Chaperone Heat Shock Protein 90 Mobilization and Hydralazine Cytoprotection against Acrolein-Induced Carbonyl Stress

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ABSTRACT

Toxic carbonyls such as acrolein participate in many degenerative diseases. Although the nucleophilic vasodilatory drug hydralazine readily traps such species under “test-tube” conditions, whether these reactions adequately explain its efficacy in animal models of carbonyl-mediated disease is uncertain. We have previously shown that hydralazine attacks carbonyl-adducted proteins in an “adduct-trapping” reaction that appears to take precedence over direct “carbonyl-sequestering” reactions, but how this reaction conferred cytoprotection was unclear. This study explored the possibility that by increasing the bulkiness of acrolein-adducted proteins, adduct-trapping might alter the redistribution of chaperones to damaged cytoskeletal proteins that are known targets for acrolein. Using A549 lung adenocarcinoma cells, the levels of chaperones heat shock protein (Hsp) 40, Hsp70, Hsp90, and Hsp110 were measured in intermediate filament extracts prepared after a 3-h exposure to acrolein. Exposure to acrolein alone modestly increased the levels of all four chaperones. Coexposure to hydralazine (10–100 μM) strongly suppressed cell ATP loss while producing strong adduct-trapping in intermediate filaments. Most strikingly, hydralazine selectively boosted the levels of cytoskeletal-associated Hsp90, including a high-mass species that was sensitive to the Hsp90 inhibitor 17-\(\text{N}\)-allylamino-17-demethoxy-geldanamycin. Biochemical fractionation of acrolein- and hydralazine-treated cells revealed that hydralazine likely promoted Hsp90 migration from cytosol into other subcellular compartments. A role for Hsp90 mobilization in cytoprotection was confirmed by the finding that brief heat shock treatment suppressed acute acrolein toxicity in A549 cells. Taken together, these findings suggest that by increasing the steric bulk of carbonyl-adducted proteins, adduct-trapping drugs trigger the intracellular mobilization of the key molecular chaperone Hsp90.

Introduction

Many degenerative diseases are accompanied by an overproduction of reactive carbonyl compounds such as acrolein and 4-hydroxynonenal (Dalle-Donne et al., 2006; Lopachin et al., 2007). As strong electrophiles, these species deplete GSH and attack the proteome to form deleterious protein adducts (West and Marnett, 2006; Negre-Salvayre et al., 2008). Seeking to offset such damage, researchers have explored the use of nucleophilic drugs to scavenge “free” carbonyls within cells, attempting to attenuate macro molecular adduction and any accompanying toxicity (Aldini et al., 2007, 2011a; Burcham, 2008). Agents that have attracted attention as “carbonyl scavengers” include the neuroprotectant evaradone (Aldini et al., 2010), the antidepressant phenelzine (Wood et al., 2006), the hypoglycemic metformin (Wood et al., 2006), the hypoglycemic metformin (Ruggiero-Lopez et al., 1999), the antimicrobial isoniazid (Galvani et al., 2008), the dipeptide carnosine (Aldini et al., 2011b), the vasodilator hydralazine (Burcham et al., 2000), the antioxidant ascorbate (Kesinger et al., 2010), and various biogenic polyphenolic compounds (Zhu et al., 2009). For some of these agents, interpreting their in vivo efficacy is complicated by “secondary” pharmacological actions unrelated to carbonyl-sequestering reactivity; hence, further medicinal chemistry workup may be needed to derive useful “pharmacologically inert” carbonyl scavengers (Burcham et al., 2008; Bouguerne et al., 2011).

Among the strongest nucleophiles in clinical use, the antihypertensive hydralazine efficiently traps traps numerous...
carbonyl compounds within the plasma of human recipients (Reece, 1981). Under test-tube or cell-free conditions, hydralazine displays pronounced reactivity toward acrolein (Kaminskas et al., 2004a). Low hydralazine concentrations also block the toxicity of acrolein in diverse cell types including hepatocytes, neuronal cells, and lung epithelial cells (Liu-Snyder et al., 2006; Burcham et al., 2007; Truong et al., 2009). Protective efficacy for hydralazine has also been shown in several animal models of carbonyl-mediated pathology, including diabetic nephropathy, allyl alcohol hepatotoxicity, spinal cord injury, atherosclerosis, and experimental autoimmune disease (Nangaku et al., 2003; Kaminskas et al., 2004b; Vindis et al., 2006; Hamann et al., 2008; Leung et al., 2011). The mechanism(s) underlying drug efficacy in these models is unclear because the direct reactivity of hydralazine with electrophilic carbonyls may be constrained within biological systems. For example, in a hepatocyte model in which acrolein was generated enzymatically from allyl alcohol, hydralazine prevented cell death without lowering either extracellular “free acrolein” levels or the formation of protein adducts (Burcham et al., 2006). Others have also observed that hydralazine fails to block protein carbonylation in cultured cells, concluding instead that antioxidant actions mediate the cytoprotection (Mehta et al., 2009; Zheng and Bizzozero, 2010).

Our laboratory has described a distinctive “protein-adduct trapping” reaction for hydralazine that involves facile reactivity with Michael adducts formed by acrolein at nucleophilic sites in proteins (Burcham et al., 2004). Using Western blotting, intense adduct trapping was detected in allyl alcohol-treated hepatocytes upon treatment with protective hydralazine concentrations (Burcham et al., 2004). Strong adduct trapping also accompanied hydralazine protection against allyl alcohol hepatotoxicity in mice (Kaminskas et al., 2004b). How adduct trapping might confer protection was unclear, but one possibility was that hydralazine inactivated carbonyl-retaining Michael adducts before their participation in cross-linking with neighboring macromolecules (Burcham and Pyke, 2006). Contrary to this expectation however, hydralazine failed to block acrolein-induced protein cross-linking in cultured lung cells (Burcham et al., 2007). Other pathways whereby adduct trapping might confer cytoprotection awaited exploration.

We identified vimentin and other intermediate filaments (IFs) as targets for acrolein modification in A549 lung cells (Burcham et al., 2010). These key cytoskeletal constituents provide tensile strength to cell networks and serve as scaffolds for diverse cell-signaling molecules (Kim and Coulombe, 2007). Consistent with the role of chaperones in helping cells withstand protein-modifying stresses, acrolein exposure triggered the redistribution of chaperones heat shock protein (Hsp) 40, Hsp70, Hsp90, and Hsp110 to IF fractions in A549 cells (Burcham et al., 2010). Moreover, the efficient acrolein scavenger bisulfite simultaneously blocked IF modification and Hsp90 redistribution, suggesting chaperone mobilization was a consequence of IF damage (Burcham et al., 2010).

This study sought to clarify the mechanisms underlying hydralazine cytoprotection against carbonyl stress. An initial goal was to determine whether antioxidant actions might explain the cytoprotective properties of hydralazine. To supplement this approach, we explored whether the covalent incorporation of hydralazine into acrolein-adducted proteins via adduct-trapping might contribute to cytoprotection by assessing the “reversibility” of the protective mechanism. Finally, we explored whether hydralazine alters the redistribution of molecular chaperones to damaged IF during acrolein toxicity. Taken together, these experiments revealed that rather than its cytoprotective efficacy being attributable to antioxidant actions alone, they instead involve a “boost” in the subcellular availability of free Hsp90 that occurs subsequent to adduct-trapping reactions with carbonyl-adducted proteins.

Materials and Methods

Reagents. Rabbit anti-Hsp90 (detects both α and β isoforms) and anti-Hsp70 were purchased from Cell Signaling Technology (Danvers, MA). Hydralazine hydrochloride, phenylmethanesulfonyl fluoride (PMSF), p-tosyl-L-arginine methylester, 17-N-allylamino-17-de-methoxygeldanamycin (17-AAG), goat anti-vimentin serum, rabbit anti-dinitrophenol, anti-Hsp40, and anti-Hsp110 sera were obtained from Sigma-Aldrich (St. Louis, MO). Peroxidase-coupled goat anti-rabbit Ig was purchased from Thermo Fisher Scientific (Waltham, MA). Acrolein was supplied by Enzo Life Sciences, Inc., Lausen, Switzerland. Dulbecco’s phosphate-buffered saline (DPBS; glucose and pyruvate-containing) was supplied by InVitrogen Australia (Mount Waverly, Victoria, Australia).

Cell Culture. All acrolein-containing solutions were prepared in a fume hood. Human A549 adenocarcinoma lung cells were grown in F12 nutrient mixture supplemented with 10% fetal bovine serum (FBS; v/v) and gentamicin (100 μg/ml) until confluence was attained. The cells were harvested by trypsin-EDTA digestion and resuspended in F12 media supplemented with 0.5% FBS before they were plated in 1.4-ml volumes on six-well plates at a density of 0.6 × 10⁶ cells/well. The plates were then maintained overnight in 5% CO₂ in a humidified environment at 37°C. Cells were rinsed twice with DPBS before commencing exposure to acrolein in DPBS at a typical final concentration of 150 μM. The use of DPBS helped minimize side-reactions of acrolein with nucleophilic media constituents (Thompson and Burcham, 2008). All acrolein solutions were prepared immediately before use. In experiments in which the effect of drug treatment during the “postadduction phase” of toxicity was studied, cells were exposed to 75 μM acrolein in the presence and absence of hydralazine (100 μM) for 60 min, after which they were rinsed twice with DPBS to remove unreacted aldehyde. Fresh solutions of F12 media containing 0.5% FBS with or without hydralazine supplementation (μM) were then added before the plates were returned to the incubator. ATP determination were made after a subsequent 3-h incubation.

Biochemical Procedures. Cell ATP levels were measured as indicators of viability using a Promega CellTiter-Glo Luminescent Cell Viability assay kit (Promega, Madison, WI) according to the manufacturer’s protocols. Luminescence readings were taken using a POLARstar Optima microplate reader (BMG Labtech GmbH, Offenburg, Germany).

Preparation of subcellular fractions (membrane, cytosol, cytoskeletal, and nuclear) was achieved using a Qproteome Cell Compartment Kit (QIAGEN, Doncaster, Victoria, Australia). Alternatively, to prepare IF extracts directly, A549 monolayers grown on 25-cm² flasks were rinsed three times with Ca²⁺- and Mg²⁺-free PBS containing 0.6 M KCl, 1% Triton X-100, 10 mM MgCl₂, 1% β-mercaptoethanol, 1 mM PMSF, and 0.5 mg/ml p-tosyl-L-arginine methylester (Burcham et al., 2010). DNase I was added to a concentration of 0.5 mg/ml, and the dishes were placed on ice for 5 min. The suspensions were then transferred to polypropylene tubes and centrifuged at 2000g for 10 min at 4°C, after which the supernatants were discarded. The IF-containing pellets were then washed via one round of centrifugation...
capped culture flasks (25 cm²) were immersed for 60 min in waterplemented with 0.5% FBS [grown on either 96-well plates or securely
were treated with an equal volume of carbonyl-derivatizing solution
protein concentrations in 5-μl aliquots were then estimated using the
were prepared for protein content, and then lysate volumes containing 15 μg of protein
were diluted to a protein concentration of 1 mg/ml. Geldanamycin-biotin (40
the resin was recovered via centrifugation and then washed four
Low levels were then estimated using the Promega CellTiter-Glo kit
Western Blotting. After exposure to acrolein or drugs, cells were
were then estimated using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Antioxidant Assays. The antioxidant efficacy of hydralazine and
reference compounds was compared in a cell-free system using the
galvinoxyl assay (Shi et al., 2001). Galvinoxyl was dissolved in methanol, whereas stock solutions of reference compounds were prepared in acetonitrile. Galvinoxyl (1 ml of a 5-μM solution) was added to a 1-cm quartz cuvette, and reactions were started by adding 10-μl volumes of test compounds to yield a final concentration of 2.5 μM. Galvinoxyl consumption was monitored at room temperature using a Hitachi U-2000 UVVIS spectrophotometer (429-nm wavelength) (Hitachi Software Engineering, Yokohama, Japan). Lipid peroxidation in cell monolayers was assessed using the thiobarbituric acid assay (Nicholls-Grzeszmi et al., 2000).

Statistics. Data were analyzed via one-way analysis of variance using SigmaStat for Windows (version 3.5) (Systat Software, Inc., San Jose, CA). The post hoc tests used to detect differences between treatment groups are specified in the legends of the relevant figures.

Results

Consistent with the induction of acute cytotoxicity, cell ATP levels were decreased by 60% after a 4-h exposure to acrolein (Fig. 1). Treatment with hydralazine (10–300 μM) strongly attenuated ATP loss (Fig. 1).

Because the protective efficacy of hydralazine against chemically induced toxicity was suggested to involve antioxidant actions (Mehta et al., 2009), the galvinoxyl assay was used to compare the radical-trapping activity of hydralazine with that of established antioxidants (Fig. 2A). Concurring with observations made by Mehta et al. (2009) using cultured cells, hydralazine displayed strong radical-quenching activity toward galvinoxyl radicals, exhibiting activity that matched or exceeded that of ascorbate or the vitamin E analog 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; Hoffmann-La Roche, Nutley, NJ) (Fig. 2A). Consistent with these properties, hydralazine suppressed the formation of lipid peroxidation products during a 3-h exposure of A549 cells to acrolein, showing comparable antioxidative efficacy in this respect to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Fig. 2B). In contrast to hydralazine however, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid did not prevent the ATP depletion that accompanied acrolein exposure, whereas ascorbate actually enhanced ATP loss (Fig. 2C). Ascorbate also increased the formation of thiobarbituric acid reactive substances (data not shown). These findings suggest antioxidant properties alone are insufficient to suppress acute acrolein-induced toxicity in A549 cells.
The reversibility of hydralazine cytoprotection was assessed in the expectation that if the covalent incorporation of hydralazine into cell proteins via adduct trapping contributed to cytoprotection, cell viability would persist if hydralazine-containing media was removed. Thus, after a 60-min concurrent exposure to acrolein in the presence and absence of hydralazine, the culture medium was removed, and monolayers were rinsed twice with buffered saline before they were incubated for a further 3 h in either hydralazine-containing or drug-free media (Fig. 2D). Consistent with the covalent incorporation of hydralazine into damaged proteins (i.e., adduct trapping) participating in cytoprotection, comparable protection was seen in washed cells maintained in drug-free media during the postadduction phase as in cells that were continuously exposed to hydralazine (Fig. 2D).

Although hydralazine displays excellent reactivity toward “free” unsaturated aldehydes in model in vitro systems, it is unclear whether these properties apply within complex intracellular environments (Burcham and Pyke, 2006). To address this issue in the lung cell model used in the present study, we determined whether hydralazine suppressed acrolein-induced adduction of IFs, which are known targets for acrolein in lung cells (Burcham et al., 2010). Concurring with earlier work that ruled out a role for carbonyl scavenging in cytoprotection, the lowest, fully protective concentrations of hydralazine (Fig. 1) protected neither vimentin nor three keratin targets against acrolein adduction (Fig. 3A). Only the highest hydralazine concentration tested, 300 µM, strongly inhibited IF modification (Fig. 3A). This suggests that within the intracellular environment, reactions between free acrolein and nucleophilic target proteins proceed rapidly, and that hydralazine suppresses these reactions only when present at very high concentrations.

Although low hydralazine concentrations failed to prevent IF carbonylation, under these experimental conditions, clear adduct trapping did occur on IF targets (Fig. 3B). Indeed, adduct trapping was detected within each of the main IF targets for acrolein in A549 cells, namely vimentin, keratin-7, keratin-8, and keratin-18 (Fig. 3B). The identity of these four IF targets was established previously via liquid chromatography/mass spectrometry (Burcham et al., 2010). Consistent with associations of adduct trapping with cytoprotection in other experimental systems (Burcham and Pyke, 2006), hydralazine demonstrated comparable radical-trapping reactivity as ascorbate and 5,7,8-tetramethylchroman-2-carboxylic acid in the galvinoxyl assay (each scavenger was tested at a 2.5-µM final concentration). Cellular thiobarbituric acid-reactive substances (TBARS; B) and ATP levels (C) were measured after a 3-h exposure of A549 cells to acrolein in the presence and absence of 100-µM concentrations of protective compounds. D, the cytoprotection afforded by hydralazine against acrolein-induced ATP loss was not lost upon washing cells and subsequent incubation in drug-free media. See Materials and Methods for experimental details. Each data point is the mean ± S.E. of four independent experiments. *, significant difference from control; #, a difference from acrolein-treated cells (p < 0.05; C: Student-Newman-Keuls test and D: Dunnett’s test). HYD, hydralazine; ASC, ascorbic acid; TROL, Trolox; ACR, acrolein.
To determine whether hydralazine altered these responses, Western blotting was used to monitor chaperone levels in IF fractions extracted from A549 cells after a 3-h exposure to acrolein in the presence and absence of hydralazine (Fig. 4). As in prior work, acrolein alone increased IF-associated levels of Hsp40, Hsp70, Hsp90, and Hsp110 relative to controls or cells that were treated with hydralazine only (Fig. 4, A–D). In the case of Hsp70 and Hsp110, the lowest concentrations of hydralazine did not alter IF-associated chaperone levels relative to the changes that accompanied exposure to acrolein alone (Fig. 4, B and D). In contrast, hydralazine elicited a concentration-dependent decrease in the levels of IF-associated Hsp40, with the 100-μM concentration suppressing Hsp40 levels below detectable levels to resemble controls (Fig. 4A).

The effects of hydralazine on Hsp90 mobilization to damaged IF were especially striking (Fig. 4C; the results obtained during densitometry of replicate experiments are shown in Fig. 4, E and F). Compared with the modest increases in IF-associated Hsp90 induced by acrolein alone, the presence of hydralazine strongly boosted Hsp90 mobilization (Fig. 4C). This effect was evident in levels of monomeric Hsp90 as well as a high-mass oligomeric Hsp90 species (~300 kDA) we observed previously in acrolein-exposed cells (Burcham et al., 2007, 2010). Because high-mass species are known to form when Hsp90 catalysis occurs in the presence of cross-linking agents (Vaughan et al., 2009), this finding suggests hydralazine boosted the intracellular availability of Hsp90 together with its catalytic activity toward damaged protein substrates. Concurring with the strong protection against ATP loss afforded by 10 and 30 μM hydralazine in Fig. 1, the effects of hydralazine on the redistribution and activation of Hsp90 were near maximal at the lowest drug concentrations tested (Fig. 4C).

The concentration dependence of drug-induced changes in IF-associated Hsp90 levels was investigated in cells exposed to a range of acrolein concentrations in the presence of 30 μM hydralazine (Fig. 5A). The 3-h treatment with hydralazine elicited an increase in the levels of monomeric and high-mass Hsp90 at the top two acrolein concentrations, 100 and 150 μM (Fig. 5A). Study of the time course of Hsp90 mobilization to IF indicated this response was rapid and essentially maximal within 1 h of commencing exposure to acrolein and hydralazine (Fig. 5B). This suggests the effect of hydralazine involved boosted mobilization of existing Hsp90, rather than an activation of chaperone synthesis as per the classic heat shock response (i.e., activation of heat shock factor 1-driven transcriptional activity) that is expected to occur over a longer experimental time frame.

Reflecting its ability to inhibit the catalytic cycle of Hsp90 by targeting the ATP-binding domain of the chaperone complex, the Hsp90 inhibitor 17-AAG abolished the increase in IF-associated Hsp90 by targeting the ATP-binding domain of the chaperone complex.
ciliated high-mass Hsp90 that accompanied coexposure to acrolein and hydralazine (Fig. 6A). In contrast, 17-AAG had no effect on the redistribution of monomeric Hsp90 to damaged IF (Fig. 6A). The Hsp90 inhibitor also failed to substantially attenuate hydralazine-induced protection against cellular ATP loss (Fig. 6B), suggesting the cytoprotection might involve boosted redistribution of monomeric Hsp90 to damaged proteins rather than any drug-induced increase in the catalytic activity of the chaperone per se (Fig. 6B).

To characterize drug-induced changes in subcellular Hsp90 distribution at the biochemical level, cells were coexposed to acrolein and a range of hydralazine concentrations for 3 h before cellular fractionation was performed to obtain cytosolic, nuclear, cytoskeletal, and membrane fractions (Fig. 7). In keeping with literature expectations, Hsp90 was primarily located within the cytosol of unstressed and hydralazine-treated control cells (Fig. 7A, left). Upon exposure to acrolein in the presence of hydralazine, a pronounced redistribution of Hsp90 to the nuclear and cytoskeletal fractions occurred above that elicited by acrolein alone, with the levels of both monomeric and high-mass Hsp90 increased by the drug (Fig. 7, B and C, left). The chaperones that relocated to these fractions most likely originated in cytosol, where hydralazine treatment elicited a clear reduction in Hsp90 levels (Fig. 7A, left).

Western blot analysis of cell extracts for the presence of hydralazine-labeled species revealed adduct trapping occurred in all fractions to which Hsp90 was mobilized (Fig. 7, A–D, right). Hydralazine also participated in “spontaneous” adduct-trapping reactions with two cytosolic proteins in controls that were not knowingly exposed to acrolein (Fig. 7A, right), indicating drug reactions with endogenous carbonyl-adducted proteins occurred in this compartment. Reactions with endogenous proteins were not detected in hydralazine-treated controls in the other cell fractions.

In cells concurrently exposed to acrolein and hydralazine, concentration dependence in the formation of trapped adducts was evident over the 10 to 100 µM drug concentration range within cytosolic (Fig. 7A), nuclear (Fig. 7C), and membrane fractions (Fig. 7D). A ~35-kDa protein sustained strong, concentration-dependent adduct trapping in the membrane fractions of acrolein/hydralazine-exposed cells (Fig. 7D, right). Concentration dependence was less obvious within cytoskeletal extracts (Fig. 7B), where adduct trapping appeared saturated at the lowest drug concentration tested (10 µM) for a ~50 kDa protein (highlighted with an arrow on Fig. 7B, right). This strong band may reflect adduct trapping on vimentin or other IF proteins that are major cytoskeletal targets for acrolein within A549 cells (Burcham et al., 2010). Given that Hsp90 mobilization was essentially maximal at the 10-µM concentration (Fig. 4, C, E, and F), this suggests adduct trapping on IF may facilitate the mobilization of subcellular Hsp90 by hydralazine.

Seeking to clarify the mechanisms underlying drug-induced Hsp90 redistribution, a new protein-capture technique using a biotin-labeled analog of the Hsp90 inhibitor geldanamycin was used to recover Hsp90 from unfractionated cell lysates prepared after a 3-h exposure of A549 cells to acrolein (150 µM). The high-mass Hsp90 reaction product is highlighted with an arrow. The immunoblot shown is representative of three independent replicates (25 µg protein/lane).

Fig. 6. A, the Hsp90 inhibitor 17-AAG inhibited hydralazine-induced Hsp90 oligomerization but not redistribution of monomeric Hsp90 to IF extracts or protection against ATP loss (B) during a 3-h exposure of A549 cells to acrolein (150 µM). The high-mass Hsp90 reaction product is highlighted with an arrow. The immunoblot shown is representative of three independent replicates (25 µg protein/lane). * * *, significant difference from acrolein-only and acrolein/17-AAG-only treated cells (p < 0.001, Holm-Sidak test, n = 4, means ± S.E.).
mass species detected within IF extracts in the previous experiments was recovered from acrolein/hydralazine-treated cells using the Hsp90 protein-capture methodology (Fig. 8A). Analysis of this recovered high-mass protein sample via electrospray ionization-mass spectrometry confirmed the presence of the α and β forms of Hsp90, but insufficient novel peptides were identified to allow identification of any putative protein cross-linking participants (data not shown).

Analysis of captured protein samples using the antibody against hydralazine/acrolein-labeled proteins revealed that adduct trapping occurred in both the monomeric and high-mass Hsp90 bands (Fig. 8B).

To test whether acute Hsp90 mobilization protects against acrolein toxicity, A549 cells were subjected to a brief 60-min heat shock treatment at 43, 44, or 45°C before commencing acrolein exposure (Fig. 9). Western blot analysis of IF extracts prepared from cells immediately after the heat treatments revealed Hsp90 mobilization to IF occurred only at the highest temperature tested (45°C; Fig. 9A). Measurement of ATP levels in control- and heat-treated cells after a subsequent 3-h exposure to 150 μM acrolein revealed that only the 45°C temperature fully protected against ATP loss (Fig. 9E).

Discussion

The present findings reveal an unexpected consequence accompanying exposure of oxidatively stressed cells to nucleophilic carbonyl-trapping drugs. Using hydralazine, an

Fig. 7. Western blot analysis of cell fractions for Hsp90 levels (immunoblots on the left) and hydralazine (HYD)-trapped adducts (immunoblots on the right) reveal that drug-induced adduct trapping likely promotes the migration of Hsp90 from cytosol (A) to nuclear (B), cytoskeletal (C), or membrane fractions (D) during a 3-h exposure of A549 cells to acrolein (ACR; 150 μM). The high-mass Hsp90 reaction product is highlighted with an arrow (left), as is a ∼50 kDa likely IF species containing hydralazine-trapped adducts in B (blots on the right). The immunoblots shown were obtained from the same cell fractions and are representative of two independent replicates (100 and 25 μg protein loaded per lane in the immunoblots on the left and right, respectively).
antihypertensive agent long known to trap biogenic carbonyl compounds (Reece, 1981), we found that the molecular chaperone Hsp90 is mobilized to subcellular locations containing damaged proteins. A key participant during the triage of abnormal, unfolded proteins, Hsp90 helps cells withstand a range of proteotoxic stresses (Taipale et al., 2010). Our finding that hydralazine selectively mobilized Hsp90 only in cells containing carbonyl-modified proteins uncovers a novel avenue of cytoprotective drug action. This mechanism appeared more directly implicated in cytoprotection than the antioxidant properties of hydralazine, because two classic antioxidants, ascorbate and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, failed to suppress ATP loss in acrolein-treated cells (Fig. 2). The finding that cytoprotection against acrolein toxicity occurred in cells in which acute intracellular Hsp90 mobilization was achieved via heat shock treatment reinforces the role of this mechanism in the suppression of cell death by hydralazine.

Although the precise mechanisms underlying hydralazine-induced Hsp90 redistribution are unclear, a role for adduct trapping seems likely (Fig. 10). We previously used mass spectrometry to study the reaction of hydralazine with an acrolein-modified peptide, confirming that hydralazine reacts with carbonyl-retaining adducts to form multiple hydration zones, which we collectively termed “trapped adducts” (Burcham et al., 2004). Such reactivity with acrolein-derived protein adducts sets hydralazine apart from other carbonyl scavengers (Burcham et al., 2004). Moreover, using a rabbit antibody raised against hydralazine/acrolein-modified protein, we detected strong adduct trapping in hepatocytes that were concurrently exposed to low concentrations of hydralazine and the acrolein precursor allyl alcohol (Burcham et al., 2004). This antibody also revealed intense adduct trapping during acrolein-mediated hepatotoxicity in mice that received hepatoprotective doses of hydralazine (Kaminskas et al., 2004b). Precisely how adduct trapping might confer such protection was unclear, because the expectation that this mechanism would suppress intramolecular protein cross-linking during acrolein toxicity was not confirmed in cellular studies (Burcham et al., 2007).

Although this study identifies Hsp90 mobilization as a novel mechanism accompanying adduct trapping by hydralazine, it is interesting that we failed to detect this mechanism previously when the effect of acrolein and hydralazine on Hsp90 expression was studied in unfractionated cell lysates (Burcham et al., 2007). Thus, drug-induced Hsp90 mobilization became evident only after fractionation techniques were used to prepare cytoskeleton-enriched cell extracts. These observations form the basis of our hypothesis concern-
that the suppression of acute cell death is associated with enhanced cellular availability of Hsp90 per se. Hsp90 complexes are thought to exist in two main forms, namely a “proteasome-targeting” species that involves the ancillary proteins Hsp70 and Hsp70-Hsp90 organizing protein and a “stabilizing” form that involves cdc37 and p23 (Sullivan et al., 1997; Aridon et al., 2011). More work is needed to clarify the protein composition and cellular fate of agglomerates that form upon recruitment of Hsp90 to “trapped adduct-containing complexes” with a view to establishing whether hydralazine promotes the formation of these known Hsp90-containing complexes. Such research should also help identify the protein constituents of the high-mass Hsp90-containing species, which is a conspicuous product of hydralazine cytoprotection against carbonyl stress.

The finding that the chaperone redistribution elicited by hydralazine in acrolein-exposed cells was selective for Hsp90 is intriguing given that Hsp90 is typically highly expressed in nonstressed cells and participates in many routine cell processes (Pearl and Prodromou, 2006). The list of Hsp90 client proteins includes kinases, transcription factors, and diverse signaling molecules (Falsone et al., 2005). The role of Hsp90 buffering in maintaining tumor cell viability in the face of high mutational load has made this chaperone a major focus of attention in cancer therapeutics (Travers et al., 2012). Perhaps one unresolved question remaining from the present study concerns the use of A549 lung cells, which as cancer cells are likely “chaperone-addicted” and thus may not predict responses in nontumor cells. Whether hydralazine elicits chaperone mobilization during carbonyl stress in primary cells that exhibit more normal patterns of chaperone expression should be explored in future work.

The possibility that adduct trapping and associated chaperone mobilization underlie the protective actions of hydralazine during carbonyl stress provides another reason to revise the conventional paradigm that covalent interactions between drugs and proteins primarily elicit harmful outcomes (Singh et al., 2011). After classic studies on the metabolic activation of such toxicants as acetaminophen, ipomeanol, furosemide, and bromobenzene, the ability of xenobiotics to form protein-modifying intermediates is typically viewed as a precursor to such unwanted outcomes as impaired macromolecular function or haptenization of tissue proteins (Mitchell et al., 1973). Although this paradigm has strongly influenced drug discovery and development, many exceptions exist to the rule that “protein adduction by drugs equals toxicity.” Indeed, the formation of stable adducts with target proteins underlies the efficacy of many medicines, including aspirin, rasagiline, phenoxybenzamine, esomeprazole, clopidogrel, and gemicitabine (Singh et al., 2011). Typically, covalent drugs are assigned to one of two classes: those containing pre-existing electrophilic functionality within the parent structure (e.g., amoxicillin) and those that undergo biotransformation to electrophilic species (e.g., acetaminophen). Our findings suggest a third category comprising nucleophilic drugs that react with electrophilic functionalities that are introduced into proteins during adduction by endogenous electrophiles (e.g., acrolein). Given the plethora of bifunctional electrophilic carbonyls that form during normal metabolism, reactions of adducted proteins with circulating nucleophilic drugs might be more extensive than currently realized.

Given the potential significance of this novel pharmacological mechanism to a range of diseases that involve carbonyl...
stress, several important avenues of future research require exploration. For example, it is unclear whether the Hsp90 redistribution elicited by hydralazine occurs selectively in response to adduct trapping on specific proteins or whether chaperone mobilization occurs indiscriminately throughout the entire cell. The use of immunocytochemical imaging to monitor changes in subcellular Hsp90 localization should resolve such questions and also clarify whether drug-induced chaperone mobilization extends to other Hsp90 family members such as Grp94 (endoplasmic reticulum) and TRAP1 (mitochondrial form). Such approaches should also allow study of these phenomena using lower acrolein and drug concentrations than those used in the present study. Moreover, because Western blotting analysis of Hsp90 captured from acrolein- and hydralazine-treated cells suggested that adduct trapping actually occurred on both the monomeric and high-mass forms of Hsp90 (Fig. 9), future work should explore whether these reactions directly participate in Hsp90 mobilization and/or activation. Another issue to be addressed is whether, after oral dosing with hydralazine, sufficient free drug survives first-pass clearance in the gastrointestinal tract and liver, as well as spontaneous carbonyl-trapping reactions in circulating blood, to permit reactions with adducted proteins within remote tissues. Such work could, therefore, establish whether adduct trapping occurs in body tissues or blood proteins during short-term or chronic dosing with low doses of hydralazine that do not induce intolerable cardiovascular changes.

In conclusion, the present study identifies chaperone Hsp90 mobilization as a novel consequence of adduct trapping by nucleophilic drugs in cells that are subjected to carbonyl stress. Our provisional hypothesis proposes that by enhancing the “steric bulk” of carbonyl-adducted proteins, adduct-trapping drugs facilitate subcellular chaperone mobilization during carbonyl stress. This novel mechanism of drug action may be relevant to diverse diseases in which protein carbonylation occurs (Dalle-Donne et al., 2006), including important neurodegenerative disorders in which molecular chaperones are already thought to mitigate pathogenic neuronal loss (Aridon et al., 2011; Salminen et al., 2011). An ability to boost chaperone availability within cells containing damaged proteins raises the possibility of novel therapeutic approaches in these conditions, thereby extending encouraging results obtained for carbonyl scavengers in animal models of human neurodegeneration (Davies et al., 2011). The currency of this novel drug mechanism might be further increased by the finding that acrolein is a major contributor to age-related protein damage within the cardiac mitochondrial proteome of aging rats (Chavez et al., 2011).

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Authorship Contributions

Participant in research design: Burcham.
Conducted experiments: Raso and Kaminskas.
Contributed new reagents or analytic tools: Burcham.
Performed data analysis: Burcham.

Wrote or contributed to the writing of the manuscript: Burcham and Kaminskas.

References

Kaminskas LM, Pyke SM, and Burcham PC (2004b) Strong protein adduct trapping actually occurred on both the monomeric and high-mass forms of Hsp90. Such approaches should also allow study of these phenomena using lower acrolein and drug concentrations than those used in the present study. Moreover, because Western blotting analysis of Hsp90 captured from acrolein- and hydralazine-treated cells suggested that adduct trapping actually occurred on both the monomeric and high-mass forms of Hsp90 (Fig. 9), future work should explore whether these reactions directly participate in Hsp90 mobilization and/or activation. Another issue to be addressed is whether, after oral dosing with hydralazine, sufficient free drug survives first-pass clearance in the gastrointestinal tract and liver, as well as spontaneous carbonyl-trapping reactions in circulating blood, to permit reactions with adducted proteins within remote tissues. Such work could, therefore, establish whether adduct trapping occurs in body tissues or blood proteins during short-term or chronic dosing with low doses of hydralazine that do not induce intolerable cardiovascular changes.

In conclusion, the present study identifies chaperone Hsp90 mobilization as a novel consequence of adduct trapping by nucleophilic drugs in cells that are subjected to carbonyl stress. Our provisional hypothesis proposes that by enhancing the “steric bulk” of carbonyl-adducted proteins, adduct-trapping drugs facilitate subcellular chaperone mobilization during carbonyl stress. This novel mechanism of drug action may be relevant to diverse diseases in which protein carbonylation occurs (Dalle-Donne et al., 2006), including important neurodegenerative disorders in which molecular chaperones are already thought to mitigate pathogenic neuronal loss (Aridon et al., 2011; Salminen et al., 2011). An ability to boost chaperone availability within cells containing damaged proteins raises the possibility of novel therapeutic approaches in these conditions, thereby extending encouraging results obtained for carbonyl scavengers in animal models of human neurodegeneration (Davies et al., 2011). The currency of this novel drug mechanism might be further increased by the finding that acrolein is a major contributor to age-related protein damage within the cardiac mitochondrial proteome of aging rats (Chavez et al., 2011).

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Authorship Contributions

Participated in research design: Burcham.
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