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HYDRALAZINE INHIBITS RAPID ACROLEIN-INDUCED PROTEIN OLIGOMERIZATION: ROLE OF ALDEHYDE-SCAVENGING AND ADDUCT-TRAPPING IN CROSS-LINK BLOCKING AND CYTOPROTECTION*

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a) Running Title: Adduct-trapping drugs and protein cross-linking by acrolein

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d) Abbreviations: AA, allyl alcohol; ACR, acrolein; ACR-RNase, acrolein-adducted ribonuclease A; 1-APH, 1-acrylaldehyde phthalazin-1-ylhydrazone; GSH, reduced glutathione; HYD, hydralazine; MeRNase A, reductively methylated ribonuclease A; MESNA, 2-mercaptoethanesulfonic acid; PBS, phosphate-buffered saline; RNase A, ribonuclease A.

Abstract

Hydralazine strongly suppresses the toxicity of acrolein, a reactive aldehyde that contributes to numerous health disorders. At least two mechanisms may underlie the cytoprotection, both of which involve the nucleophilic hydrazine possessed by hydralazine. Under the simplest scenario, hydralazine directly scavenges free acrolein, decreasing intracellular acrolein availability and thereby suppressing macromolecular adduction. In a second "adduct-trapping" mechanism, the drug forms hydrazones with acrolein-derived Michael adducts in cell proteins, preventing secondary reactions of adducted proteins that may trigger cell death. To identify the most important mechanism, we explored these two pathways in mouse hepatocytes poisoned with the acrolein precursor allyl alcohol. Intense concentration-dependent adduct-trapping in cell proteins accompanied the suppression of toxicity by hydralazine. However, protective concentrations of hydralazine did not alter extracellular free acrolein levels, cellular glutathione loss or protein carbonylation, suggesting the cytoprotection is not due to minimization of intracellular acrolein availability. To explore ways whereby adduct-trapping might confer cytoprotection, the effect of hydralazine on acrolein-induced protein cross-linking was examined. Using bovine pancreas ribonuclease A as a model protein, acrolein caused rapid time- and concentration-dependent cross-linking, with dimerized protein detectable within 45 min of commencing protein modification. Lysine adduction in monomeric protein preceded the appearance of oligomers, while reductive methylation of protein amine groups abolished both adduction and oligomerization. Hydralazine inhibited cross-linking if added 30 min after commencing acrolein exposure but was ineffective if added after a 90 min delay. Adduct-trapping closely accompanied the inhibition of

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cross-linking by hydralazine. These findings suggest cross-link blocking may contribute to hydralazine cytoprotection.

Acrolein (2-propenal) is the smallest and most reactive α,β–unsaturated aldehyde formed during lipid peroxidation cascades (Esterbauer et al., 1991; Uchida, 1999). It also forms during the incomplete combustion of a diverse range of organic matter including timber, plastics and other construction materials (Vermeire, 1992). Oxidative metabolism of allyl alcohol in hepatocytes also generates acrolein, accounting for the overt periportal hepatotoxicity of this substance (Belinsky et al., 1985). A powerful electrophile, the toxicity of acrolein reflects the pronounced reactivity of its unsaturated bond, which is readily attacked by nucleophilic centres in protein and DNA to form carbonyl-retaining adducts (Michael adducts) (Uchida et al. 1998a; Burcham & Fontaine, 2001). Such reactivity ensures that acrolein disrupts a wide range of cellular and molecular processes in exposed tissues, including alterations in the activity of various transcription factors, enzyme activities, mitochondrial processes and redox regulatory pathways (Biswal et al., 2002; Ranganna et al., 2002; Luo and Shi, 2004; Yang et al., 2004). Consistent with endogenous acrolein production via lipid peroxidation cascades, acrolein-adducted proteins have been detected in the affected tissues of a number of degenerative conditions known to involve oxidative stress, including Alzheimer's disease, atherosclerosis, dermal photodamage and spinal cord trauma (Uchida et al., 1998b; Calingasan et al., 1999; Tanaka et al., 2001; Luo et al., 2005).

Given the role of acrolein in a wide range of health conditions, we commenced a search for low molecular weight, nitrogen-containing nucleophiles that block the toxicity of this substance. An expectation was that such compounds would intercept free acrolein within the intracellular environment, preventing adduction of macromolecules and the progression of cell death (Shapiro, 1997). Our efforts identified the vasodilatory antihypertensive hydralazine as a potent inhibitor of

acrolein-mediated toxicity in isolated murine hepatocytes (Burcham et al., 2002). These properties were consistent with the carbonyl-trapping actions of hydralazine, since the drug is known to scavenge pyruvate and other endogenous carbonyl compounds in humans (Reece et al., 1985). Accordingly, we recently characterised two isomeric hydrazones formed during interactions between acrolein and hydralazine (Kaminskas et al., 2004a).

In addition to scavenging free acrolein, hydralazine also readily forms hydrazones with acrolein-adducted cell proteins, a reaction we term “adduct-trapping” (Burcham et al., 2004). Using electrospray ionisation mass spectrometry to characterise products formed upon exposure to an acrolein-modified model peptide, we found that hydralazine readily trapped several species of carbonyl-retaining adducts formed by acrolein at lysine (Burcham et al., 2004). Using rabbit antiserum against drug-labelled adducts, we also detected strong adduct-trapping in a wide range of cell proteins from mouse hepatocytes treated briefly with allyl alcohol and cytoprotective concentrations of hydralazine (e.g. 2 to 50 μM, see Burcham et al., 2004). Moreover, intense protein adduct-trapping occurred in the livers of allyl alcohol-intoxicated mice that received hepatoprotective doses of hydralazine (Kaminskas et al, 2004b).

Collectively, these findings imply two distinct mechanisms may contribute to hydralazine cytoprotection against acrolein-mediated cellular injury (Figure 1). In the first instance, direct acrolein-scavenging is expected to diminish the intracellular availability of acrolein, thereby reducing alkylation of intracellular targets such as proteins, glutathione and DNA. In the alternative mechanism, hydralazine targets carbonyl-retaining Michael adducted-proteins, forming hydrazones that may prevent

participation by modified proteins in nucleophilic additions that generate inter- and intra-molecular cross-links (Figure 1).

The present experiments were intended to assess the relative importance of these competing mechanisms of drug action. Firstly, the contributions of acrolein-scavenging and adduct-trapping to hydralazine cytoprotection were addressed in a cellular model of acrolein-mediated toxicity, using glutathione depletion, extracellular acrolein concentrations and protein carbonylation to assess any drug effects on intracellular acrolein availability. A clinically-used acrolein-scavenging drug, MESNA, served as a positive control in these experiments. Secondly, the protein cross-linking properties of acrolein were explored to clarify the kinetics of these reactions as well as any involvement of carbonylated lysine residues in protein oligomerization. Bovine pancreas ribonuclease A (RNase A) served as a model protein in these experiments since it's low mass (12.4 kDa) permits ready detection of oligomeric forms via polyacrylamide gel electrophoresis (Liu *et al*, 2003; Gotte and Libonati, 2004). Thirdly, having established conditions for the induction of protein cross-links by acrolein, the effect of hydralazine on protein oligomerization was explored using Western blotting to assess the role of adduct-trapping in any inhibitory outcomes. Finally, the efficacies of two endogenous compounds as inhibitors of acrolein-induced protein cross-linking were explored, namely glutathione and carnosine. Both agents trap a range of α,β–unsaturated aldehydes including acrolein (Burcham et al., 2002; Carini et al., 2003).

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Materials and Methods

Materials. Allyl alcohol, Ribonuclease A (Type III-A, bovine pancreas, EC 3.1.27.5), *EZBlue* Gel Staining Reagent, anti-dinitrophenyl serum, hydralazine and carnosine were purchased from the Sigma-Aldrich Chemical Company (St. Louis, MO, USA). 1-Acrylaldehyde phthalazin-1-ylhydrazone (1-APH, mixture of *E*- and *Z*- isomers) was synthesised as described previously and its chemical purity was confirmed by ^{1}H- and ^{13}C-NMR spectroscopy (Kaminskas et al., 2004a). Adult male Swiss mice (5 to 6 weeks old) were obtained from Animal Services at the Waite Institute of the University of Adelaide 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 compliant with principles outlined in the *Guide for the Care and Use of Laboratory Animals* published 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). Freshly isolated cells were washed four times in collagenase-free Krebs-Henseleit buffer and then resuspended at 1 x 10^6 cells per ml in RPMI 1640 media supplemented with 0.2 % BSA, 0.03 % L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). The cells were then layered on collagen-coated 60-mm polystyrene dishes and placed in a humidified CO_2 incubator at 37 °C (5 % CO_2). After 2 h, the dishes were washed twice with phosphate-buffered saline (PBS) to remove nonadherent cells before fresh aliquots of Krebs-Henseleit buffer (pH 7.3) supplemented with glucose (5 g/L) and pyruvate (1 mM) were added to each dish. The use of this amino acid-free media was intended to minimize extracellular side-reactions between free acrolein and nucleophilic buffer constituents. Hydralazine, MESNA and allyl alcohol were

dissolved directly in culture media immediately prior to use. Following the addition of reagents, dishes were returned to the incubator and 100 μL media samples were taken at 30 min (for the assessment of extracellular acrolein) or at 180 min (for the estimation of lactate dehydrogenase (LDH) leakage). Some dishes collected at 30 min were also used for the immunochemical detection of hydralazine-trapped adducts and protein carbonyls as described below. Cell lysates used in these immunochemical assays were prepared using recently described procedures (Burcham et al., 2004).

Biochemical Procedures. LDH activity was measured using a spectrophotometric procedure based on the enzymatic reduction of NAD^+ in the presence of l-lactate (Burcham and Fontaine, 2001). For the estimation 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 seconds using a Labsonic 1510B Cell Disrupter (Braun Melsungen AG, Germany). Separate dishes were used for glutathione determination via the method of Saville (Saville, 1958). Briefly, cell monolayers that had been exposed to allyl alcohol in the presence and absence of various concentrations of hydralazine were washed three times with cold PBS. Next, 1 mL cold trichloroacetic acid (6.5 %) was added to each plate and then the contents were transferred to 1.5 mL Eppendorf tubes. Following centrifugation at 3,000 x g for 10 min at 4 °C, the glutathione content of 0.25 mL aliquots of supernatant were measured against a standard curve constructed using reduced glutathione dissolved in 6.5% trichloroacetic acid (standards spanned a 10 to 200 μM concentration range).

Acrolein Determination. Acrolein concentrations were determined in aliquots of culture media or RNase A reaction mixtures (see below) using a HPLC-based procedure described recently (Kaminskas et al., 2004a). Depending on the acrolein

concentration present within the sample, sample aliquots were diluted with from 4 to 249 volumes of mobile phase prior to HPLC analysis. The HPLC system comprised an ODS Hypersil column (150 x 4.6 mm, 5μm, Keystone Scientific Inc, PA, USA) connected to a GBC LC1150 pump (Dandenong, Australia), fitted with an online ERC 3415 degasser and a Hewlett Packard series 1100 UV detector that monitored the absorbance of column eluate at 210 nm. Output from the detector was collected using Delta *Junior* chromatography analysis software (Version 5009, Digital Solutions, Queensland). The mobile phase (20 % aqueous methanol, *v/v*) was maintained at a flow rate of 1 mL/min, with acrolein eluting from the column at a retention time of 3.0 min. Aldehyde concentrations were determined by comparing sample peak areas to those obtained by analysing standard solutions of acrolein prepared in mobile phase over a concentration range of 1 to 16 μM.

RNase A Oligomerization Assay. Protein cross-linking **r**eactions were performed in 50 mM sodium phosphate buffer (pH 7.0) in a final volume of 0.2 mL using 0.2 mL polypropylene PCR tubes (the use of maximal reaction volumes helped minimise acrolein loss into headspace at 37° C). The RNase A concentration was 2 mg/mL, conferring a final lysine concentration of 1.5 mM. In reactions exploring the concentration-dependence of RNase A cross-linking by acrolein, the latter was added to achieve aldehyde:lysine ratios of 0.5, 1, 2, 4 and 8 (i.e. final concentrations of 0.75, 1.5, 3, 6 and 12 mM). In related experiments, the time-course of protein cross-linking and adduction by acrolein was studied using 5 mL glass vials fitted with a rubber septum, enabling aliquot collection without undue loss of acrolein vapours. Briefly, vials containing 9 mg RNase A dissolved in 4.9 mL of 50 mM phosphate buffer (pH 7.0) were prewarmed at 37 °C. A 100 μL aliquot of acrolein was then added to give a final concentration of 3 mM (i.e. achieving a 2:1 acrolein:lysine ratio).

At various time intervals (0, 15, 30, 45, 60, 90, 120, 150 and 180 min), 100 μL aliquots of reaction mixture were removed and diluted 3:1 with 5X Gel Electrophoresis Sample Buffer (Bollag et al, 1996). The samples were then chilled on ice until 20 μL volumes (i.e. 30 μg protein) were resolved via gel electrophoresis as outlined below.

In experiments in which the role of primary amines in protein adduction and oligomerization were explored, reductively methylated RNase A (Me-RNase A) was substituted for native protein. Me-RNase A was prepared using a half-scale version of a procedure described by others (Xu et al*.,* 1999). Briefly, RNase A (17 mg, 1.25 μmol) was dissolved in 490 μL sodium phosphate buffer (0.1 M, pH 7.2). A 100-fold molar excess of formaldehyde was then added (125 μmol), followed by 7.9 mg sodium cyanoborohydride (125 μmol). After 18 h at room temperature, unreacted reagents were removed via dialysis for 24 hours against 2 changes of 0.1 M sodium phosphate buffer (pH 7.2, 500 mL) using Pierce *Slide-A-Lyser* cassettes (3.5 kDa cut-off). Confirmation that the number of accessible primary amines was diminished by the formaldehyde/cyanoborohydride treatment was obtained by comparing Me-RNase A to native protein using a spectrophotometric assay for protein amine groups (Spadaro et al., 1979).

Oligomerization Blocking Experiments. To examine hydralazine's efficacy as an inhibitor of oligomerization, and also any time-dependence of susceptibility to the drug, assay substrate was prepared by reacting RNase A (2.1 mg/mL) with 3.2 mM acrolein in 2 mL volumes of buffer in Eppendorf tubes at 37 °C. At either 30 or 90 min, 190 μL aliquots were removed and added to 0.2 mL PCR tubes containing either 0, 1, 3 or 10 μL volumes of 60 mM hydralazine (diluted to a 10 μL volume with

buffer), achieving respective hydralazine concentrations of 0, 0.3, 1 and 3 mM. Reactions were allowed to proceed for a further 120 minutes at 37 °C after which the reaction mixtures were diluted 3:1 with 5X Sample Buffer. Aliquots containing 30, 5 or 3 μg protein were then resolved on separate 14% acrylamide gels for respective analysis of protein oligomerization via Coomassie blue staining (Gel 1), lysine adduction (Gel 2) and adduct-trapping (Gel 3).

In related experiments, the effects of various reagents (e.g. sodium borohydride, sodium cyanoborohydride, glutathione and carnosine) on protein oligomerization were examined as described for hydralazine, using RNase A that had been pre-modified by acrolein for 30 minutes only. Following the secondary incubation, reaction mixtures were assessed for oligomers and lysine adducts as described below. In addition, the protein carbonylation status of 2.5 μg aliquots of modified protein were assessed using an immunochemical 2,4-dinitrophenylhydrazine-based procedure (Keller et al., 1993).

Mechanism of Formation of Hydralazine-Adducts in Protein. To assess the reactivity of 1-APH with the model protein, RNase (2 mg/mL final concentration) was treated with 0.1, 0.3 or 1 mM concentrations of 1-APH in 50 mM sodium phosphate buffer (pH 7.0). The final reaction volume was 90 μL. 1-APH solutions were prepared freshly in methanol, and added to tubes to obtain a final methanol concentration of 16.6%. Control tubes received equivalent concentrations of neat methanol. Tubes were then placed in an incubator at 37 °C for 30 min, after which RNase was processed for the presence of hydralazine adducts via Western blotting as outlined below. To prepare acrolein-adducted RNase A that was free of unbound acrolein for use as a positive control in these experiments, 1.5 mg RNase was dissolved in 500

µL sodium phosphate buffer (50 mM, pH 7.0) and then treated with an equimolar concentration of acrolein (relative to the lysine concentration of 2.25 mM) for 30 min at 37 °C. Unincorporated acrolein was then removed via dialysis for 18 hours against 50 mM sodium phosphate buffer (pH 7.0, 3 X 1L) using Pierce *Slide-A-Lyser* cassettes (3.5 kDa cut-off). The resulting modified protein was then treated with 0.1, 0.3 or 1 mM concentrations of hydralazine under the same conditions outlined above for the reaction with 1-APH. The presence of trapped-adducts was then assessed via Western blotting.

Gel Electrophoresis and Western Blotting. Following heat denaturation for 6 to 8 min at 90 °C, protein samples were loaded onto a 14 % acrylamide minigel (4 % stacking gel) before electrophoresis at 200V for 45 minutes. Samples comprising cell lysates (i.e. used to detect trapped-adducts and protein carbonyls) were resolved overnight at 120 V on a large gel (12% acrylamide, 4% stacking gel) using a BioRad *Protean II* apparatus. After transfer to nitrocellulose (100 V, 30 min), membranes were blocked with 7.5 % nonfat milk in PBS for 30 min and then treated for 60 min with 1/1000 dilutions of rabbit antiserum (raised against either acrolein-modified hemocyanin (Burcham et al., 2003), hydralazine-stabilised acrolein-modified hemocyanin (Burcham et al., 2004) or commercial anti-dinitrophenyl serum (Keller et al., 1993)). Following washing and exposure to horseradish peroxidase-coupled goat anti-rabbit IgG serum (*Pierce Immunopure*, 1/10,000 dilution, 30 min), membranes were developed using Pierce *Super Signal West Pico* chemiluminescence reagent and Kodak *BioMax Light* film. The resulting images were analyzed via densitometry using Kodak *Digital Science* software.

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Statistical Analysis. Relevant experimental data (LDH leakage, acrolein concentrations, glutathione contents) were analyzed by 1-way ANOVA followed by Tukey's Multiple Comparison Test using *GraphPad Prism 4.01* software.

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Results

Status of Competing Protective Mechanisms in Cells. Isolated mouse hepatocytes were exposed to the acrolein precursor allyl alcohol in the presence of 0, 10, 25, 50 or 100 μM hydralazine (Figure 2). For comparative purposes, cells in separate dishes were exposed to equivalent concentrations of MESNA, a thiol compound used clinically as an acrolein-scavenger in chemotherapy recipients. Hydralazine afforded strong concentration-dependent protection against cell killing, indicated by the diminished leakage of lactate dehydrogenase (LDH) into the culture media after a 3 h incubation (Fig. 2A). Consistent with its ionic character and inability to penetrate cell membranes, MESNA did not protect against allyl alcohol-induced LDH leakage (Fig. 2B). The two drugs also differed strongly in their effects on extracellular acrolein levels (Fig. 2C and D). While MESNA produced strong concentration-dependent declines in extracellular acrolein (Fig. 2D), a range of cytoprotective hydralazine concentrations did not significantly alter these values (Fig. 2C).

Despite the lack of effect upon extracellular acrolein levels, hydralazine produced strong concentration-dependent adduct-trapping in many cell proteins at the 30 min point (Fig. 2E). In keeping with prior observations (Burcham et al., 2004), adduct-trapping was detected in a ≈130 kDa mass protein in control cells exposed to 100 μM hydralazine only, indicative of hydralazine reactions with endogenously-carbonylated proteins (Fig. 2E, Lane 2). At low drug concentrations in cells co-exposed to allyl alcohol and hydralazine, adduct-trapping reactions predominately involved high mass proteins, although smaller proteins (~30 to 50 kDa) were targeted at higher drug concentrations (Fig. 2E). Despite the strong adduct-trapping produced

by hydralazine, the drug did not minimize the extensive protein carbonylation occurring in cells during a 30 min co-exposure to allyl alcohol (Fig. 2F). Protein carbonyl groups were detected by derivatizing cell proteins with 2,4-dinitrophenylhydrazine followed by denaturing polyacrylamide electrophoresis and Western blotting using anti-dinitrophenyl serum (Keller et al., 1993). Protective concentrations of hydralazine also failed to prevent the marked depletion of hepatocyte glutathione occurring during a 30 min exposure to 100 μM allyl alcohol (Fig. 2G). The fact that protective concentrations of hydralazine had no effect on extracellular acrolein concentrations, glutathione depletion or protein carbonylation suggests acrolein scavenging and minimization of the adduction of intracellular targets is not central to the cytoprotective actions of the drug. This conclusion focused attention on possible ways the adduct-trapping reactivity of hydralazine might contribute to its cytoprotective efficacy. The next phase of the project thus explored the cross-linking properties of acrolein as well as hydralazine's ability to disrupt these reactions.

Protein Cross-linking by Acrolein. Coomassie blue staining of gels obtained following electrophoresis of acrolein-modified ribonuclease A (RNase A) allowed facile detection of oligomeric species formed via cross-linking reactions (Fig. 3A). Exposure for 3 h at 37 °C to 0.75, 1.5, 3, 6 and 12 mM acrolein resulted in concentration-dependent accumulation of RNase A dimers to respective values of 11, 32, 41, 52 and 51% of starting material (Fig. 3A, Lanes 2 to 6). Masses corresponding to RNase A trimers represented 7.7, 21 and 22% of starting material at 3, 6 and 12 mM concentrations of acrolein (Lanes 4 to 6). Protein cross-linking was accompanied by extensive protein modification, as detected using rabbit

antiserum selective for acrolein-adducted lysine residues (Figure 3B). Immunorecognition of oligomeric RNase A species was particularly intense (Figure 3B, Lanes 4 to 6), suggesting acrolein-adducted lysine residues are key participants in the formation of aggregated proteins in this system. Moreover, subjecting the model protein to reductive methylation prior to acrolein exposure abolished both the accumulation of oligomeric RNase A (Fig. 3A, Lanes 8 and 9) and the immunodetection of acrolein-adducted protein (Figure 3B, Lanes 8 to 9). Reductive methylation reduced the primary amine content of the RNase A by over 90% relative to native protein, as determined using a picrylsulfonic acid-based spectrophotometric assay (Spadaro et al., 1979). The finding that methylated RNase A resisted cross-linking indicates a role for carbonyl-retaining adducts at the ε–amine group of lysine in protein oligomerization. This was reinforced by the finding that delayed (30 min) addition of the strong, carbonyl-reducing reagent sodium borohydride diminished oligomer levels after a subsequent 120 min reaction (compare Lanes 5 to 7 with Lane 4). In contrast, a milder reducing agent which is inactive at carbonyl groups (sodium cyanoborohydride) was less effective in this regard (Fig. 3E, Lanes 8 to 10)).

The time-dependence of RNase A oligomerization by acrolein was then studied under conditions in which the aldehyde was present at a 2-fold molar excess relative to the concentration of lysine in the reaction mixture (Figure 3C and B). This ratio was based on the stoichiometry of lysine adduction by acrolein, which involves sequential addition of 2 acrolein molecules to a given lysine sidechain (Uchida et al., 1998b). Densitometry of Figure 3C revealed that dimeric RNase A comprised 8.4% of starting material at 45 min, increasing to 29% by 180 min (Fig. 3C Lanes 4 and 9). The generation of oligomeric species was again accompanied by extensive lysine adduction (Fig. 3D). Consistent with a role for lysine adducts in the cross-linking

chemistry, adducts were detected in monomeric RNase A at early time points that preceded the appearance of oligomeric RNase species (e.g. at 15 min after commencing acrolein exposure as per Lane 2 in Figure 3D).

Inhibition of Acrolein-Induced Protein Oligomerization by Hydralazine.

Added concurrently with acrolein, hydralazine (0.1 to 3 mM) produced strong concentration-dependent inhibition of acrolein-induced RNase A oligomerization during a 3 h reaction at 37 °C (Figure 4). Densitometry of Coomassie blue-stained proteins (Panel A) revealed hydralazine concentrations of 0.3, 1 and 3 mM decreased dimer abundance by 25, 56 and 100% respectively (Fig. 4A). Hydralazine's effects on levels of RNase A trimers were particularly striking, since levels of this product were reduced by 39%, 100% and 100% at these drug concentrations (Fig. 4A). Hydralazine also produced concentration-dependent declines in the immunoreactivity of acrolein-modified lysine residues within each form of RNase A (Fig. 4B) and these effects were accompanied by parallel formation of hydralazine-trapped protein adducts (Fig. 4C). Acrolein modification strongly increased the carbonyl content of monomeric, dimeric and trimeric forms of RNase A (Fig. 4D), with hydralazine suppressing this effect only at the 3 mM concentration (Lane 7). Consistent with it's efficient acrolein-scavenging properties, 0.1, 0.3, 1 and 3 mM hydralazine decreased free acrolein concentrations by 18%, 31%, 65% and 100% respectively 30 min after commencing concurrent exposure to acrolein and hydralazine (Fig. 4E). This indicates both acrolein-scavenging and adduct-trapping probably contributed to the inhibitory effects of hydralazine on protein oligomerization during co-exposure of RNase A to acrolein and the drug.

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Hydralazine Traps "Early" Acrolein-Protein Adducts. To clarify the role of adduct-trapping in hydralazine's effects on acrolein-mediated protein oligomerization, the time-dependence of susceptibility to the drug was examined (Fig. 5). By delaying hydralazine addition until 30 min, reactivity with adducts present on monomeric RNase A could be explored. Fig. 5A indicates that 0.3 to 3 mM hydralazine elicited a concentration-dependent inhibitory effect when added 30 min following the commencement of RNase A modification by acrolein (compare Lanes 4 to 6 with Lane 3). For example, 1 mM hydralazine (Lane 5) diminished dimer abundance by 67% relative to acrolein-modified RNase alone (Lane 3). However, if drug addition was delayed until 90 min after starting acrolein exposure, hydralazine had negligible effect upon levels of oligomeric RNase A present after a secondary 120 min incubation (Fig. 5A, Lanes 7 to 9). This suggests hydralazine targets Michael adducts formed during the early stages of protein modification by acrolein, but that cross-links formed during subsequent reaction of these species with neighboring nucleophiles resist attack by the drug.

In keeping with a role for acrolein-adducted lysine residues in protein oligomerization, hydralazine produced concentration-dependent effects on these species that paralleled its effects on the abundance of RNase A dimers and trimers (Fig. 5B). When added at 30 min, 3 mM hydralazine strongly suppressed both the appearance of oligomers (Fig. 5A, Lane 6) and the immunodetection of acrolein-lysine adducts (Fig. 5B, Lane 6). Yet this concentration of hydralazine had little effect on either oligomer abundance or acrolein-lysine modification when added 90 min post-commencement of protein modification (Fig. 5). Consistent with a role for adduct-trapping in hydralazine's effects on oligomer abundance and lysine adduction, concentration-dependent adduct-trapping accompanied the inhibitory effects of

hydralazine when added at the 30 min time point (Fig. 5C, Lanes 4 to 6). Intriguingly, despite its lack of effect on oligomer abundance or lysine adduction when added at 90 min, hydralazine did participate in adduct-trapping reactions when added at this time (Fig. 5C, Lanes 8 to 10). This indicates that at this later time-point, not all carbonyl-retaining adducts have been consumed via the formation of cross-linked RNAse species, hence they remain free to react with nucleophilic drugs.

Mechanism of Formation of Hydralazine-Adducts in Protein. The main isolable product of reactions between free acrolein and hydralazine, 1-acrylaldehyde phthalazin-1-ylhydrazone (1-APH), (Kaminskas et al., 2004a), possesses an α,β-unsaturated bond that might be attacked by protein nucleophiles to form hydralazine-labelled adducts (Scheme 1). To assess this possibility, the yield of adducts generated during reactions of RNase A with 1-APH was compared to those obtained via the "adduct-trapping" route (i.e. where hydralazine reacts directly with acrolein-adducted protein). To assess the latter under conditions which precluded 1-APH formation, acrolein-adducted RNase A was subjected to extended dialysis to remove unreacted acrolein (i.e. prior to the addition of hydralazine). The resulting "unbound acrolein-free" modified RNase A was then reacted with a range of hydralazine concentrations (0.1, 0.3 and 1 mM) for 30 min at 37 °C. Results obtained during immunochemical analysis of the resulting protein, as well as RNase A that was treated with a range of concentrations of 1-APH, are shown in Fig. 6. The data confirm that the reaction of hydralazine with acrolein-adducted protein (Fig. 6, Lanes 4 to 6) was by far the favoured route for the introduction of hydralazine moieties into the model protein. In contrast, comparable concentrations of 1-APH exhibited no reactivity with the protein (Fig. 6, Lanes 7 to 9).

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Glutathione and Carnosine As Endogenous Cross-Link Blockers. We next explored whether endogenous substances with known carbonyl-trapping properties can act as “cross-link blockers” during protein modification by acrolein (Figure 7). For these studies, the effect of adding 1, 3 or 10 mM concentrations of either glutathione or carnosine to RNase A that had been pre-modified during a 30 min incubation with 3 mM acrolein were assessed following a subsequent 120 min incubation. The end-points assessed were again oligomer abundance (Fig. 7A), acrolein-lysine adduction (Fig. 7B) and protein carbonylation (Fig. 7C). The thiol-containing tripeptide glutathione strongly inhibited oligomer formation and the immunorecognition of lysine adducts, although its effects on protein carbonyls were less pronounced (in Figure 7, compare Lanes 5 to 7 with Lane 4 in each panel). The inhibitory efficacy of glutathione against dimer formation was comparable to that of hydralazine in this system, with the 1 mM concentration diminishing dimer levels by 70 % (Fig. 7A, Lane 5, c.f. the 67% decrease produced by the same concentration of hydralazine (Fig. 5A, Lane 6). The effects of the dipeptide carnosine on protein oligomerization were less striking, although the two highest concentrations produced minor reductions in dimer abundance and the immunoreactivity of acrolein-lysine adducts (Lanes 7 to 9 in Fig. 7A and 7B).

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Discussion

The search for drugs that counteract oxidative cell injury has typically focussed on radical scavenging antioxidants (Salvemini et al., 2002), but an alternative strategy involves using nucleophilic drugs to intercept toxic carbonyls (Shapiro, 1998). Our group has recently explored the latter option, giving attention to drugs that neutralize acrolein, the most reactive carbonyl compound formed during oxidative stress. We identified the antihypertensive hydralazine, among the strongest nucleophiles in clinical use, as a powerful inhibitor of acrolein toxicity (Lalich et al., 1975; Burcham *et al*., 2002). Although its protective efficacy appeared related to its acrolein scavenging properties in cell-free systems (Kaminskas et al., 2004b), we found that hydralazine also reacts extensively with adducts formed during protein modification by acrolein (Burcham et al., 2004; Kaminskas et al., 2004b). The present work breaks new ground by establishing that the latter "adduct-trapping" reactivity is more closely related to hydralazine cytoprotection than the direct acrolein-scavenging reaction (Fig. 1). This finding was not a foregone conclusion, since our previous studies in hepatocytes were optimized to detect "adduct-trapping" events (Burcham et al, 2004). Thus the cells were preloaded with acrolein adducts and then, immediately prior to the irreversible loss of membrane integrity, they were washed free of acrolein precursor before subsequent incubation with fresh media containing a range of drug concentrations. This ensured hydralazine was added to cells under conditions where levels of adducted proteins were high relative to concentrations of free, unadducted acrolein (Burcham et al., 2004). Although extensive adduct-trapping occurred under such conditions, it was unknown whether adduct-trapping reactions also occur where ongoing acrolein generation would ensure direct acrolein-scavenging reactions would compete for hydralazine alongside trapping reactions with adducted proteins. Indeed,

if the former was highly efficient within the cellular environment, it is conceivable that this reaction might entirely prevent protein modification by acrolein, thereby precluding protein adduct-trapping by hydralazine. The current findings resolve this issue by confirming that intense adduct trapping occurred in cells exposed simultaneously to allyl alcohol and hydralazine, indicating the reaction with adducted proteins is a significant intracellular fate of hydralazine.

The finding that acrolein levels in the media of allyl alcohol-treated cells were unaffected by cytoprotective hydralazine concentrations reinforces the conclusion that reactions with unadducted aldehyde are not a major cellular fate of hydralazine. This conclusion concurs with our recent finding of only low levels of the major hydrazone formed during reactions between free acrolein and hydralazine, 1-acrylaldehyde phthalazin-1-ylhydrazone (1-APH), in the culture media of hepatocytes following a 30 min incubation with allyl alcohol and the same drug concentrations used in the present study (Kaminskas *et al*., 2004a). For example, the concentration of 1-APH in cells after a 30 min exposure to 100 μM hydralazine and 100 μM allyl alcohol was 0.38 ± 0.1 nmol/mL - an order of magnitude lower than the level of free acrolein measured under the same conditions in our present study (3.6 ± 0.8 nmol/mL – see Figure 2C). Thus even at this high drug concentration, direct acrolein-scavenging cannot account for the cytoprotection afforded by hydralazine.

While the present work confirmed that adduct-trapping occurred under conditions of concurrent exposure to hydralazine and allyl alcohol, the experimental design that was employed raised the possibility of alternative mechanisms for the incorporation of drug moieties into protein. One possibility was that the hydralazine-containing adducts might have been formed via the intermediacy of 1-APH, the product of scavenging reactions between hydralazine and free acrolein (Scheme 1).

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For example, attack on the α,β-unsaturated bond of 1-APH would yield ternary protein-aldehyde-drug complexes that structurally resemble species formed by hydralazine when attacking Michael adducts in acrolein-modified proteins. The present findings discount this mechanism, since exposure of RNase A to high concentrations of 1-APH (0.1 to 1 mM) for 30 min resulted in no detectable adducts (Figure 6). The 30 min reaction duration was used since this was the time after which extensive adduct-trapping was detected in hepatocytes (Fig. 2). Moreover, repeating the experiment using 3 to 30 mM concentrations of 1-APH failed to generate detectable hydralazine-containing adducts (*data not shown*). Consequently, the intense adduct-trapping occurring in hepatocytes exposed simultaneously to allyl alcohol and hydralazine in Fig. 2 almost certainly reflects trapping of Michael-adducted proteins by free hydralazine, rather than protein adduction by 1-APH.

Exactly how adduct-trapping reactivity confers cytoprotective efficacy on hydralazine is unclear, although the possibility that it involves interference with cross-link formation is strengthened by the present work. The protein cross-linking reactivity of acrolein is poorly characterised, and the chemistry underlying such reactions is unknown. Since the present work established a role for lysine in protein cross-linking by acrolein, future work should clarify whether these residues account for both nucleophilic centres participating in the formation of a given cross-link (i.e. LYS-ACR-LYS), or whether other nucleophiles known to be modified by acrolein also participate (e.g. CYS-ACR-LYS, HIS-ACR-LYS, etc). In analogous work, clarification of the chemistry of protein cross-linking by the lipid peroxidation product 4-hydroxynonenal facilitated development of antibody-based assays to detect cross-linking in oxidised low density lipoproteins (Xu et al., 2000). An immunochemical

strategy was also used to detect cross-linking of cytoskeletal proteins in neuroglial cells after brief (3 h) exposures to 10 to 1000 μM concentrations of 4-hydroxynonenal (Montine et al., 1996). Since the cross-linking reactivity of acrolein towards RNase A in the present study resembled that described for 4-hydroxynonenal under comparable conditions (Liu et al., 2003), it is likely that acrolein will facilitate protein cross-linking in the cellular environment in a similar manner to 4-hydroxynonenal (Montine et al., 1996). Ongoing work in our laboratory is aimed at establishing the identity of the major protein targets for acrolein adduction in exposed tissues, using a proteomics-based approach comparable to that which recently enabled identification of targets for 4-hydroxynonenal in rodent liver (Carbone et al., 2004). Since adducted proteins are precursors to cross-link formation, identifying proteins most prone to adduction by acrolein will allow development of immunochemical approaches to detect aggregated proteins in acrolein-exposed tissues.

The finding that physiological concentrations of glutathione inhibited acrolein-mediated protein oligomerization raises the possibility that this tripeptide acts as an endogenous “adduct-trapping” reagent. Although glutathione is a highly efficient scavenger of free acrolein, the delayed administration of glutathione to the assay system in this study (i.e. 30 min post-commencement of acrolein modification) ensured that adduction had already occurred on the model protein. This rules out the possibility that scavenging of free acrolein explains the low yield of protein oligomers seen in the presence of glutathione. The chemistry underlying the “adduct-trapping” efficacy of glutathione is unclear, but it is noteworthy that Uchida and associates found that glutathione adds rapidly to the cyclic unsaturated bond of FDP-lysine, the product of sequential addition of 2 molecules of acrolein to a given lysine side-chain (Furuhata et al., 2002). This implies FDP-lysine is a reactive intermediate able to

participate in reactions with nucleophiles in addition to glutathione (e.g. the thiolate anion of cysteine residues within proteins might react with FDP-lysine to generate inter- or intra-molecular cross-links). However, another explanation for the cross-link blocking efficacy of glutathione could be that the tripeptide's terminal amine participates in Schiff reactions with carbonyl adducts generated by acrolein. Work is underway in our laboratory to characterise the relative contributions of *N*- versus *S*-glutathiolation pathways in reactions of glutathione with acrolein-adducted proteins.

As a mechanism of drug action, the "cross-link blocking" action of hydralazine documented in this study resembles the "cross-link breaker" compounds that are under exploration for efficacy against the role of toxic carbonyls in diabetic complications (Zieman and Kass, 2004). The chemical diversity of AGE production is striking, but a major consequence of these reactions is the cross-linking of collagen molecules in connective tissues and especially vascular tissue, a key factor in the arterial inelasticity accompanying diabetes (Thorpe and Baynes, 2003). The prototype compounds in the "cross-link breaker" class, N-phenacylthiazolium bromide and 4,5-dimethyl-3-(2-oxo-2-phenylethyl)-thiazolium chloride (ALT-711), improve a number of disease end-points in animal models of diabetes as well as aged nondiabetic animals, producing reductions in AGE levels in renal and vascular tissue, declines in myocardial and aortic stiffness, and diminished collagen deposition in a range of tissues (Cooper et al., 2000; Vaitkevicius et al., 2001). At present, the extent to which "cross-link cleavage" contributes to the clinical improvements in these animal models is subject to debate, since these compounds improve a number of biological end-points in target tissues (e.g. reduction in inflammatory markers, profibrotic cytokines, etc) but may not cleave cross-linked proteins in diabetic tissues (Yang et al., 2003). Nevertheless, since carbonyl-retaining protein adducts

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contribute to collagen cross-linking during AGE reactions, the possibility that adduct-trapping compounds might produce beneficial outcomes in these conditions is worthy of attention.

In conclusion, this work sheds light on the mechanisms underlying protection by hydralazine against acrolein-mediated toxicity, and confirms that the drug is able to readily “trap” reactive Michael adducts in proteins. More work is needed to establish how such adduct-trapping might suppress the onset of cell death, but the present work indicates that prevention of protein cross-linking is one outcome of this mechanism of drug action.

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Footnotes.

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Figure Legends

Figure 1. Fate of hydralazine during acrolein toxicity showing the role of aldehyde-scavenging to form 1-APH (1), protein adduct-trapping (2), and the potential reaction of 1-APH with proteins (3).

Figure 2. Cytoprotective and acrolein-scavenging efficacies of hydralazine and MESNA in isolated hepatocytes. *Panels A and B:* LDH leakage after a 3 hour incubation in the presence of 0, 10, 25, 50 or 100 μM hydralazine (Panel A) or MESNA (Panel B). *Panels C and D*: Extracellular acrolein concentrations 30 min after commencing exposure to 100 μM allyl alcohol in the presence of the above concentrations of either hydralazine (C) or MESNA (D). *Panels E and F* : Western blots obtained during the analysis of hydralazine-trapped protein adducts (Panel E, 40 μg protein/lane) or protein carbonyl groups (Panel F, 10 μg protein/lane). The lane contents in panels E and F are the same as the X-axis labels on panel G. Immunoblots are representative of results obtained during 3-4 independent determinations. *Panel G*: Glutathione (GSH) levels in cells after a 30 min exposure to allyl alcohol and hydralazine under the conditions specified for Panel A. In Panels A to D and G, each data point represents the mean ± S.E. of 4 independent determinations (*p<0.05).

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Figure 3.Time- and concentration-dependent RNase A adduction and cross-linking by acrolein and the role of lysine groups. *Panel A:* Coomassie stained gel showing concentration-dependent RNase A oligomerization of native protein but not Me-RNase A after a 3 h incubation with increasing concentrations of acrolein (30 μg protein/lane). *Panel B:* Concentration-dependent formation of acrolein-modified lysine groups in native RNase A but not Me-RNase A (5 μg/lane). *Panels C and D*; Time course of protein oligomerization (assessed via Coomassie staining - Panel C) and lysine adduction (Panel D). Each immunoblot is representative of results obtained during 3 independent determinations. *Panel E:* Sodium borohydride ($NaBH_4$) attenuates acrolein-induced RNase A oligomerization when added after a 30 min delay more strongly than sodium cyanoborohydride ($NaBCNH_3$, Coomassie stained gel, 30 μg/lane). The various treatments are: 1) unmodified RNase A; 2) RNase A + 60 mM $NaBH_4$; 3) RNase A + 60 mM $NaBCNH_3$; 4) RNase A + 3 mM ACR; 5) ACR-RNase A + 6 mM $NaBH_4$; 6) ACR-RNase A + 30 mM $NaBH_4$; 7) ACR-RNase A + 60 mM $NaBH_4$; 8) ACR-RNase A + 6 mM $NaBCNH_3$; 9) ACR-RNase A + 30 mM $NaBCNH_3$; 10) ACR-RNase A + 60 mM $NaBCNH_3$.

Figure 4. Concurrent exposure to hydralazine attenuates acrolein-induced RNase A oligomerization. Panel A: Coomassie stained gel (30 μg/lane) Panel B: Acrolein-modified lysine residues (5 μg/lane) Panel C: Hydralazine-trapped adducts (3 μg/lane) Panel D: protein carbonyl groups (2.5 μg/lane). Panel E: Free ACR concentrations 30 min after commencing exposure to ACR in the presence of various concentrations of hydralazine. Each data point represents the mean ± S.E. of 4

independent determinations (*p<0.05). Each immunoblot is representative of results obtained during 2-3 independent determinations.

Figure 5. Hydralazine blocks acrolein-induced RNase A oligomerization when added 30 min after commencing acrolein modification, but not after a 90 min delay. *Panel A:* Coomassie stained gel (30 μg/lane) *Panel B*: Acrolein-modified lysine residues (5 μg/lane) *Panel C*: Hydralazine-trapped adducts (3 μg/lane). Each immunoblot is representative of 2 independent determinations.

Figure 6. Drug-retaining adducts are introduced during reaction of acrolein-adducted RNase A with a range of concentrations of hydralazine, but not during exposure of native protein to 1-acrylaldehyde phthalazin-1-yl hydrazone (1-APH). Immunoblot obtained using rabbit antiserum raised against hydralazine/acrolein-modified hemocyanin (3 μg protein/lane). Results typical of 2 independent determinations.

Figure 7. Efficacy of glutathione (GSH) and carnosine (CARN) as endogenous "cross-link blockers." *Panel A:* Coomassie stained gel (30 μg/lane) *Panel B*: Acrolein-modified lysine residues (5 μg/lane) *Panel C*: Protein carbonyl groups (2.5 μg/lane). Each immunoblot is representative of 3 independent determinations.

Figure 1

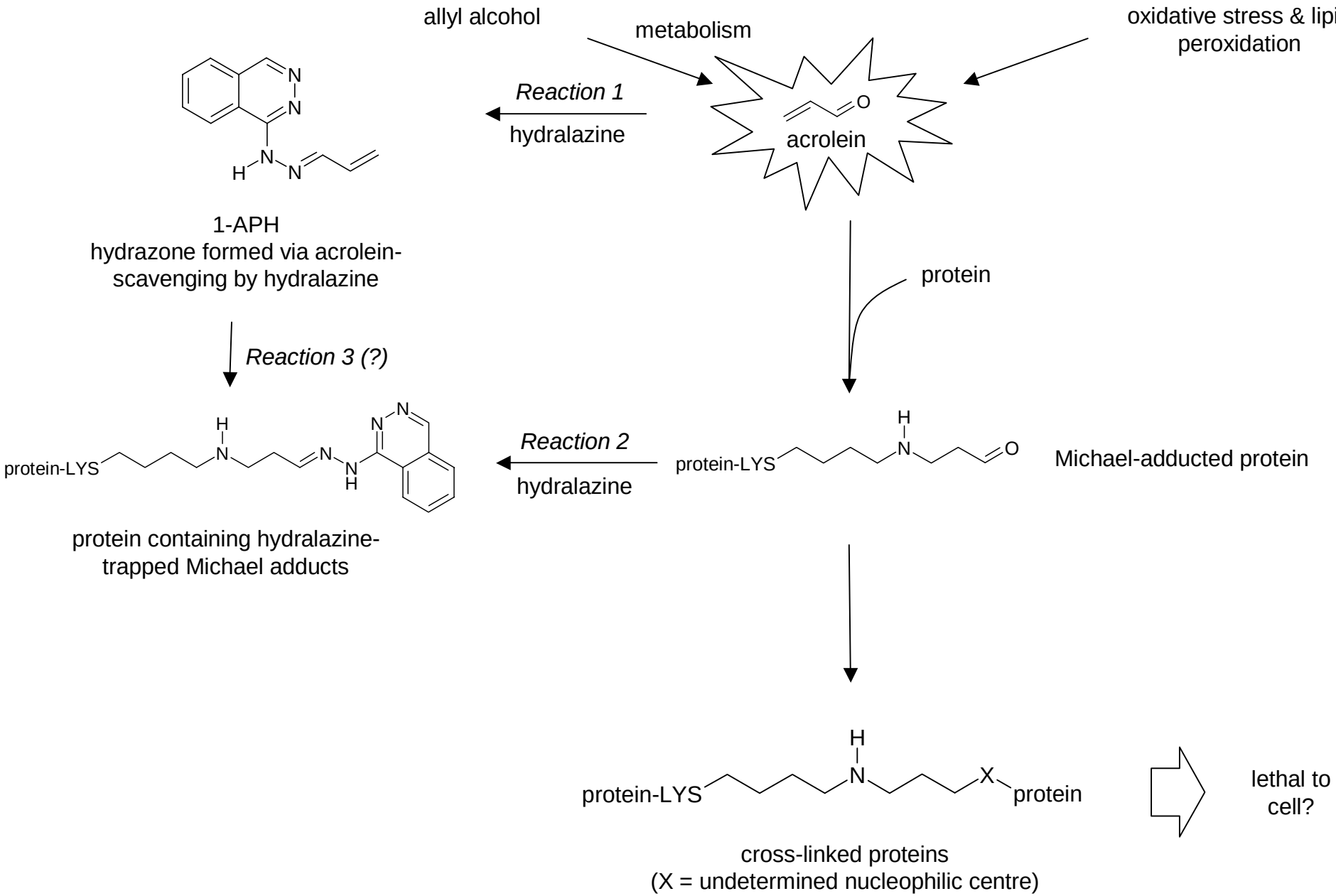

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Figure 2

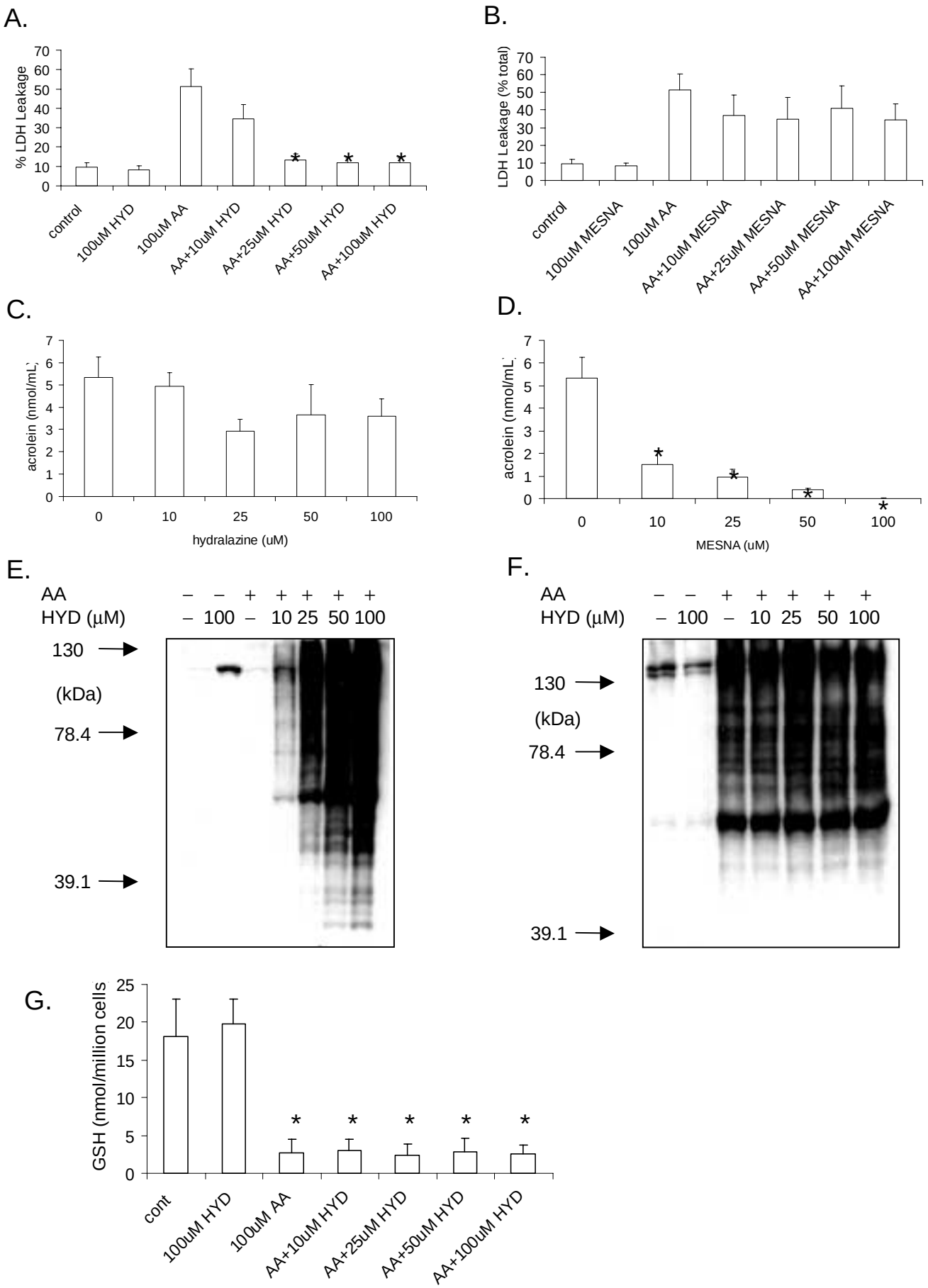

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Figure 3

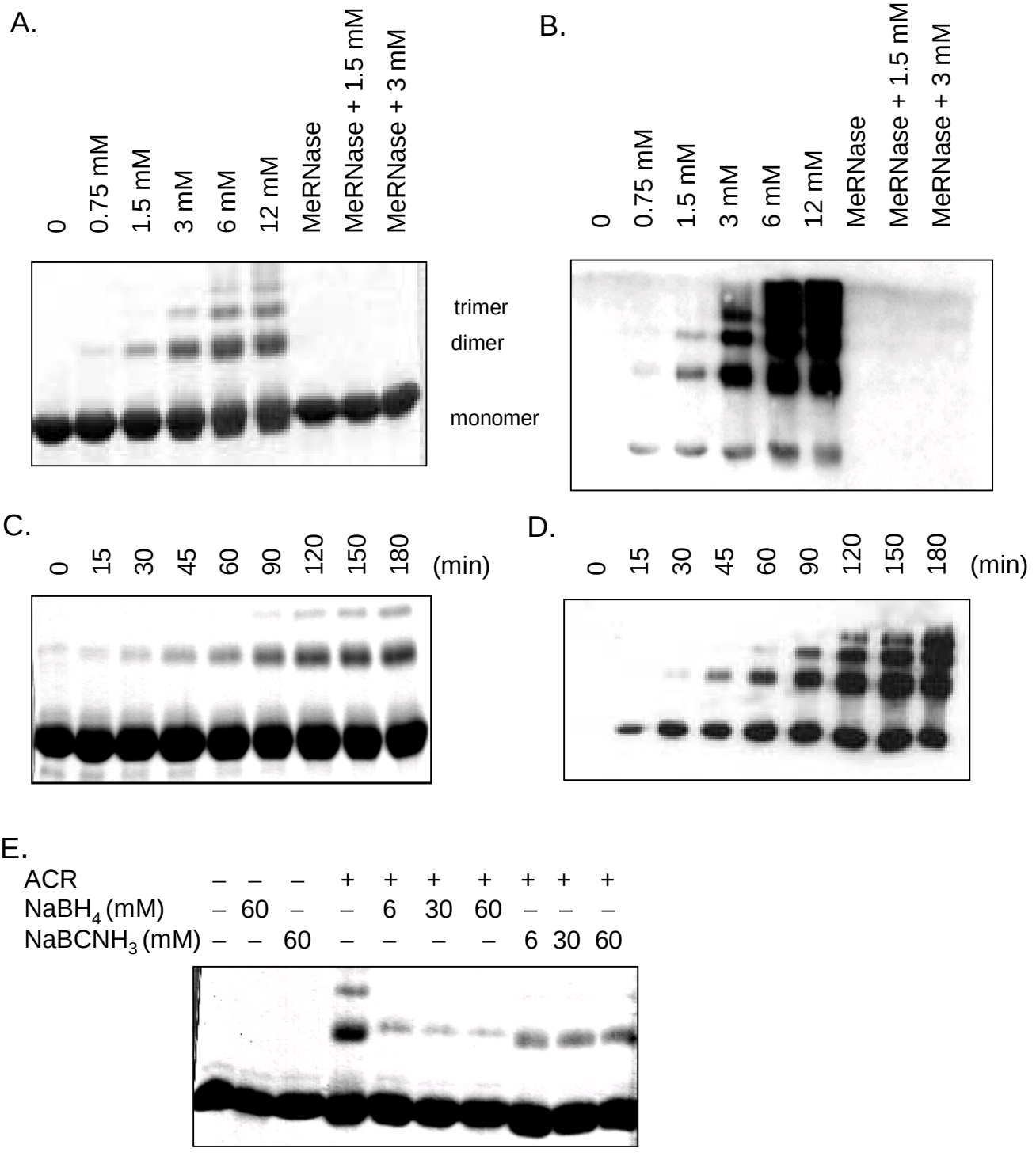

Figure 4

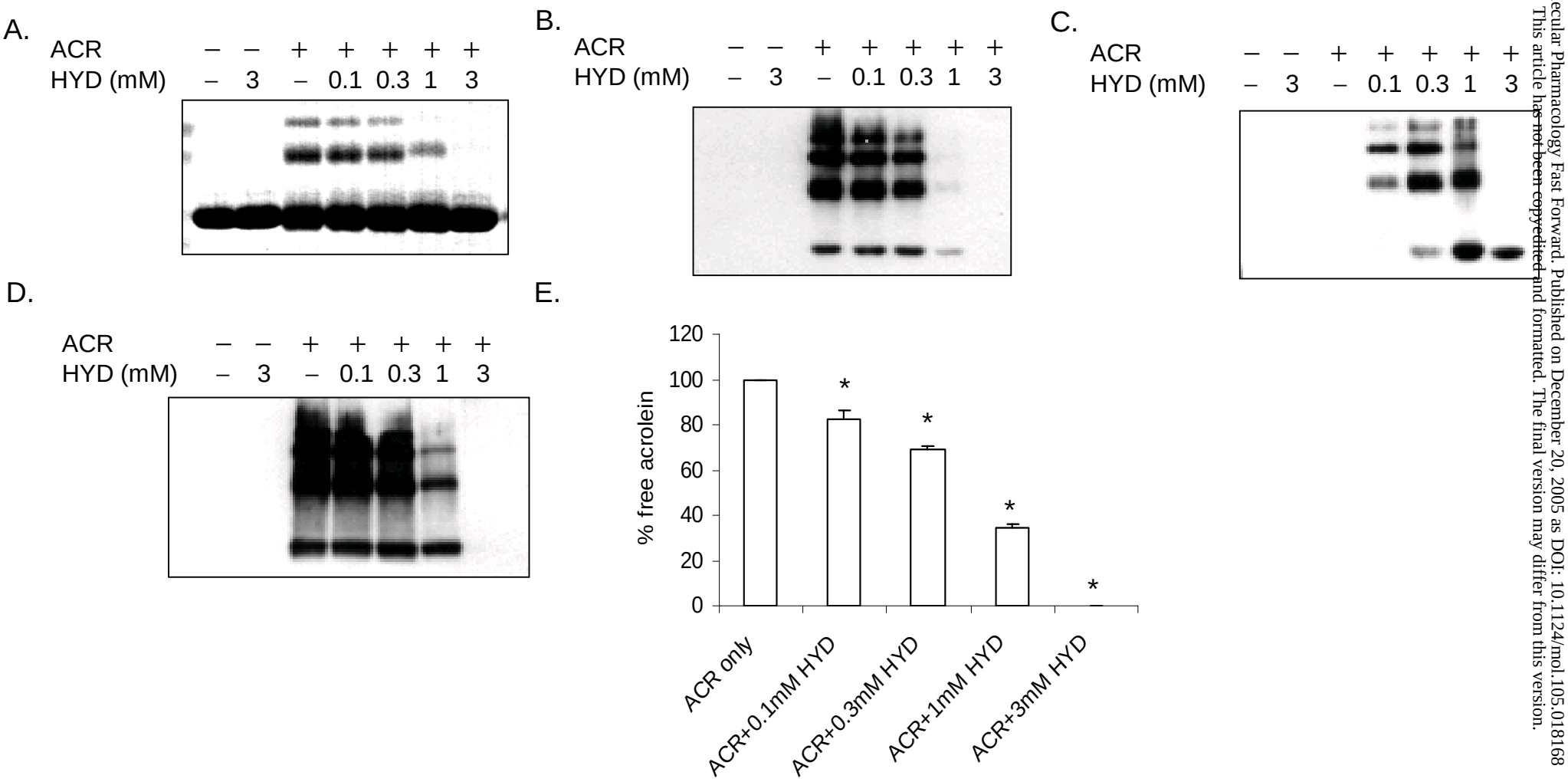

Figure 5

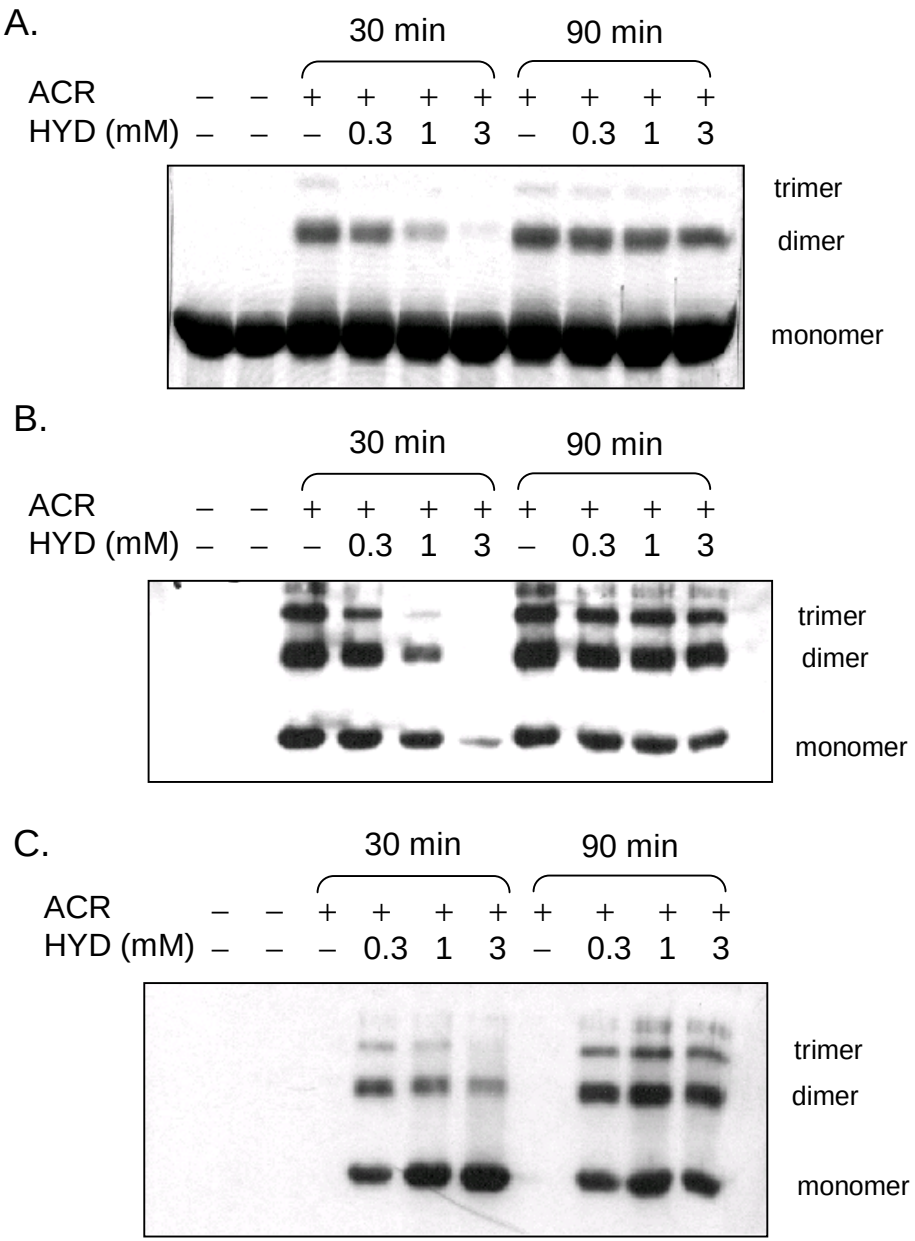

Figure 6

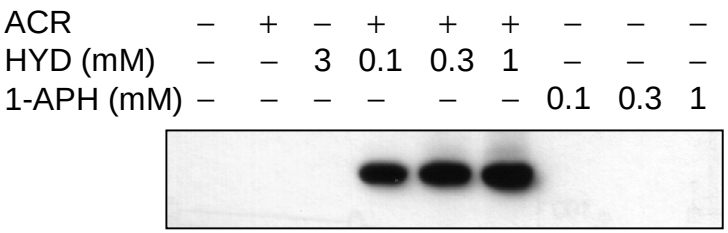

Figure 7