016 & 1190 & 7.3 \times 10^2 \\
15. trans,trans-2,4-Nonadienal & 0.41 & 1030 & 2.5 \times 10^4 \\
16. Cinnamaldehyde & 0.075 & 940 & 1.2 \times 10^4 \\
17. α-Methylcinnamaldehyde & 1.7 & 786 & 4.7 \times 10^3 \\
18. Benzylidene acetone & 0.039 & 3280 & 8.4 \times 10^2 \\
19. Chalcone & 0.18 & 1940 & 1.1 \times 10^3 \\
20. 15-Oxoprostaglandin E1 & 0.001 & 2400 & 2.4 \times 10^4 \\
\hline
\end{tabular}
\caption{Antioxidative Function of Alkenal/one Oxidoreductase}
\end{table}

3A). A trans-4-phenyl-3-buten-2-ol standard was synthesized and found to have an 8.2-min retention time (Fig. 3C). Equimolar (15 nmol) amounts of standards and substrate were used for each HPLC run. No trans-4-phenyl-3-buten-2-ol peak was detected in the benzylidene acetone metabolite chromatogram. The UV-visible absorbance spectra of the metabolite and benzyl acetone were identical (Fig. 3D). The spectra of benzylidene acetone (Fig. 3F) and trans-4-phenyl-3-buten-2-ol (Fig. 3H) were also obtained. Notice the absence of the 250 and 285 nm peaks present in the spectra of trans-4-phenyl-3-buten-2-ol and benzylidene acetone, respectively, in the metabolite spectrum. These absorbance peaks are associated with extensions of the π-structure of the phenyl ring through the α,β-double bond. Benzylidene acetone chromatographs (Fig. 3, A and E) were monitored at 285 nm; benzyl acetone chromatographs (Fig. 3, B and D) were monitored at 220 nm; and the trans-4-phenyl-3-buten-2-ol chromatograph (Fig. 3C) was monitored at 250 nm. Together, these data demonstrate that AO catalyzes the NADPH-mediated reduction of the α,β-double bond rather than the carbonyl group.

A similar experiment was performed using HNE as the substrate. First, to establish that only a hydrogen molecule (H2) is added to the substrate, HNE was metabolized to completion and analyzed on a mass spectrometer. Large peaks at m/z 181.0 and 213.0 were apparent when the metabolite was
lyze. Once the $\alpha,\beta$-double bond is reduced, the molecule has freedom of rotation about the 2,3-single bond, and the alcohol of C-4 can attack the carbonyl carbon to form the cyclic hemiacetal (Fig. 5). This isomerization is physiologically useful in that it reversibly sequesters the aldehyde group, which has the potential to react further, especially with primary amines, and thus abolishes the apparent toxicity of the parent molecule. However, it is experimentally problematic because it eliminates its ability to react with DNPH under standard conditions. Oligomerization was also observed when solutions of the HNE metabolite were concentrated for $^1H$ NMR. Additionally, as mentioned previously, the polarity of the HNE metabolite differed very slightly from that of HNE. This made the metabolite extremely difficult to purify from residual HNE using silica-based methods.

**Protective Effects against 4-Hydroxy-2-nonenal in Cells Overexpressing AO**—Rat liver AO cDNA was cloned into an episomal mammalian expression vector (pCEP4) and transfected into human embryonic kidney EBNA-293 cells. As shown in Fig. 6A, pCEP4/AO-transfected cells had a 100-fold greater AO specific activity compared with cells transfected with an empty vector. Cells were then plated in 96-well plates and challenged with graded concentrations (0–500 $\mu$M) of HNE. The concentration of HNE at which 50% of the cells were killed ($LC_{50}$) after 24 h increased from $\sim15\, \mu$M in control cells to 70 $\mu$M in AO-transfected cells (Fig. 6B). All control cells were killed by 50 $\mu$M HNE, whereas concentrations $>200\, \mu$M were required to kill all of the AO-transfected cells. Plated cells were also challenged with 50 $\mu$M HNE, and cell viability was measured as a function of time (Fig. 6C). All control cells were killed by 24 h, whereas 70% of the AO transfectants remained viable at this time. HNE-treated AO-overexpressing and control cells were then assayed for HNE Michael addition–protein adducts (Fig. 6D). Cells were exposed to 0, 15, or 30 $\mu$M HNE for 2 h to minimize cell detachment from the culture dishes and then harvested, lysed, and sonicated. An immunoblot analysis was performed on whole cell sonicates using a slot-blot apparatus and an anti-HNE Michael addition–protein adduct primary antibody. The results demonstrate that HNE–protein adducts increased manyfold following treatment of control cells with 30 $\mu$M HNE. Band intensity did not increase significantly with 15 or 30 $\mu$M HNE treatment of AO-overexpressing cells. Thus, AO overexpression seems to protect cells from HNE protein damage.

**DISCUSSION**

Dithiolethiones belong to a structurally diverse family of compounds that are known to up-regulate the expression of phase 2 and antioxidative enzymes and to inhibit chemically induced carcinogenesis (27, 28). Members of this class of compounds, including isothiocyanates, mercaptans, trivalent arsenicals, $\alpha,\beta$-unsaturated carbonyls, quinones, and heavy metal salts, are sulfhydryl-reactive and thought to activate transcription of carcinogen detoxication enzymes via reaction with the cysteine-rich sensor protein Keap1 (25, 29, 30). This interaction may release the cytoplasmically sequestered transcription factor Nrf2, which then translocates into the nucleus and binds to the antioxidant-responsive element enhancer (31). Several enzymes, including GST, UDP-glucuronosyltransferases, NAD(P)H:quinone reductase, ferritin, manganese-superoxidemutase, catalase, and $\gamma$-glutamylcysteine synthetase, are up-regulated by dithiolethiones (1, 2). Classical phase 2 enzymes detoxify electrophilic molecules by conjugation to polar compounds that serve to increase solubility and facilitate elimination (32). Antioxidative enzymes play an important role in cellular defense against reactive oxygen species (ROS) by scavenging oxygen radicals and peroxides, preventing ROS generation, and repairing or detoxifying products of the ROS reaction with cellular organic molecules such as lipids and DNA (33).

A subtractive hybridization screen was performed to identify 3H-1,2-dithiole-3-thione-inducible genes of rat liver with the hope of identifying gene products with novel phase 2 or antioxidative capabilities (4). Several previously unidentified gene products were found and named dithiolethione-inducible genes. One gene, DIG-1, was later identified as an NADP$^+$-dependent leukotriene B$_{4}$ 12-hydroxydehydrogenase that is capable of inactivating the pro-inflammatory lipid LTB$_{4}$ (5, 9). A porcine homolog of this enzyme was found to have NADP$^+$-dependent leukotriene B$_{4}$ 12-hydroxydehydrogenase and NAD(P)H-dependent 15-oxoprostaglandin 13-reductase/15-oxolipoxin A$_{4}$ reductase activities (7, 8).

The leukotriene B$_{4}$ 12-hydroxydehydrogenase activity of DIG-1 was originally thought to provide a rationale for the
Antioxidative Function of Alkenal/One Oxidoreductase

coordinate regulation of DIG-1 with phase 2 and antioxidative enzymes. LTB₄ is a potent chemoattractant at subnanomolar concentrations and a stimulator of neutrophil degranulation and superoxide generation at higher concentrations (34). Pretreatment of LTB₄ with DIG-1 and NADP⁺ was shown to abate its ability to stimulate superoxide generation by neutrophils (9). Thus, the antioxidative role of DIG-1 was considered novel in that it served to inactivate eicosanoid inducers of inflammation.

In this study, we chose to investigate the substrate specificity of the more vigorous NADPH-dependent reductase activity of DIG-1. We found that it is capable of reducing the α,β-double bond of a wide variety of electrophilic α,β-unsaturated aldehydes and ketones at appreciable rates (Fig. 1). This activity, which is consistent with 15-oxoprostaglandin and 15-oxolipoxin A₄ reductase activities of porcine homologs, was verified using the substrates trans-2-nonenal and benzylidene acetone (Figs. 3 and 4). Many of these α,β-unsaturated aldehydes (trans-2-alkenals, trans,trans-2,4-alkadienals, and trans-4-hydroxy-2-nonenals) are common products of lipid peroxidation (10). Thus, we propose a different antioxidative role for DIG-1 in which it serves to protect cells from the cytotoxic products of the ROS reaction with their main cellular targets, polyunsaturated fatty acids. This hypothesis was supported by transfecting cells with a DIG-1 expression plasmid and challenging them with the most abundant and cytotoxic product of lipid peroxidation, HNE. DIG-1 has the ability to protect cells from HNE-induced cell death and protein alkylation.

Because of this potent antioxidative activity, we propose the renaming of this enzyme to NADPH-dependent alkenal/one oxidoreductase. This name best suits the in vitro characteristics of this enzyme because its NADPH-dependent reductase activity is up to 3 orders of magnitude greater than its NADP⁺-dependent dehydrogenase activity, and it is relatively nonspecific in its ability to reduce α,β-unsaturated ketones and aldehydes.

HNE has been implicated in the etiology of many diseases, including Alzheimer’s disease, atherosclerosis, myocardial ischemia-reperfusion injury, and cancer (15–17, 35). For this reason, the metabolism of this molecule has been extensively studied. From these widespread studies, three major routes of detoxification have been elucidated. They are the conjugation of glutathione to C-3 via a Michael addition, the oxidation of the aldehyde group to a carboxylic acid, and the reduction of the aldehyde to an alcohol. Although glutathione conjugation is catalyzed by GSTs, class A4-4 in particular (36, 37), oxidation of the aldehyde is catalyzed by aldehyde dehydrogenase. Reduction of the aldehyde is catalyzed by several aldoketo reductases and alcohol dehydrogenase. No study has reported HNA, the product of HNE reduction by AO, as a metabolite. We believe that this is due to the chemical properties of HNA rather than the absence of metabolism by AO. HNA does not have an appreciable UV absorbance; it does not react with DNPH under standard conditions; and it has a polarity that is nearly identical to that of HNE. Haynes et al. (38) have synthesized HNA and reported no apparent cytotoxicity at concentrations up to 2 mM. Extensive metabolism of HNE to 4-hydroxynonanoic acid has been reported in erythrocytes, endothelial cells, and liver (39–41). This metabolite may be the result of an initial reduction of the 2,3-double bond by AO and subsequent oxidation of the aldehyde by aldehyde dehydrogenase. HNE is either inhibitory or a very poor substrate for most aldehyde dehydrogenase isozymes (42–46). The reduction of the α,β-double bond could create a suitable substrate for these enzymes.

Of the enzymes currently identified as having HNE-reducing or -oxidizing capabilities, AO has a superior catalytic rate constant, but a slightly higher Kₘ (Table II). Its catalytic efficiency is similar to those of alcohol dehydrogenases classes II and IV and 7 times greater than the better HNE-metabolizing members of the aldoketo reductase family. However, AO has a much lower catalytic efficiency than GST A4-4 due to both a higher Kₘ and a lower kₗ for AO. Whereas GST A4-4 appears to be the best HNE-detoxifying enzyme, at least kinetically, reductive metabolism and oxidative metabolism are thought to be necessary for cellular survival, as they preserve GSH levels and thus the redox state of cells. Extensive GSH depletion by α,β-unsaturated carbonyl compounds can lead to apoptotic cell death (47).

Although the Kₘ for the AO reduction of HNE is slightly higher compared with several other HNE-metabolizing enzymes, AO expression did impart a significant protective effect at physiological HNE concentrations (1–20 μM) (Fig. 6, B and D). This outcome may be attributed to the relatively high kₗ for AO.
for HNE metabolism by AO or to the fact that since HNE is a lipophilic molecule, it tends to accumulate in hydrophobic cellular membranes. Local membrane concentrations have been estimated to exceed 300 μM (48). In the case of membrane accumulation, AO would provide a relatively unsaturable system for HNE detoxication due to its higher $K_m$ and $V_{max}$ values. Preliminary data suggest that a significant portion of AO is membrane-associated (data not shown). HNE-metabolizing enzymes with significantly lower $K_m$ values (and $V_{max}$ values) such as members of the aldoketo reductase family would presumably saturate very quickly if they served to scavenge membrane aldehydes. Their predominant role may involve the detoxication of lower HNE concentrations found in the cytoplasm.

Several other α,β-unsaturated carbonyl-containing com-

![Fig. 5. Scheme for toxic actions of HNE and for its detoxication by AO.](image-url)

![Fig. 6. Protective effects of AO when overexpressed in human 293 cells and challenged with HNE.](image-url)

**TABLE II**

| Enzyme                                | $K_m$ (μM) | $V_{max}$ (nmol/min/mg) | $k_{cat}$ (min⁻¹) | $k_{cat}/K_m$ (min⁻¹ μM⁻¹) | Ref. |
|---------------------------------------|------------|------------------------|-------------------|---------------------------|-----|
| AO                                    | 0.12       | 12,600                 | 4040              | 3.3 x 10⁷                  | This study |
| Aldehyde dehydrogenase                | 0.017      | 4.51                   |                   |                           | 44  |
| Glutathione S-transferase (A4–4)      | 0.049      | 7950                   |                   | 1.62 x 10⁸                 | 37  |
| Aldoketo reductase (FR-1)              | 0.009      | 22.7                   |                   | 2.5 x 10⁶                  | 53  |
| Aldose reductase                       | 0.022      | 102                    |                   | 4.6 x 10⁶                  | 54  |
| AKR1B1                                | 0.032      | 2.7                    |                   | 8.4 x 10⁴                  | 55  |
| Alcohol dehydrogenase                 |            |                        |                   |                           |     |
| Class I                               | 1.4        | 3570                   |                   | 2.6 x 10⁵                  | 56  |
| Class II                              | 0.097      | 2700                   |                   | 2.8 x 10⁵                  | 56  |
| Class IV                              | 0.038      | 1510                   |                   | 3.9 x 10⁵                  | 57  |
pounds proved to be good-to-excellent substrates for AO. A number of these have been shown to be cytotoxic, mutagenic, and/or carcinogenic (18, 19). Short chain aliphatic 2-alkenals (such as acrolein), alkenones (such as methyl vinyl ketone), 2,4-alkadienones (such as 2,4-decadienal), and 4-hydroxy-2-alkenals (such as HNE and 4-hydroxy-2-hexenal) are thought to readily form exocyclic DNA adducts under physiological conditions (49, 50). These adducts lead to mutations and are formed through the epoxidation of the α,β-double bond and subsequent reaction with nucleophilic atoms of the DNA bases (12, 51). Furthermore, most α,β-unsaturated aldehydes and ketones are capable of covalently binding to other cellular nucleophiles, including lysine, cysteine, and histidine residues of proteins, via a Michael addition mechanism (Fig. 5). This reaction is also dependent on the α,β-double bond. Thus, reduction of this double bond abolishes the ability of the parent molecule to react through the two mechanisms thought to be responsible for its cytotoxicity.

AO belongs to a growing family of α,β-unsaturated carbonyl oxidoreductases. Homologs have been cloned from six mammalian species and a marine sponge. A related plant enzyme, NADPH:azodicarbonyl/quinone oxidoreductase, readily reductively forms through the epoxidation of the α,β-double bond. These adducts lead to mutations and are formed through the epoxidation of the α,β-double bond and subsequent reaction with nucleophilic atoms of the DNA bases (12, 51). Furthermore, most α,β-unsaturated aldehydes and ketones are capable of covalently binding to other cellular nucleophiles, including lysine, cysteine, and histidine residues of proteins, via a Michael addition mechanism (Fig. 5). This reaction is also dependent on the α,β-double bond. Thus, reduction of this double bond abolishes the ability of the parent molecule to react through the two mechanisms thought to be responsible for its cytotoxicity.

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Acknowledgments—We are grateful to Dr. P. Talalay for help in naming AO, Dr. P. Cole for thoughtful comments on the manuscript, Dr. C. Gross for training on 1H NMR and electrospray ionization mass spectrometry, Jennie Chin for support, and especially Patrick Dolan for technical support.

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J. Biol. Chem. 2001, 276:40803-40810.
doi: 10.1074/jbc.M105487200 originally published online August 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105487200

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