Protein Alkylation, Transcriptional Responses and Cytochrome c Release during Acrolein Toxicity
in A549 Cells: Influence of Nucleophilic Culture Media Constituents.

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Short title: Acrolein side-reactions in A549 cells

Abstract

Acrolein is a smoke constituent that elicits apoptotic and/or necrotic cell death depending on the exposure conditions. As a strong electrophile, side-reactions with nucleophilic media constituents seem likely to accompany study of its toxicity in vitro, but these reactions are poorly characterised. We thus examined the influence of media composition on the responses of A549 cells to acrolein. Cells were exposed to acrolein in either Dulbecco's buffered saline (DBS) or F12 media supplemented with various concentrations of fetal bovine serum. Cell viability was assessed using the MTT assay, while heme oxygenase-1 (HO-1) and cytoplasmic cytochrome c were measured as respective markers of transcriptional response and apoptosis. Protein damage was evaluated using the protein carbonyl assay. Compared to F12 media (with or without serum), maximal cell death as well as carbonylation of intracellular proteins, occurred when cells were exposed to acrolein in DBS. Cytochrome c release also occurred in the presence of DBS, but only at low acrolein concentrations. In contrast, the presence of serum-containing F12 during exposure to acrolein inhibited protein modification, cytochrome c release and overt cell death. These findings highlight the need for careful selection of culture media when conducting in vitro toxicological studies of reactive substances.

Keywords: Acrolein, protein adducts, protein carbonyls, Antioxidant Response Element, HO-1.

Abbreviations: ARE, antioxidant response element; Arg, arginine; BCA, bicinchoninic acid; BSA, bovine serum albumin; Cys, cysteine; DBS, dulbecco's buffered saline; FBS, Fetal Bovine Serum; F12, F-12K nutrient mixture (Kaighns modification); F12/0.5% FBS, F-12K nutrient mix supplemented with 0.5% FBS; F12/5% FBS, F-12K nutrient mix supplemented with 5% FBS; F12/10% FBS, F-12K nutrient mix supplemented with 10% FBS; His, histidine; HO-1, heme-oxygenase 1; Lys, lysine; PBS, phosphate-buffered saline; RIPA, Radioimmunoprecipitation Buffer; TBS, tris-buffered saline.

Introduction

Acrolein is a highly toxic three-carbon unsaturated aldehyde produced during the incomplete combustion of organic matter (Hartzell, 1996). Human exposure to low level acrolein is largely unavoidable due to its production in everyday activities (car exhaust fumes, cigarette smoke, etc.). High concentrations of acrolein in smoke produced upon combustion of timber, plastics and other building materials ensures it is a key contributor to lung damage in smoke inhalation victims (Hales 1988, 1992). In addition to foreign sources, acrolein forms in the body during lipid peroxidation and polyamine metabolism and such endogenous exposure has been implicated in the pathogenesis of several degenerative diseases (Finkel and Holbrook, 2000). The high toxic potential of acrolein reflects its possession of two strongly electrophilic centres which ensure it readily reacts with nucleophilic groups on biological molecules including glutathione (Miccadei *et al*, 1988), DNA (Chung *et al*, 1984) and proteins (Esterbauer *et al*, 1991). These reactions typically proceed via Michael addition of nucleophiles to the α,β -unsaturated bond of acrolein, generating carbonyl-retaining adducts (Burcham & Fontaine, 2001). Adduction of a diverse range of targets, in addition to disruption of the cellular redox balance, appears to underlie the disruption of multiple biochemical pathways in acrolein-exposed cells (Kehrer & Biswal, 2000). Such events can trigger the death of exposed cells via either apoptosis and/or necrosis, depending on such factors as the level of acrolein exposure, the degree of caspase inhibition elicited by acrolein, and the cell type involved (Kern and Kehrer, 2002).

While the chemical reactivity of acrolein is important in understanding the molecular events underlying its toxicity, it also raises the prospect of side-reactions with cell media constituents during *in vitro* studies employing cultured cells. Such experiments typically involve acrolein addition to nucleophile-containing culture media; hence, any side-reactions with media constituents may diminish the bioavailability of acrolein and thus influence experimental outcomes. Such a scenario was suggested by early studies of acrolein mutagenicity (Grafstom, 1990) and also during exploration of acrolein teratogenicity in rat embryos (Slott and Hales 1986). More recently,

different laboratories have used quite dissimilar experimental conditions in terms of concentrations of nucleophilic constituents in the culture media during cellular exposure to acrolein. For example, to minimize unwanted side-reactions during a series of studies in alveolar Type 2-derived A549 lung tumor cells, Kehrer and associates employed amino acid- and serum-free Earl's Balanced salt solution, a medium that is essentially free of nucleophilic constituents. This allowed detection of acrolein effects at such molecular targets as the transcription factors AP-1 (Biswal et al., 2002) and NF- κ B (Horton et al. 1999); the tumor suppressor protein p53 (Biswal et al., 2003) and the antioxidant thioredoxin (Yang et al, 2004). During related studies of the effect of acrolein and cigarette smoke extracts on apoptotic endpoints in A549 cells, others favored the use of a serum-free yet amino acid-containing medium, DMEM (Hoshino et al., 2001). Likewise, a recent study of the effect of acrolein on cytokine expression in A549 cells employed F12K media supplemented with a very low concentration of BSA (Doyle et al, 2006). In contrast, during characterization of the effect of acrolein on Nrf2-regulated genes, A549 cells were exposed to acrolein solutions prepared in amino acid-containing media (NMEM) supplemented with 10 % FBS (Tirumalai et al., 2002).

To date, no study of acrolein toxicity in an in vitro model has addressed the impact of culture media composition on the extent of damage occurring at protein targets within acrolein-exposed cells, or the degree to which the culture media constituents influence pathways of cell death elicited by acrolein. To address these issues, we explored the effect of media composition on the toxicity of acrolein towards A549 cells, using Dulbecco's buffered saline as a reference nucleophile-free medium. Amino acid-containing F12 media (Kaighn's modification, supplemented with different levels of FBS) was used to investigate the effect of nucleophilic media constituents on cellular responses to acrolein. Protein damage was detected via immunoblotting for carbonylated proteins (Burcham and Fontaine, 2001). Cellular viability was assessed using the thiazolyl blue tetrazolium bromide (MTT) assay (Alley *et al* 1988), while the onset of apoptotic cell death was assessed via measurement of cytochrome c levels in cytoplasm (Tanel and Averill-Bates, 2005; Liu et al 1996). Transcriptional responses to acrolein were detected via immunodetection of

heme oxygenase-1 (HO-1), one of a number of Nrf2-regulated genes that are up-regulated in acrolein-exposed cells (Kehrer and Biswal, 2000; Tirumalai *et al*, 2002; Wu et al., 2006).

Materials and Methods

Materials. F12 nutrient mixture (Kaighn's modification), Dulbecco's phosphate buffered saline (glucose- and pyruvate-containing), fetal bovine serum (FBS), Trypsin-EDTA and gentamicin were manufactured by Gibco and purchased from Invitrogen Australia Pty. Ltd. (Mount Waverly, VIC, Australia). *N,N* – dimethyl-formamide, acrolein, rabbit anti-DNP sera and alkaline phosphatase-coupled anti-rabbit IgG were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Heme oxygenase-1 polyclonal antibody was supplied by Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), while the BCA Protein Assay kit was obtained from Pierce (Rockford, IL, USA). Mouse anti-cytochrome c monoclonal antibody and HRP goat anti-mouse polyclonal antibody was supplied by BD Pharmingen (San Jose, CA, USA). Nitrocellulose Trans-blot[®] Transfer Medium was purchased from Biorad (Hercules, CA, USA). Acrolein was purchased from Alexis Biochemicals (Lausen, Switzerland).

Cell culture. Human A549 adenocarcinoma lung cells were grown in F12 nutrient mixture supplemented with 10% FBS (v/v) and gentamicin (100 mg/L) until a confluent state was observed. The cells were harvested by trypsin-EDTA digestion and resuspended in F12 media supplemented with 0.5% FBS. Cell number was determined using a hemocytometer and cells were plated in 1.4 mL volumes on 6-well plates at a density of 0.6×10^6 cells per well. Plates were maintained in 5% CO₂ at 37 °C overnight prior to the commencement of exposure to acrolein. Immediately before use, stock dilutions of acrolein were first prepared in Dulbecco's phosphate-buffered saline (DBS) followed by a second dilution in the appropriate culture medium to obtain the final concentrations required for each experiment. Following acrolein addition the plates were returned to the incubator for the duration of the experiment.

Cell viability assay. Following exposure to acrolein, 0.1 volumes of MTT solution (5 mg/mL, prepared in double-distilled water) were added to each well and plates were returned to the incubator for 2 h. Following the removal of 0.75 mL of media the same volume of Lysis buffer (comprising 20% SDS (*w/v*) and 50% *N,N* – dimethylformamide (*v/v*)) was added to each well. The dishes were then placed on a shaking platform until the formazan crystals were dissolved. A 1/10 dilution of each sample was prepared and the optical density of the resulting solution was determined at 570 nm using a BMG *POLARstar Optima* spectrophotometer.

Protein carbonyl assay. Upon completion of acrolein exposure the cell media was discarded and the cell monolayers were washed three times with DBS. The cells were then lysed using a cell scraper following the addition of 50 μ L of 6M urea solution to each well. The lysates were transferred to an Eppendorf tube and then 10 μ L aliquots were removed for protein determination using the Pierce BCA Protein Assay Kit. Next, lysate volumes comprising 15 μ g protein were transferred to fresh tubes that contained an equal volume of DNPH solution (0.5% 2,4-dinitrophenyl hydrazine (*w/v*) in 10% trifluoroacetic acid (*v/v*)). After allowing carbonyl derivatization to proceed for 20 minutes at room temperature, 2 volumes of neutralizing solution were added to each tube (2M Tris in 40% glycerol (*v/v*)). One volume of (5 $\times$) SDS-PAGE sample buffer (free of reducing agents) was then added to each tube (Bollag, 1996). The samples were then resolved on a 14% polyacrylamide gel for 45 minutes at 200 V. Following transfer to nitrocellulose (100 V, 30 minutes), the membrane was blocked in PBS/5% nonfat milk overnight followed by a 1 hour incubation in PBS/5% nonfat milk containing rabbit anti-DNP sera (1/1000 dilution). The membrane was washed in PBS and incubated with the secondary antibody (alkaline phosphatase-coupled anti-rabbit IgG) in TBS/5% nonfat milk for 1 hour. Finally, the membrane was washed in TBS and then incubated with substrate (Pierce Super Signal[®] West Pico Chemiluminescent Substrate) before exposure to Kodak Biomax Light film. Relative intensities of protein bands were determined by scanning densitometry analysis using Kodak *Molecular Imaging* software (Version 4.0).

HO-1 Western blot analysis. For plates that were used for HO-1 analysis, upon completion of a 6 hour exposure to acrolein the media was removed and the cells were washed three times with DBS. Monolayers were then lysed using RIPA buffer and the protein concentration of the resulting lysate was determined using the BCA assay. Fifty μ g of protein was then loaded onto a 14% polyacrylamide gel and the proteins were resolved as outlined above. The resulting immunoblots were processed as described above except that the primary antibody solution (containing a 1/500 dilution of rabbit anti-HO-1 serum) was prepared in PBS/5% BSA.

Preparation of the cytosolic fraction and cytochrome c detection. Following exposure to acrolein, cell monolayers were washed three times with DBS and then harvested by trypsinization. After a further three washes with DBS, cells were resuspended in sucrose-containing buffer (250 mM sucrose containing 20 mM Tris [pH 8.0], 1.0 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM PMSF) and then disrupted with twenty strokes using a hand homogenizer. The lysates were then centrifuged for 10 minutes at $700 \times g$ to remove unbroken cells and cell debris. Following centrifugation of supernatants for 20 minutes at $15,000 \times g$, the protein content of the resulting postmitochondrial supernatants (the cytoplasmic fraction) were assessed via the BCA assay. Forty μ g of protein were loaded onto a 14% polyacrylamide gel and the gels were processed as outlined above. Following immunoblotting to nitrocellulose, membrane handling was as described above except that primary and secondary antibody solutions (containing 1/500 dilutions of mouse anti-cytochrome c and 1/10,000 dilutions of goat anti-mouse serum respectively) were prepared in PBS/5% BSA.

Results

Media Composition and Acrolein Toxicity in A549 cells. To evaluate the influence of media composition on the susceptibility of A549 cells to the acute toxicity of acrolein, cells were incubated for 3.5 or 18 h with 0, 10, 25, 50, 100 or 200 μ M acrolein prepared in either DBS or F12K/0.5% FBS (Figure 1A). Under the experimental conditions used, these correspond to acrolein doses of 24, 60, 120, 240 and 480 fmol acrolein/cell. The functional integrity of the cells was assessed using the MTT viability assay. After exposure of cells to acrolein for 3.5 h in F12K/0.5% FBS, cell viability was essentially maintained at control values at all acrolein concentrations studied up to and including 200 μ M (Figure 1A). In contrast, all concentrations of acrolein above 50 μ M were toxic at this time point when exposure took place in DBS, with the 200 μ M concentration reducing viability to 67% of controls (Figure 1A). Similar differences between toxic responses in the two types of media were evident after 18 h, with the 200 μ M concentration of acrolein alone inducing cytotoxicity in F12K/0.5% FBS, reducing cell viability to 64% of control. In DBS, all acrolein concentrations induced cell death, with the top concentration (200 μ M) reducing cell viability to 39% of controls (Fig. 1A).

The subsequent experiments assessed the effect of a broader range of cell media compositions on A549 cell viability following 3.5 or 18 h exposure to a single concentration of acrolein (200 μ M). The media conditions included DBS, serum-free F12K, and F12K supplemented with several concentrations of serum (0.5, 5 and 10%). As in the preceding experiment, acrolein toxicity at both 3.5 h (Figure 1B) and 18 h (Figure 1C) was more pronounced in DBS-maintained cells compared to those exposed to acrolein in the presence of either serum-free or serum-containing F12K. Notably, the degree of cell death induced by 200 μ M acrolein in serum-free F12K resembled that produced in cells exposed to acrolein in serum-containing F12K (Figure 1C), suggesting that although serum constituents may participate in trapping extracellular acrolein, F12K components appear to play the major role in attenuating acrolein toxicity.

To clarify the mechanisms involved in the induction of cell death under different exposure conditions, we assessed cytosolic levels of cytochrome c following exposure to acrolein (5 to 100

μM) for 6 h in the presence of either DBS or F12/0.5% FBS (Figure 1D-F). Cytochrome c is released during the mitochondrial permeability transition and is a useful marker of apoptotic cell death (Weinberg, 2007). When exposure occurred in DBS, 5 to 50 μM concentrations of acrolein increased cytosolic cytochrome c levels over control, in contrast to the 100 μM concentration which diminished cytochrome c below basal levels (Figure 1D and F). When exposure occurred in F12/0.5% FBS, cytoplasmic cytochrome c levels increased only in cells exposed to the 100 μM concentration of acrolein (Figure 1E and 1F). These results indicate that mitochondrial integrity was maintained at low acrolein concentrations when exposure occurred in nucleophile-containing media, yet in the presence of DBS the same acrolein concentrations elicited cytochrome c release.

Media Composition and Protein Carbonylation in A549 Cells. Western blotting revealed that media composition strongly influenced levels of carbonylated proteins in A549 cells following 60 min exposure to acrolein (0 to 200 μM) in either DBS (Figure 2A); F12K media supplemented with 0.5% FBS (Figure 2B), or 10% FBS (Figure 2C). Under all three conditions, acrolein induced concentration-dependent damage to cell proteins over a broad mass range, although the degree of damage elicited by the lowest concentrations of acrolein varied between the three media types. When acrolein was delivered in DBS, intense adduction occurred at the two lowest acrolein concentrations (i.e. 25, 50 μM), and damage to most targets appeared saturated at 100 μM acrolein (Figure 2A). In contrast, minimal protein modification was evident at the lowest concentrations of acrolein in cells maintained in either of the F12K-based media (Figure 2B and 2C). Such differences in the extent of protein damage occurring between DBS and F12K-based media are evident upon densitometric analysis of the 20-kDa band, a conspicuous target for acrolein in each of the experimental conditions (Figure 2D, where the net intensity of the 20-kDa band for each treatment is expressed as a fold increase compared to the corresponding control). A threshold for adduction of the 20-kDa protein was clearly evident for cells maintained in the two serum-containing F12K media (Fig. 2B and 2C) compared to DBS (Fig. 2A), again suggesting that

nucleophilic media constituents competed for available acrolein within the extracellular culture media.

To further clarify the effect of media composition on the degree of damage occurring at intracellular targets, protein carbonyls were assessed in A549 cells following a 1 hour exposure to a single acrolein concentration (50 μ M) in the presence of range of media conditions (Figure 3A). Again, densitometric analysis of the 20-kDa band revealed that protein damage was most intense (6-fold increase relative to controls) when acrolein was delivered to cells in DBS (Figure 3B). Irrespective of the serum concentration employed, the 20-kD band intensity was diminished when cells were exposed to acrolein in FBS-containing F12K media (Figure 3A, Lanes 4-6 and Figure 3B). Moreover, in keeping with the MTT viability data obtained under equivalent conditions (Figure 1), adduction of the 20-kDa protein was attenuated in serum-free F12K media (Figure 3A, Lane 3 and Figure 3B), further suggesting that F12K constituents other than serum proteins contribute to suppressing cell injury by acrolein in this experimental model.

Carbonylation of Extracellular Serum Proteins. Since FBS is rich in albumin and other plasma proteins it is likely these species scavenge free acrolein within culture media. To assess such reactions, serum constituents were evaluated for carbonyl adducts following a 1 hour exposure of A549 cells to various concentrations of acrolein in F12 media supplemented with 10% FBS (Figure 4). The resulting immunoblot (Figure 4A) reveals concentration-dependent carbonylation of at least 6 serum proteins, with the results obtained during densitometric analysis of a representative 50-kDa band shown in Figure 4B (open squares). The concentration response curve for carbonylation of the 20-kDa intracellular target under equivalent exposure conditions is also included on Figure 4B (solid squares). Notably, a clear threshold was evident in the carbonylation of the 20-kDa cellular protein relative to the 50-kDa serum constituent at the 100 μ M concentration of acrolein, although at lower acrolein concentrations little carbonylation occurred in either protein compartment. This

finding is further consistent with the conclusion drawn from Figure 3, namely that F12K media constituents other than serum proteins dominate acrolein trapping at low acrolein concentrations.

Nucleophilic Amino Acids as Acrolein Scavengers. Although serum-free F12K contains numerous nutrients and metabolic substrates, nucleophilic amino acids seem the most likely constituents in terms of mediating side reactions with acrolein. To identify the amino acid most responsible for scavenging free acrolein, protein carbonylation was assessed in A549 cells following a 60 min exposure to 50 μ M acrolein in DBS that was supplemented with one of the four amino acids most likely to react with acrolein, namely Cys, Lys, His or Arg (Figure 5). Each of the amino acids was tested at two concentrations, one of which equalled the concentration present in F12K media (the latter is designated with an asterix in the labels on Figure 5). Since the 20-kDa protein band was particularly responsive to changes in acrolein availability, the carbonylation status of this band is depicted in Figure 5. Although His and Lys are known to be capable of forming adducts with acrolein (Esterbauer et al, 1991), under the conditions of these experiments only Cys-containing media suppressed carbonylation of the 20-kDa protein (Figure 5A and 5B). Indeed, Cys completely suppressed carbonylation even when present at a concentration that was 25% of that present in F12K media (i.e. 100 μ M, Figure 5). Arg had little or no effect on carbonylation of the 20-kDa protein even when present at twice the concentration present in F12K (i.e. 5 mM). These findings reveal that Cys is the critical constituent within F12 media that is responsible for attenuating cellular responses to acrolein.

Media Composition and HO-1 Induction. To determine whether media composition influences transcriptional responses to acrolein, the expression of the inducible antioxidant response protein HO-1 was examined in A549 cells exposed to 50 μ M acrolein for 6 h in the presence of either DBS, serum-free F12K, or F12K media containing 0.5, 5 or 10% FBS (Figure 6). Significantly, while the 50 μ M acrolein/DBS combination elicited strong protein carbonylation and a loss of cell viability

(Figures 1A and 2A), it failed to increase HO-1 expression over controls (Figure 6A, Lanes 1 and 2), suggesting that under these conditions overt cytotoxicity precluded Nrf2 activation and HO-1 overexpression. In contrast, the same concentration of acrolein did cause HO-1 induction in serum-free F12K as well as F12K containing 0.5 or 5% FBS (Figure 6A, Lanes 3 to 5). As carbonylation and cytotoxicity were attenuated under these conditions in the earlier experiments (Figures 1A and 2A), this further suggested that antioxidant response activation only occurred under exposure conditions not associated with overt macromolecular adduction and cell death. HO-1 induction was diminished at the maximal FBS concentration of 10% (Figure 6A, Lane 6), suggesting aldehyde scavenging at high serum concentrations inhibited activation of Nrf2-dependent pathways.

To further characterize the effect of serum components on transcriptional responses to acrolein, HO-1 expression was assessed in A549 cells following a 6 hour exposure to a range of acrolein concentrations (5 to 100 μ M) in both DBS and F12/0.5% FBS. In DBS-treated cells, HO-1 induction occurred only at low acrolein concentrations (5 μ M and 10 μ M acrolein produced 1.8- and 2.9-fold increases in HO-1 levels respectively), and at higher acrolein concentrations HO-1 expression decreased to control levels (Figure 7A-C). In contrast, the two lowest concentrations of acrolein (5 and 10 μ M) failed to elevate HO-1 levels when exposure occurred in F12/0.5% FBS, although induction did occur at higher acrolein concentrations, with the 100 μ M concentration producing a 2.8-fold elevation over controls (Figure 7B and 7C). As in the preceding experiments, these findings indicate that induction of Nrf2-regulated pathways only occurred under conditions of acrolein exposure not associated with extensive protein modification and cell death.

Discussion

Interest in the molecular actions of acrolein is increasing in light of growing knowledge that it participates in a wide range of diseases (Janssens et al., 1994; Luo et al., 2005; Montine et al., 2002; Zarkovic, et al., 2006). As with many chemicals, a considerable proportion of our knowledge concerning the toxicology of acrolein is derived from cell-based observations. Yet the extreme reactivity of acrolein raises a number of distinctive issues during such in vitro studies, not least of which is the probability of side-reactions with media constituents. As highlighted in the Introduction, it appears that little standardization exists among laboratories in regard to culture media selection during in vitro studies of acrolein toxicity. Thus the aim of this study was to systematically explore the effect of media composition on the extent of protein adduction, cytotoxicity, HO-1 induction and cytochrome c release during acrolein toxicity in A549 cells. The resulting data confirms that media selection is of high importance since acrolein produced greater protein modification and overt cytotoxicity when cells were exposed to the aldehyde in Dulbecco's buffered saline than in F12K media that contained nucleophilic constituents. In contrast, in the presence of nucleophile-containing F12K media, high concentrations of acrolein were required to elicit changes in cytosolic cytochrome c levels or HO-1 expression. Given that elucidating transcriptional changes elicited by reactive carbonyl compounds is currently an area of active interest in a number of laboratories (West and Marnett, 2005; Doyle et al., 2006), our findings highlight the need to carefully clarify relationships between macromolecular adduction, cytotoxicity and gene dysregulation during such studies.

The finding that strongest HO-1 induction by acrolein occurred under conditions where overt cell death was minimized is consistent with recent findings by Wu et al (2006). These investigators studied the role of protein kinases in acrolein-induced HO-1 induction in bovine aortic endothelial cells, with the experiments conducted in serum-free NMEM culture media. Notably, 100 and 300 μ M concentrations of acrolein that induced cytotoxicity and reactive oxygen production failed to increase cellular levels of HO-1, although the latter was achieved by lower

acrolein concentrations (1 to 50 μ M) that produced neither cell killing nor oxidative stress (Wu et al., 2006). This observation that the cellular events triggered by high dose acrolein that elicit oxidative stress and cytotoxicity are distinct from those altering gene expression at lower doses concurs with our findings and implies a discontinuous spectrum of cellular responses to acrolein that vary according to the exposure level. The finding that a nontoxic structural analogue of acrolein, cinnamaldehyde, was a potent HO-1 inducer provides further evidence of a discontinuity between gene expression changes and overt cytotoxicity in acrolein-exposed cells (Wu et al., 2006).

The pathways mediating acrolein-induced cell death were strongly influenced by media composition (Figure 1E to F). Exposure to 100 μ M acrolein in the absence of nucleophilic media constituents produced extensive protein modification (Figure 2A) and decreased cell viability in the MTT assay (Figure 1) but did not elicit cytochrome c release, a marker of apoptotic cell death. The latter form of cell death can proceed via two pathways; the so-called intrinsic mitochondrial pathway and the extrinsic death receptor-mediated pathway. The role of these two pathways in acrolein toxicity was recently studied in CHO cells, and while acrolein mainly activated the mitochondrial pathway, death-receptor signaling through the cross-talk pathway to form the proapoptotic protein tBID also occurred (Tanel and Averill-Bates, 2005, 2007a). In the present study we confirmed that release of mitochondrial cytochrome c occurred in A549 cells exposed to acrolein, and that this response was strongly media-dependent (Figure 8). When acrolein exposure occurred in nucleophile-free DBS, cytochrome c release was elicited at concentrations up to 50 μ M, but suppression of cytosolic cytochrome c levels by 100 μ M acrolein suggests cell death proceeded via a different mechanism at this concentration, presumably oncosis/necrosis. Our findings are similar to those of Liu-Snyder and co-workers (2006), who found that necrotic cell death followed the collapse of mitochondrial function when PC12 cells were exposed to 100 μ M acrolein in nucleophile-free PBS. The role of cytochrome c release in acrolein-mediated cell death remains unclear given that during a recent study, pretreatment of CHO cells with N-acetylcysteine (NAC) which elevated intracellular glutathione levels and decreased acrolein-induced cytotoxicity, did not

alter acrolein-induced cytochrome c release (Tanel and Averil-Bates, 2007). The authors concluded that inhibition of post-mitochondrial events (e.g. caspase activation) was the key cytoprotective and antiapoptotic mechanism exerted by NAC. However based on our present observations concerning the efficiency of cysteine as an inhibitor of protein carbonylation by acrolein, another possible contributing factor could be that low levels of NAC remained within cells following the pretreatment regimen, and thus the excellent acrolein-scavenging properties of NAC contributed to attenuating acrolein toxicity.

In contrast to patterns of cell death seen in the presence of DBS, in serum-containing F12 media, cytochrome c release was suppressed at acrolein concentrations below 100 μ M (Figure 1E and F), indicating mitochondrial integrity was preserved under these conditions. Since protein modification was also diminished over this acrolein concentration range in serum-containing F12K (Figure 2), it is likely that attenuated acrolein bioavailability due to side-reactions with media nucleophiles precluded activation of cellular machinery involved in triggering cytochrome c release.

Such conclusions may also be relevant to results obtained during a recent study of genes that govern the sensitivity of *Saccharomyces cerevisiae* to acrolein (Trotter *et al.*, 2006). In contrast to the finding by Wu *et al.* that high concentrations of acrolein triggered oxidative stress in aortic endothelial cells, this yeast screening study did not identify any antioxidant or stress-responsive pathways as determinants of cellular sensitivity to acrolein (Trotter *et al.*, 2006). Instead, loss of an NADPH-dependent oxidoreductase (old yellow enzyme 2, *Oye2*) with acrolein-reducing activity was the major factor that increased yeast sensitivity to acrolein, indicating that the cellular capacity to detoxicate acrolein rather than reactive oxygen species was the key factor influencing susceptibility to toxicity. However, it seems possible that the inability to identify a role for antioxidant pathways in yeast defenses against acrolein may have been influenced by the cellular exposure to acrolein under conditions which provided ample opportunities for side-reactions of acrolein with media nucleophiles, since the acrolein-sensitive yeast were selected on agar plates that contained rich YEPD (yeast extract peptone dextrose) media. Although high concentrations (3 to 5

mM) of acrolein were added to the plates, the probability of extensive acrolein-trapping by media nucleophiles suggests the actual free concentrations to which cells were exposed was likely to have been much lower, perhaps accounting for the inability to identify antioxidant pathways as determinants of yeast sensitivity to acrolein.

Although culture media contains many constituents that seem likely to react with strong electrophiles such as acrolein, Cys was the primary participant in acrolein-scavenging reactions within the extracellular phase (Figure 5). Other amino acids with nitrogen-containing nucleophilic side-chains, namely His, Lys and Arg, displayed little tendency to suppress protein carbonylation by acrolein. These observations are consistent with the knowledge that Michael addition of acrolein to the thiol group possessed by Cys is a particularly rapid reaction (Esterbauer et al., 1976; 1991). Serum proteins were a secondary contributor to acrolein-trapping reactions within the extracellular phase, with concentration-dependent accumulation of carbonyl adducts evident in several serum proteins (Figure 4). The chemistry underlying modification of both extracellular (serum) and intracellular proteins is likely to involve Michael additions to a range of amino acids including Cys, Lys and His. To date, most research investigating protein modification by acrolein has focused on Lys adduction, and several adducts that form via stepwise condensation of two or more acrolein molecules at a given Lys side-chain have been identified, some of which can be detected within biological samples using antibodies (Uchida et al., 1998; Furuhashi et al., 2003). However, given that Cys residues are likely to be favored over Lys side-chains during protein modification by acrolein, it is possible that existing antibody-based strategies that are directed against Lys adducts underestimate protein adduction in acrolein-exposed cells and tissues. The protein carbonyl assay used in the present study allowed sensitive detection of acrolein-adducted proteins, but the lack of specificity of this approach ensures that Michael adducts at a range of nucleophilic residues are detected in addition to those involving Cys groups (Burcham and Fontaine, 2001). Given the growing awareness of the significance of protein adduction at Cys residues to a range of toxicological phenomena involving soft electrophiles (Barber and LoPachin, 2004; Carbone et al.,

2005; Dennehy et al., 2006; LoPachin and Barber, 2006), it is clear that a need exists for new methodologies to facilitate detection of acrolein-Cys adducts in proteins from biological samples. One possible strategy could involve the use of thiolating reagents to introduce Cys-like residues into carrier proteins prior to antigen preparation (Duncan et al, 1983; Hartley et al., 1997).

In conclusion, the aim of this work was to explore the impact of different media/serum combinations on the toxicological responses of A549 cells to acrolein. The finding that media composition strongly influenced cellular responses to a given concentration of acrolein indicates that media selection requires careful consideration during the development of in vitro test systems that investigate the properties of reactive chemicals such as acrolein.

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FIGURE LEGENDS

Figure 1. Acrolein is more toxic towards A549 cells when exposure occurs in Dulbecco's Buffered saline (DBS) than in F12K media containing 0.5% FBS. Panel A. Cellular viability in A549 cells following either 3.5 or 18 h exposure to various concentrations of acrolein in DBS (*triangles*) or F12/0.5% FBS (*squares*). Data obtained at 3.5 h is indicated with *solid* symbols, while 18 h data is shown with *open* symbols. Panels B & C. Viability of A549 cells exposed to 200 μ M acrolein for 3.5 h (Panel B) or 18 h (Panel C) in either DBS or F12K supplemented with various concentrations of FBS (0, 0.5, 5 or 10%). In Panels A to C, controls were incubated in acrolein-free F12K/0.5% FBS for the appropriate time indicated. Each data point represents the mean $\pm$ SE of 4 independent observations. Panels D to F. Acrolein-mediated cytochrome c release is strongly influenced by the presence or absence of nucleophile-containing media. Panels D and E depict representative immunoblots (40 μ g protein/lane) obtained following 6 h incubations of A549 cells with 0 to 100 μ M concentrations of acrolein in the presence of either DBS (Panel D) or F12K/0.5% FBS (Panel E). In Panel F, the intensity of the cytochrome c band from Panel D (closed bars) and Panel E (open bars) is shown.

Figure 2. Carbonylation of a range of cell proteins (15 μ g/lane) is more intense when a 60 min exposure to various concentrations of acrolein (0 to 200 μ M) occurred in DBS (Panel A) compared to F12K containing 0.5% (Panel B) or 10% FBS (Panel C). In Panel D, the intensity of the 20-kDa protein in cells exposed to acrolein (0 to 200 μ M) in F12K/0.5% FBS (■), F12K/10.0% FBS (▲) or DBS (◆).

Figure 3. Carbonylation of cell proteins (15 μ g/lane) during a 60 min exposure to a single concentration of acrolein (50 μ M) was suppressed in the presence of F12K media alone or

containing various concentrations of FBS. In Panel A, the lane contents are: *Lane 1*, Control (F12/0.5% FBS, no acrolein exposure); *Lane 2*, DBS + acrolein; *Lane 3*, F12K + acrolein; *Lane 4*, F12K/0.5% FBS + acrolein; *Lane 5*, F12K/5% FBS + acrolein; *Lane 6*, F12K/10% FBS + acrolein. Panel B depicts the intensity of the highlighted 20-kDa band as determined via scanning densitometry.

Figure 4. Representative immunoblot showing concentration-dependent carbonylation of serum proteins (15 µg/lane) in the extracellular media phase following a 60 min incubation of A549 cells with a range of acrolein concentrations (0 to 200 µM) in F12K/10% FBS (Panel A). In Panel B, the relative band intensity of the highlighted 50 kD serum protein (■) is compared to that of the 20-kDa cellular target highlighted in Figure 2C (□).

Figure 5. Cysteine but not other nucleophilic amino acids (histidine, lysine or arginine) strongly inhibits carbonylation of the 20-kDa protein target by acrolein (50 µM) during incubation of A549 cells in DBS for 60 min. In Panel A, the concentration of each amino acid present in F12K media is highlighted with an asterisk (*). In Panel B, the relative intensity of the 20-kDa target for acrolein is shown for all four amino acids when tested at the concentration present in F12K media.

Figure 6. HO-1 induction accompanies exposure of A549 cells to 50 µM acrolein for 6 h in F12K-based media but not in DBS. In the representative immunoblot shown in Panel A, the various lane contents are: *Lane 1*, control, F12/0.5% FBS only, no acrolein exposure); *Lane 2*, DBS + acrolein; *Lane 3*, F12K + acrolein; *Lane 4*, F12K/0.5% FBS + acrolein; *Lane 5*, F12K/5% FBS + acrolein; *Lane 6*, F12/10% FBS + acrolein. In Panel B, the intensity of the HO-1 band is shown as determined via scanning densitometry.

Figure 7. Patterns of acrolein-mediated HO-1 induction are strongly influenced by the presence or absence of nucleophile-containing culture media. Panels A and B depict representative immunoblots (50 μ g protein/lane) obtained following 6 h incubations of A549 cells with 0 to 100 μ M concentrations of acrolein in the presence of either DBS (Panel A) or F12K/0.5% FBS (Panel B). In Panel C, the intensity of the HO-1 band from Panel A (closed bars) and Panel B (open bars) is shown.