Protein Adduct-Trapping by Hydrazinophthalazine Drugs: Mechanisms of Cytoprotection Against Acrolein-Mediated Toxicity

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ABSTRACT

Acrolein is a highly toxic aldehyde involved in a number of diseases as well as drug-induced toxicities. Its pronounced toxicity reflects the readiness with which it forms adducts containing an electrophilic center that can participate in secondary deleterious reactions (e.g., cross-linking). We hypothesize that inactivation of these reactive protein adducts with nucleophilic drugs may counteract acrolein toxicity. Because we previously observed that 1-hydrazinophthalazine (hydralazine) strongly diminishes the toxicity of the acrolein precursor allyl alcohol, we explored the possibility that hydralazine targets reactive acrolein adducts in proteins. We report that hydralazine abolished the immunoreactivity of an acrolein-modified model protein (bovine serum albumin), but only if the drug was added to the protein within 30 min of commencing modification by acrolein. The ability of a range of carbonyl-trapping drugs to interfere with “early” events in protein modification strongly correlated with their protective potencies against allyl alcohol toxicity in hepatocytes. In mass spectrometry studies using a model lysine-containing peptide, hydralazine rapidly formed hydrazones with Michael adducts generated by acrolein. Using an antibody raised against such ternary drug-acrolein-protein complexes in Western blotting experiments, clear adduct-trapping was evident in acrolein-preloaded hepatocytes. In mass spectrometry studies using a model lysine-containing peptide, hydralazine rapidly formed hydrazones with Michael adducts generated by acrolein. Using an antibody raised against such ternary drug-acrolein-protein complexes in Western blotting experiments, clear adduct-trapping was evident in acrolein-preloaded hepatocytes. In mass spectrometry studies using a model lysine-containing peptide, hydralazine rapidly formed hydrazones with Michael adducts generated by acrolein. Using an antibody raised against such ternary drug-acrolein-protein complexes in Western blotting experiments, clear adduct-trapping was evident in acrolein-preloaded hepatocytes.

The highly reactive α, β-unsaturated aldehyde acrolein (2-propenal) participates in many important pathological states. Formed during the combustion of organic matter, it is implicated in the pathogenesis of smoke inhalation injury to the lung (Hales et al., 1988). Acrolein also forms via hepatic biotransformation of the chemotherapeutic drugs cyclophosphamide and ifosfamide (Ludeman, 1999). In addition, there is growing recognition that endogenous acrolein, formed via lipid peroxidation, mediates cell damage in various diseases of old age, including Alzheimer’s disease (Uchida et al., 1998b; Uchida, 1999; Lovell et al., 2001).

Acrolein’s pronounced toxicity reflects its reactivity as a bifunctional electrophile, ensuring that it readily attacks electron-dense centers in DNA and protein (Esterbauer et al., 1991). This reactivity underlies most of the cellular effects of acrolein, including alterations in the activity of transcription factors such as AP-1, nuclear factor κB, and Nrf2 (Horton et al., 1999; Biswal et al., 2002; Tirumalai et al., 2002); inhibition of cytokine production (Li et al., 1997); and cell death (Li et al., 1997; Kern and Kehrer, 2002).

Typically, cellular nucleophiles target acrolein’s β-carbon, generating carbonyl-retaining Michael adducts (Esterbauer et al., 1991; Uchida et al., 1998a; Burcham and Fontaine, 2001). The reactive carbonyl may then react with neighboring nucleophiles to form inter- or intramolecular cross-links (Esterbauer et al., 1991; Permana and Snapka, 1994; Kurtz and Lloyd, 2003). During reactions with protein, acrolein readily carbonylates lysine, cysteine, and histidine side
chains (Esterbauer et al., 1991; Uchida, 1999). Uchida and associates (1998a) have shown that lysine modification involves sequential addition of two acrolein molecules to a given residue, followed by ring fusion and dehydration to form a six-membered heterocycle, N^\prime-(3-formyl-3,4-dehydropiperidino)lysine (FDP-lysine) (Fig. 1). Antibodies raised against FDP-lysine have revealed elevated levels of acrolein-adducted proteins in the affected tissues of several degenerative diseases (Uchida et al., 1998a,b; Calingasan et al., 1999; Tanaka et al., 2001).

With increasing evidence for an involvement of toxic carbonyls in human disease, we began a search for nucleophilic compounds that attenuate the toxicity of acrolein and related lipid-derived aldehydes (Burcham et al., 2002). This effort is part of a wider search for “carbonyl-trapping” drugs to counteract the toxicity of endogenous carbonyls (Shapiro, 1998).

In simple terms, the goal is to identify nucleophiles that react with carbonyls at a faster rate than do cell macromolecules, thereby ensuring carbonyls are safely excreted as drug-carbonyl conjugates (Shapiro, 1998). Efforts have typically focused on scavengers of sugar-derived carbonyls, although such agents may also trap lipid-derived substances (Burcham et al., 2002). Thus the advanced glycation end-product inhibitor aminoguanidine has been found to trap malondialdehyde and 4-hydroxy-nonenal (Al-Abed and Bucala, 1997), carnosine can trap 2-trans-hexenal, 4-hydroxy-nonenal, and malondialdehyde (Hipkiss et al., 1998; Zhou and Decker, 1999; Aldini et al., 2002), and pyridoxamine readily traps 9- and 13-keto-octadecadienoic acids (Onorato et al., 2000). The structure of these various nucleophiles are shown in Fig. 2.

Recently, we identified 1-hydrazinophthalazine (hydralazine) as an efficient acrolein scavenger and a powerful inhibitor of acrolein-mediated toxicity in hepatocytes (Burcham et al., 2002). The drug afforded strong cytoprotection at concentrations that were several orders of magnitude lower than those of the other scavengers tested, including aminoguanidine, carnosine, and pyridoxamine (Burcham et al., 2002). These findings concurred with an early demonstration that hydralazine protects rats against the cardiovascular toxicity of allylamine, a syndrome involving acrolein formation via vascular amine oxidases (Lalich and Paik, 1974). In pharmacological terms, hydralazine is a vasodilatory antihypertensive agent, but its ability to suppress acrolein toxicity is more likely caused by its chemical properties. Possessing a strongly nucleophilic hydrazine, it readily scavenges several biogenic keto compounds; e.g., formation of a pyruvic hydrazone is a key systemic fate in humans (Reece, 1981). We have recently isolated two novel hydrazones formed during acrolein scavenging by hydralazine (L. M. Kaminskas, S. M. Pyke, and P. C. Burcham, unpublished observations).

Although many substances act as carbonyl-trapping agents in cell-free systems, evidence that they exert such actions in biological systems is often limited. Ideally, such proof requires confirmation that candidate nucleophiles attenuate adduction of cellular targets by toxic carbonyls in cells. This goal formed the basis for the present work, which sought to determine whether hydralazine’s cytoprotective potency against acrolein-mediated toxicity in hepatocytes involved protection against protein adduction. Unexpectedly, the results indicate that rather than simply preventing protein modification by free acrolein, hydralazine actively targets protein-bound acrolein. We hypothesize that this novel property underlies the cytoprotective potency against acrolein toxicity, because by inactivating reactive centers in the proteome, hydralazine may prevent secondary reactions that trigger cell death.

Fig. 1. Chemistry of reaction of acrolein with lysine. See Uchida (1999) for details.

**Materials and Methods**

Materials. Allyl alcohol, poly-l-lysine-HBr (molecular weight laser light-scattering detector, 19.6 kDa), poly-l-histidine-HCl (molecular weight laser light-scattering detector, 16.1 kDa), bovine serum albumin (fraction V, essentially globulin-free, >99%), hydralazine, dihydralazine, carnosine, aminoguanidine, methoxyamine, and pyridoxamine were purchased from Sigma-Aldrich (St. Louis, MO). Keyhole limpet hemocyanin, secondary antibody, and Freund’s Adjuvant came from Pierce (Rockford, IL). Adult male Swiss mice (5–6 weeks old) were obtained from Animal Services at the Waite Institute of the University of Adelaide (Adelaide, SA, Australia) and housed under normal conditions until use as hepatocyte donors. All procedures involving animal use were approved by the institutional Animal Ethics Committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals promulgated by the National Institutes of Health.

**Cell-Culture Experiments.** Hepatocytes were prepared by collagenase digestion of mouse livers as outlined previously (Burcham and Fontaine, 2001). Cells were washed four times in collagenase-free Krebs-Henseleit buffer before they were resuspended at a density of 1 × 10^6 cells per ml in RPMI 1640 media supplemented with 0.2% bovine serum albumin (BSA), 0.03% l-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml). Hepatocytes were then layered on collagen-coated 60-mm polystyrene dishes before they were placed in a humidified CO_2 incubator at 37°C (5% CO_2). After 2 h, nonadherent cells were removed by washing the dishes twice with phosphate-buffered saline (PBS) before fresh aliquots of RPMI 1640 medium (not supplemented with BSA) were added. The various nucleophilic drugs (e.g., hydralazine and dihydralazine) or toxic treatments (i.e., acrolein and allyl alcohol) were added to media immediately before use. For the experiments involving direct addition of acrolein to cell media (Fig. 1), Krebs-Henseleit buffer, pH 7.3, supplemented with glucose (5 g/l) and pyruvate (1 mM) was used in place of RPMI 1640 medium, in an effort to minimize extracellular side-reactions between free acrolein and nucleophilic buffer constituents (Horton et al., 1999). RPMI 1640 medium was used in all experiments that involved the use of allyl alcohol as an intracellular acrolein precursor. Dishes were returned to the incubator, and samples of culture media were taken for the assessment of lactate dehydrogenase (LDH) leakage at 0, 30, 60, 120, and 180 min or as indicated in the appropriate figure legends.

In some experiments, hepatocytes were exposed to a toxic concentration of allyl alcohol (100 μM) for 25 min, and then the culture media were removed and the monolayers were washed once with...
PBS. The cells were then layered with fresh solutions of culture media containing 5 to 50 μM hydralazine. The cells were returned to the incubator, and aliquots of media were removed for the determination of LDH activity at the same time points specified above. LDH activity was measured using a spectrophotometric procedure described elsewhere (Burcham and Fontaine, 2001). For the determination of total LDH activity, cells were lysed by adding 250 μl of 5% Triton X-100 to each dish before they were sonicated for 15 s using a Labsonic 1510B Cell Disrupter (B. Braun Melsungen AG, Melsungen, Germany).

**Antibody Production and Characterization.** Antiserum against acrolein-modified protein was obtained as outlined recently (Burcham et al., 2003). To prepare antiserum against hydralazine-stabilized acrolein-adducted protein, keyhole limpet hemocyanin (KLH; 5 mg/ml) was modified with 5 mM acrolein for 25 min, and then hydralazine was added to a final concentration of 10 mM. After 4 h at 37°C, the mixture was diluted 1:9 with PBS and then 3:1 with Freund's Complete Adjuvant before it was administered to an adult male New Zealand white rabbit (1 mg over 10 injection sites). The rabbit received two subsequent booster injections at 3-week intervals using freshly prepared antigen diluted in Freund's Incomplete Adjuvant. Ten days after the final boost, the rabbit was anesthetized, and whole blood was collected via cardiac puncture. Serum was prepared and analyzed for cross-reactivity against unmodified BSA or BSA that had been modified by acrolein in the presence and absence of hydralazine using a competitive ELISA similar to that described recently (Burcham et al., 2003).

Epitope characterization was carried out using acrolein/hydralazine-adducted poly-L-lysine and poly-L-histidine in antigen-competition ELISA experiments (Chen et al., 1992). To make the inhibitors, aminoacyl polymers were dissolved in 50 mM sodium phosphate buffer, pH 7.0, to a final concentration of 1 mg/ml, and then acrolein was added to give a 1:1 aldehyde/monomer molar ratio. Reactions were allowed to proceed for 30 min at 37°C, and then hydralazine was added to give a 2:1 M ratio relative to acrolein. After reaction overnight at 37°C, the inhibitors were stored at −20°C until use in competitive ELISA experiments using methods described recently (Burcham et al., 2003).

**SDS-PAGE and Western Blotting.** Cell monolayers were rinsed with PBS and then resuspended in 0.3 ml of ice-cold lysis buffer (containing 0.25% SDS, 30% glycerol, 50 mM Tris-HCl, pH 6.8, 0.5% Triton X-100, Sigma Protease Inhibitor cocktail, 5 μg/ml, 0.2 mg/ml phenylmethylsulfonyl fluoride, and 1 mM benzamidine). In experiments in which cell proteins were assessed for acrolein adducts, 50 mM phosphate buffer, pH 7.0, was substituted for Tris-HCl (Burcham et al., 2003). Lysates were prepared by sonicating the samples (60 s) on ice and then centrifuging the resulting suspensions at 5,000g for 10 min at 4°C. After protein estimation (Pierce BCA Kit), 50 μg of protein was resolved overnight at 4 V/cm on either a 4 to 20% gradient acrylamide gel (Jule Inc. Biotechnologies, Milford, CT) or a 10% acrylamide gel. After transfer to nitrocellulose (100 V, 30 min), membranes were blocked with 5% nonfat milk in PBS and then reacted for 60 min with 1/1000 dilutions of respective rabbit antisera (raised against either acrolein-modified KLH or hydralazine-stabilized acrolein-modified KLH). After washing and exposure of membranes to horseradish peroxidase-coupled goat anti-rabbit IgG serum (Immunopure, 1/1000 dilution for 30 min; Pierce), membranes were washed and developed using Super Signal West Pico chemiluminescence reagent (Pierce) and Kodak BioMax Light film (Eastman Kodak, Rochester, NY). In some instances, the resulting images were analyzed via densitometry using Kodak Digital Science software (Eastman Kodak).
ducts in a model peptide, preproenkephalin fragment 128 to 140 (PPE). PPE is a 13-mer peptide that possesses a single central lysine residue (GGEVLGLRYGGFM; molecular weight, 1370). PPE and acrolein were dissolved in H₂O to give final concentrations of 100 and 1000 μM, respectively. After 30 min at 37°C, an equivalent volume of hydralazine solution was added to give a final drug concentration that was 10-fold in excess relative to acrolein. The samples were returned to the incubator for a further 30 min. Immediately before injection into the MS, samples were diluted 1:1 with an aqueous solution comprising 2% glacial acetic acid and 50% acetonitrile. MS analyses were performed using a Finnigan LCQ mass spectrometer in positive ESI mode (Thermo Finnigan, San Jose, CA). Samples were introduced into the electrospray source using a syringe pump at a flow rate of 8 μl/min. The spray voltage was set at 4.8 kV with a capillary temperature of 200°C and a cylinder gas (nitrégé) pressure of 100 psi. Mass spectra were collected by scanning a m/z range of 1000 to 2000.

**Statistical Analysis.** Cell toxicity data (i.e., LDH leakage time course curves) were analyzed via two-way analysis of variance followed by Bonferroni’s post-test using GraphPad Prism 4.01 for Windows software (GraphPad Software Inc., San Diego, CA).

**Results**

Because the readiness with which acrolein modifies cell macromolecules underlies its toxicity, an efficient acrolein-trapping agent should ideally prevent such damage. To determine whether hydralazine protects cell proteins against acrolein addition, we used Western blotting to detect acrolein-lysine adducts in mouse hepatocyte proteins after a 15-min exposure to a cytotoxic concentration of acrolein. The experiment was performed in amino acid-free buffer, thereby minimizing side reactions involving acrolein (Horton et al., 1999). The antibody used to detect adducted proteins was raised against acrolein-modified KLH, and it is highly selective for acrolein-adducted lysine residues (Burcham et al., 2003). Because of high basal levels of adducts in controls and concerns over adduct stability during SDS-PAGE, a high concentration of acrolein (0.5 mM) was used in this experiment, necessitating high concentrations of hydralazine to afford cytoprotection. The latter was confirmed by following the time course of LDH leakage into culture media (Fig. 3A). Protein adducts were assessed after a 15-min exposure period to avoid any loss of adducted proteins via a ruptured cell membrane that was evident at later time points (Fig. 3B). Compared with levels in control cells (lane 1, Fig. 3B), exposure to 0.5 mM acrolein increased adduction for a number of proteins in the 20- to 85-kDa range (lane 3, Fig. 3B). Hydralazine had a striking effect on the immunoreactivity of proteins in both control (lane 2) and acrolein-exposed cells (lanes 4–6, Fig. 3B), with the drug decreasing levels of adducted proteins below control values. This finding implied that the drug did not simply prevent protein adduction by free acrolein, but instead it interfered with the immunoreactivity of acrolein-adducted proteins.

Because hydrazine compounds readily form hydrazones with carbonyl groups, the known chemistry of lysine addition by acrolein (Fig. 1) suggested that “early” acyclic Michael adducts might be key targets for hydralazine. To explore this in a cell-free system, we examined the time-dependence of the reactivity of acrolein adducts in a model protein (BSA) with hydralazine. BSA was modified with 1 mM acrolein for 15, 30, 60, or 120 min. At each time point, an aliquot of reaction mixture was treated with 1 mM hydralazine for a further 30 min at 37°C. The BSA was then analyzed for acrolein-lysine adducts via Western blotting (Fig. 4A). Consistent with the observations in cells, adducts generated during brief incubations with acrolein (15 and 30 min) were very susceptible to hydralazine, yet those formed during extended reactions (60 and 120 min) resisted attack by the drug (Fig. 4A). These findings are consistent with our recent observation that early adducts formed during protein modification by acrolein are sensitive to the nucleophilic buffer triis(hydroxymethyl)aminomethane (Burcham et al., 2003).

We reported recently that dihydralazine, a close analog of hydralazine, is a highly efficient acrolein-trapping reagent and displays 2-fold greater cytoprotective potency against acrolein-mediated toxicity in hepatocytes compared with hydralazine (Burcham et al., 2002). In the same study, we established that 1000-fold higher concentrations of the versatile carbonyl-scavenger methoxyamine were required to achieve comparable cytoprotection, whereas other carbonyl-trapping compounds (aminoguanidine, carnosine, and pyridoxamine) (Fig. 2) were not protective even at 10 mM concentrations (Burcham et al., 2002). To determine whether unequal abilities to interfere with early acrolein adducts contribute to these different protective potencies, we compared hydralazine, dihydralazine, or methoxyamine (Fig. 4B), and aminoguanidine, carnosine, or pyridoxamine (Fig. 4C) for their ability to attack early acrolein adducts in BSA. For this experiment, BSA was treated with 1 mM acrolein for 30 min, then for an additional 30 min with 50 to 500 μM concentrations of each carbonyl-trapping compound. In keeping with their strong cytoprotective potencies, hydralazine and especially dihydralazine strongly diminished the immunoreactivity of acrolein-modified BSA (Fig. 4B), whereas the nonprotective agents (aminoguanidine, carnosine, and pyridoxamine) had no effect on adduct immunoreactivity (Fig. 4C). The moderately cytoprotective carbonyl scavenger, me-

**Fig. 3.** Loss of acrolein-lysine adducts in mouse hepatocytes accompanies protection against acute acrolein toxicity by hydralazine. A, cells were exposed to 0.5 mM acrolein in the presence and absence of 0.3 to 3 mM hydralazine, with aliquots of culture media removed for LDH determinations at the times shown. Each data point represents the mean ± S.E. of three independent observations. The various treatments are: , control cells; ○, 3 mM hydralazine; A, 0.5 mM acrolein; ●, acrolein plus 0.3 mM hydralazine; ○, acrolein plus 1.0 mM hydralazine; □, acrolein plus 3.0 mM hydralazine.***, significant difference between acrolein-treated cells and other treatments at the time point indicated (Bonferroni’s post test, p < 0.001). B, acrolein-lysine adducts were measured at 15 min, before overt loss of membrane integrity. Lane 1, control cells; lane 2, 3 mM hydralazine only; lane 3, 0.5 mM acrolein only; lane 4, 0.5 mM acrolein plus 0.3 mM hydralazine; lane 5, 0.5 mM acrolein plus 1.0 mM hydralazine; and lane 6, 0.5 mM acrolein plus 3.0 mM hydralazine. The depicted blot is representative of results obtained in two independent experiments.
to diminish adduct levels (Fig. 4B). These findings reinforce the conclusion that aryldiazino drugs such as hydralazine and dihydralazine display heightened reactivity toward acrolein-derived protein adducts.

To explore the possible chemical basis for these observations, we used ESI-MS to monitor reaction products during modification of a lysine-containing model peptide by acrolein and also to identify species formed upon the addition of hydralazine. The peptide used, PPE, was selected because it possesses a single central lysine, which others have shown is a key target during reactions of acrolein with proteins (Uchida et al., 1998a). Furthermore, PPE’s suitability for use in this study was enhanced by the fact that it lacks other residues known to react with acrolein (e.g., cysteine and histidine). For these experiments, PPE (100 µM in H2O) was reacted with a 10-fold excess of acrolein for 30 min (37°C). The mixtures were then divided into two portions before they were subjected to a second 30-min reaction in the presence and absence of a 10-fold molar excess of hydralazine (compare acrolein). Finally, the respective samples were analyzed via ESI-MS, with representative MS spectra shown in Fig. 5, which also shows the structures of the main species detected during these analyses. Analysis of native PPE revealed a dominant MH+ ion corresponding to unmodified peptide at m/z 1370 (data not shown). The spectrum obtained during MS analysis of peptide that had been treated with hydralazine alone was essentially identical with that obtained from unmodified PPE (data not shown). In Fig. 5A, acrolein’s diverse reactivity with lysine is evident: a minor MH+ ion corresponding to a Schiff base product formed during reaction of acrolein’s carbonyl group with PPE is evident at m/z 1408 (1), whereas the expected MH+ ions for the mono- and bis-Michael adducts are detected at m/z 1426 (2) and 1482 (4) (Fig. 5A). An MH+ ion corresponding to formation of the condensed-ring product FDP-lysine in PPE is evident at m/z 1464 (3) (Fig. 5A). The addition of hydralazine to acrolein-modified PPE generated several new ions (Fig. 5B). The relative abundance of the mono adduct (m/z 1426) seemed to be diminished, with the expected hydrazone reaction product evident at m/z 1568 (5). A single hydralazine molecule also reacted with the bis-acrolein adduct, generating a hydrazone at m/z 1624 (7) (Fig. 5B). Finally, an ion corresponding to the reaction of hydralazine with the formyl group of FDP-lysine is detected at m/z 1606 (6). Because of inherent limitations of ESI-MS analysis, strict quantitative conclusions cannot be drawn from the data in Fig. 5B, but the findings clearly confirm the formation of hydralazine species during adduct-trapping reactions by hydralazine at carbonyl-retaining acrolein adducts in PPE.

These findings suggested the possibility of raising antibodies against “hydralazine-trapped” acrolein-protein adducts, in the expectation that such a tool would be very useful for exploring the biological significance of hydralazine’s reactivity with acrolein-adducted proteins. We thus immunized a rabbit with antigen that had been prepared by sequentially modifying KLH with acrolein and hydralazine as was outlined under Materials and Methods. Testing of the resulting antiserum in an ELISA procedure revealed that it is highly reactive toward BSA that was modified by acrolein and hydralazine but not toward unmodified BSA or BSA that was modified by acrolein or hydralazine alone (Fig. 6A). Furthermore, during competitive ELISA studies using acrolein and hydralazine-modified polyamino acids as immunoinhibitors, it was established that the antiserum strongly recognized hydralazine-acrolein-lysine species, with lesser activity at hydralazine-acrolein-histidine complexes (Fig. 6B). Using the antiserum, we then verified that “adduct-trapping” accompanied the abolition of acrolein-lysine adducts by hydralazine under the conditions used in Fig. 3B. Thus, BSA was exposed to 1 mM acrolein for 30 min and then to 50 to 500 µM concentrations of hydralazine for 30 min. The Western blot shown in Fig. 6C reveals strong adduct-trapping by hydralazine over the concentration range that abolished acrolein-lysine adducts in Fig. 3B.

To determine whether hydralazine’s adduct-trapping actions accompany the protection it affords against acrolein-mediated toxicity, we conducted experiments in mouse hepatocytes using the acrolein precursor allyl alcohol, which causes time- and concentration-dependent toxicity in these...
Fig. 5. ESI-MS spectra obtained during analysis of acrolein- and hydralazine-modified PPE. A, effect of a 30-min reaction with acrolein (ACR), showing new ions caused by the formation of a Schiff base adduct (1), mono- (2) and bis-Michael (4) addition adducts, and the cyclised adduct FDP-lysine (3). B, addition of hydralazine (HYD) generated several new ions caused by hydrazone formation, namely ions 5 (formed from the mono-Michael adduct), 6 (derived from FDP-lysine), and 7 (derived from the bis-Michael adduct). Suggested structures for each species are shown at the bottom. The spectra shown are representative of results obtained during five independent replicates of the experiments.
cells via alcohol dehydrogenase-catalyzed conversion to acrolein (Burcham and Fontaine, 2001). Allyl alcohol is useful for exploring the biological consequences of adduct-trapping drugs because its toxicity is separable into distinct “metabolism/adduction” and “postadduction” phases (Rikans and Cai, 1994). We hypothesized that if protein adduct-trapping played any role in cytoprotection, hydralazine should protect cells when present only during the secondary postadduction phase. To assess this possibility, mouse liver cells were treated briefly with allyl alcohol (100 μM) to allow metabolism and protein adduction to occur (Rikans and Cai, 1994). Before the onset of cell death (25 min), the culture media were removed and the cells were washed with PBS. They were then exposed to fresh media containing 5 to 50 μM hydralazine for up to 3 h, over which time the onset of cell death was assessed at regular intervals via measurements of LDH leakage. Consistent with the hypothesis that cytoprotection involves an ability to interfere with events that are “downstream” of metabolism and adduction, Fig. 7A shows that hydralazine was essentially as protective during the secondary postadduction phase as when it was present during the entire phase of toxicity (Fig. 7B).

Western blotting was then used to determine whether adduct-trapping occurred under conditions in which cytoprotection is afforded by hydralazine during the postadduction phase of allyl alcohol toxicity. Figure 7C shows drug-trapped adducts in proteins from allyl alcohol-pretreated cells after a secondary 30-min incubation in the presence and absence of hydralazine. No immunoreactivity was evident in control cells (lane 1, Fig. 7C) or in cells pretreated only with allyl alcohol (lane 3), although trapped adducts were detected in two ~130-kDa proteins in control cells exposed only to hydralazine (50 μM), presumably reflecting reactions of hydralazine with carbonyl-retaining protein adducts of endogenous origin (lane 2). In striking contrast, intense, concentration-dependent adduct-trapping occurred in allyl alcohol-pretreated cells that were subjected to a second incubation with a range of cytoprotective hydralazine concentrations (5–50 μM, lanes 4–6). For the data shown in Fig. 7D, a greater amount of protein was analyzed to determine whether adduct-trapping also occurred in the postadduction phase at low hydralazine concentrations (2–10 μM) that are of greater relevance to human pharmacology. Clear concentration-dependent trapping was evident over this range, with densitometric analysis of several midsize proteins (37, 40, and 43 kDa, indicated with arrows in E) revealing 12- to 200-fold increases in band intensity over the drug concentration range of 4 of 10 μM (Fig. 7E). Faint adduct-trapping reactions involving the ~130-kDa proteins are evident even at the lowest hydralazine concentration studied (2 μM, lane 1). This concentration is close to peak plasma levels of hydralazine in hypertensive human subjects after a 50-mg oral dose of hydralazine (Hanson et al., 1983).

**Discussion**

The present work breaks new ground by showing that the strongly nucleophilic antihypertensive drug 1-hydrazinophthalazine (hydralazine) is highly reactive with acrolein-modified proteins, readily forming hydrazone adducts. Most importantly, concentration-dependent adduct-trapping reactions occurred at drug concentrations that provided strong cytoprotection in mouse hepatocytes, suggesting that inactivation of protein adducts by hydralazine attenuates acrolein-induced cell death pathways. This conclusion was strengthened by the finding that the ability of a range of carbonyl-trapping re-

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**Fig. 6.** Immunochemical detection of hydralazine-trapped acrolein adducts in BSA. A, immunoreactivity of hydralazine/acrolein/KLH antiserum in a direct ELISA using either unmodified BSA (■), acrolein-modified BSA (□), hydralazine-modified BSA (●), or acrolein/hydralazine-modified BSA (▲) as absorbed antigen. Acrolein/hydralazine-modified BSA was prepared by reacting BSA (2 mg/ml) with 5 mM acrolein (25 min) before 10 mM hydralazine was added for an additional 4 h. B, competitive ELISA using polyamino acid inhibitors to facilitate epitope characterization. The inhibitors were prepared as described under Materials and Methods. The treatments were: ●, unmodified polylysine; ■, unmodified polyhistidine; □, acrolein/hydralazine-modified polylysine; and ▲, acrolein/hydralazine-modified polyhistidine. Data are expressed as a percentage of control. In A and B, the depicted data (mean ± S.E.) were obtained during two independent experiments performed in triplicate. C, concentration-dependent adduct-trapping by hydralazine in acrolein-premodified BSA. BSA (10 mg/ml) was treated with 1 mM acrolein for 25 min at 37°C in 50 mM sodium phosphate, pH 7.0, before the addition of hydralazine to give 0 (lane 1), 50 (lane 2), 100 (lane 3), 250 (lane 4), or 500 μM (lane 5). After an additional 30-min reaction at 37°C, aliquots containing 20 μg of BSA were resolved via SDS-PAGE and assessed via Western blot analysis.
agents to abolish the immunoreactivity of acrolein-adducted protein correlated closely with their cytoprotective potency against allyl alcohol toxicity in hepatocytes. These collective findings suggest that adducts formed in the early stages of protein modification by acrolein are chemically reactive entities that participate in downstream reactions that otherwise trigger cell death (e.g., protein-protein or protein-DNA cross-linking reactions). Intervention in these downstream reactions with nucleophilic adduct-trapping or adduct-deac-
tivating drugs may explain the suppression of toxicity by these compounds.

These findings reinforce the notion that hydralazine possesses a range of diverse chemicopharmacological properties. Indeed, despite clinical use of hydralazine for more than 40 years, the mechanisms underlying its major pharmacological action—arteriolar vasodilatation—remain subject to dispute (Vidrio et al., 2003). Thus, hydralazine’s main pharmacological effects have been variously attributed to guanylate cyclase activation (Schultz et al., 1977), inhibition of inositol triphosphate-induced calcium release from the sarcoplasmic reticulum (Ellershaw and Gurney, 2001), and increased ion flux at high-conductance Ca²⁺-activated K⁺ channels (Bang et al., 1998). In addition, hydralazine also strongly inhibits copper-containing semicarbazide-sensitive amine oxidases (Lyles et al., 1983). Our present findings indicate that in addition to the reversible interactions with various subcellular targets that underlie these diverse pharmacological actions, hydralazine’s chemical properties (i.e., nucleophilicity of hydrazine substituent) allow it to form stable, covalent adducts with reactive centers introduced into proteins by toxic bifunctional aldehydes. Whereas more work is needed to evaluate the in vivo relevance of this mechanism of drug action, it is significant that an early study demonstrated protective actions for hydralazine against the cardiovascular toxicity of allylamine in rats, a syndrome in which acrolein plays a central role (Lalich and Paik, 1974). More recently, evidence that hydralazine reacts with carbonyl-containing modified proteins in vivo emerged during study of the drug’s effect on the progression of diabetic nephropathy in spontaneously hypertensive rats (Nangaku et al., 2003). After 20 weeks’ administration at a dose rate of 5 mg/kg/day, hydralazine strongly diminished the severity of proteinuria and glomerular lesions in the animals. Most importantly, the renoprotection was accompanied by diminished levels of the advanced glycation end-product pentosidine (Nangaku et al., 2003). Because protein glycation/glycoxidation involves the formation of a number of reactive bifunctional carbonyls, it is feasible that hydralazine may target carbonyl-retaining adducts formed during the course of macromolecular modification by these toxic species. Ongoing work in our laboratory is addressing whether hydralazine targets protein adducts formed by biogenic carbonyl compounds in addition to those formed by short-chain 2-alkenals such as acrolein.

The present findings contrast with results obtained by others during study of the reactivity of hydrazinio compounds with oxopropenyl adducts formed by malondialdehyde, a three-carbon dialdehyde that exhibits strong chemical and toxicological similarities to acrolein (Giloni et al., 1981). Indeed, Ottender and associates (2002) recently demonstrated that pentafluorobenzylhydrazine efficiently “strips” malondialdehyde adducts from such model nucleophiles as DNA bases and lysine, thereby restoring the structural integrity of the target molecule. We initially hypothesized that such an adduct-stripping mechanism might underlie the apparent disappearance of acrolein-adducted proteins from hydralazine-treated cells in Fig. 3B. However, because MS analysis of acrolein-modified peptide revealed conspicuous hydrazono formation upon exposure to hydralazine, it seems likely that adduct-trapping rather than adduct-stripping actions are the predominant reactions involving hydralazine at acrolein-modified lysine residues. This concurs with the structural

Fig. 7. Adduct-trapping accompanies cytoprotection against acrolein-mediated toxicity by hydralazine. A, attenuation of LDH leakage during simultaneous exposure to allyl alcohol and hydralazine. B, attenuation of LDH leakage by hydralazine when present only during the postadduction phase of allyl alcohol toxicity. The treatments in A and B are as follows: ○, controls; ●, 100 μM allyl alcohol; ▲, 50 μM hydralazine; ◇, allyl alcohol plus 5 μM hydralazine; ■, allyl alcohol plus 10 μM hydralazine; △, allyl alcohol plus 25 μM hydralazine; and ○, allyl alcohol plus 50 μM hydralazine. In A and B, each data point represents the mean ± S.E. of three independent observations. In A and B, differences between allyl alcohol only-treated cells and other treatments at various time points are indicated with asterisks (*, p < 0.05; **, p < 0.01; ***, p < 0.001, Bonferroni’s post test). In C and D, cells were pretreated for 25 min with 100 μM allyl alcohol and then subsequently with hydralazine for 30 min. Next, cell lysates were prepared and 40 (C) or 60 μg (D) of protein was resolved via SDS-PAGE (12.5% acrylamide gel). Western blot analysis was performed as described under Materials and Methods. C, the relevant lane designations are as follows: 1, control—no allyl alcohol pretreatment; 2, no allyl alcohol pretreatment, and 50 μM hydralazine in second phase; 3, allyl alcohol-pretreated only; 4, allyl alcohol-pretreated, then 5 μM hydralazine; 5, allyl alcohol-pretreated, then 10 μM hydralazine; 6, allyl alcohol-pretreated, then 25 μM hydralazine; and 7, allyl alcohol-pretreated, then 50 μM hydralazine. D, detection of adduct-trapping at low hydralazine concentrations after loading 50% more protein per lane during SDS-PAGE. The lane contents are as follows: 1, allyl alcohol-pretreated, then 2 μM hydralazine; 2, allyl alcohol-pretreated, then 4 μM hydralazine; 3, allyl alcohol-pretreated, then 6 μM hydralazine; 4, allyl alcohol-pretreated, then 8 μM hydralazine; and 5, allyl alcohol-pretreated, then 10 μM hydralazine. The blots in C and D are representative of results obtained during two to three independent replicates of the experiment. E, results obtained during densitometric analysis of three hydralazine-labeled proteins highlighted in D (arrows).
differences between mono adducts formed by acrolein and malondialdehyde at primary amines in biological targets. In the case of acrolein, the product of Michael addition reactions is a saturated propanal analog (Uchida, 1999), in contrast to the unsaturated oxopropanyl species formed by malondialdehyde. Whereas the latter contain a reactive center (double bond) that can further react with the second electron-dense center possessed by hydrazino compounds as bifunctional nucleophiles (Otteneder et al., 2002), the saturated acrolein-derived counterpart lacks this capability. Thus, because drug-trapping (hydrzone formation) is expected to predominate during reactions of hydrazines with acrolein-derived propanal adducts, the loss of immunostaining in hydrazine-treated cells (Fig. 3B) most likely reflects the conversion of carbonyl-retaining adducts to trapped species that are not recognized by the anti-acrolein/KLH serum. Ongoing work in our laboratory is seeking to establish the chemistry underlying the adduct-trapping reactions in cell proteins, including the identity of the adducted amino acids involved in reactions with hydrazine.

The present findings suggest that trapping of reactive species formed during protein modification by acrolein underlies the cytoprotection afforded by hydrazine, but more work is needed to substantiate this possibility. Unfortunately, progress toward such a goal is hampered by the fact that the precise molecular mechanisms underlying acrolein toxicity are poorly defined. However, new insights into the pathways of cell damage by acrolein in rat hepatocytes came with the finding that the acute cytotoxicity of allyl alcohol and acrolein was abolished by rottlerin, a specific inhibitor of the δ-isofrom of protein kinase C (PKC) (Maddox et al., 2003). Furthermore, immunochemical studies revealed that PKC-δ activation and translocation occurred within just 10 min of exposing cells to allyl alcohol (Maddox et al., 2003). Because our work was conducted in mouse hepatocytes, whether the same PKC isoform participates in acrolein-mediated death pathways in these cells remains to be determined. If so, whether adduct-trapping drugs such as hydrazine prevent activation of PKC-dependent pathways would emerge as an intriguing research question.

Other questions arising from the present work relate to the biological and toxicological consequences of drug-trapping reactions within the proteome. For example, whereas inactivation of reactive adducts may be beneficial during short-term exposure to toxic aldehydes because it might prevent immediate cross-linking reactions, the presence of drug-labeled adducts may have deleterious cellular consequences in the longer term. For instance, ternary drug-aldehyde-adduct complexes might act as haptons, thereby triggering immune responses. Because immunotoxicity is a serious problem plaguing the use of hydrazine in humans (systemic lupus erythematosus), the contribution of drug-trapped species should be explored in relation to other known pathways of hydrazine immunotoxicity (e.g., drug bioactivation to free radical intermediates by neutrophils) (Jiang et al., 1994).

In conclusion, our present findings concerning trapping of acrolein adducts by hydrazine raise intriguing questions concerning the role of protein modification in the acute toxicity of acrolein. Although specific mechanisms underlying the cytoprotective effects of hydrazine in acrolein toxicity remain to be defined, the novel findings presented here raise the prospect of innovative chemopharmacological interventions in diseases that involve reactive bifunctional carbonyl compounds such as acrolein.

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