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Acrolein Oxidizes the Cytosolic and Mitochondrial Thioredoxins in Human Endothelial Cells

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Abstract

Acrolein is a reactive aldehyde that is a widespread environmental pollutant and can be generated endogenously from lipid peroxidation. The thioredoxin (Trx) system in endothelial cells plays a major role in the maintenance of cellular thiol redox balance, and is critical for cell survival. Normally, cells maintain the cytosolic (Trx1) and mitochondrial (Trx2) thioredoxins largely in the reduced state. In human microvascular endothelial cells, Trx1 was more sensitive than Trx2 to oxidation by acrolein. A 30-minute exposure to 2.5 μM acrolein caused partial oxidation of Trx1 but not Trx2. The active site dithiol of Trx1 was essentially completely oxidized by 5 μM acrolein whereas 12.5 μM was required for complete oxidation of Trx2. Partial recovery of the Trx1 redox status was observed over a 4 hour acrolein-free recovery period, with increases in the reduced form and decreases in the fully oxidized form. For cells treated with 2.5 or 5 μM acrolein the recovery did not require protein synthesis, whereas protein synthesis was required for the return of reduced Trx1 in cells treated with 12.5 μM acrolein. Pretreatment of cells with *N*-acetylcysteine (NAC) resulted in partial protection of Trx1 from oxidation by acrolein. In cells treated with acrolein for 30 min, followed by a 14- to 16-hr acrolein-free period, small but significant cytotoxic effects were observed with 2.5 μM acrolein whereas all cells were adversely affected by $\geq 12.5 \mu\text{M}$. NAC pretreatment significantly decreased the percentage of stressed cells subsequently exposed to 5 or 12.5 μM acrolein. Given the critical role of the thioredoxins in cell survival, the ability of acrolein to oxidize both thioredoxins should be taken into account for a thorough understanding of its cytotoxic effects.

Keywords

Acrolein; Thioredoxin-1; Thioredoxin-2; Redox blot; Endothelial cells; *N*-acetylcysteine

1. Introduction

Acrolein is a reactive α,β -unsaturated aldehyde; it is a strong electrophile and potentially reactive with sulfhydryl groups (Uchida et al., 1998). Acrolein is a toxic product of the *in vivo* activation of the anti-neoplastic drug cyclophosphamide. Other sources of acrolein exposure include cigarette smoke, exhaust from internal combustion engines, wood combustion, overheated cooking oils, and the manufacture of acrylate polymers. Inhaled volatile aldehydes such as acrolein can be carried through the blood as protein-carbonyl adducts (Uchida et al., 1998) and released at distant sites in the circulation where they can act directly on vascular

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endothelial cells (Barnoya and Glantz, 2005). Lipid peroxidation has negative consequences for endothelial cell health, and significant amounts of acrolein can be generated during lipid peroxidation (Uchida et al., 1998).

The redox balance of endothelial cells is critical for their normal function and viability. Glutathione and the thioredoxins are major players in the maintenance of cellular thiol redox balance, although these systems are not in redox equilibrium with each other (Nkabyo et al., 2002; Hansen et al., 2006a). Thioredoxins are largely responsible for maintaining intracellular proteins in their reduced state (Arner and Holmgren, 2000). The redox status of the thioredoxin (Trx) system may be more critical to cell survival than is glutathione. Metals that oxidize Trx tend to cause cell death, whereas metals that oxidize GSH, but not Trx, do not tend to cause cell death (Hansen et al., 2006b).

All mammalian cells have cytosolic (Trx1) and mitochondrial (Trx2) forms of thioredoxin (Powis and Montfort, 2001). These 12 kDa proteins are encoded by distinct genes, but they share a conserved active site (Trp-Cys-Gly-Pro-Cys-Lys) that is cycled between the reduced (dithiol) and oxidized (disulfide) forms (Watson et al., 2003). Trx1 has two active site cysteines (C32/C35) and three other cysteines (Watson et al., 2003) of which two are in a dithiol motif (C62/C69). The C32/C35 active site is more readily oxidized than is the C62/C69 dithiol (Watson et al., 2003). Trx2 has only the two active site cysteines. Under normal conditions, cells maintain Trx1 and Trx2 largely in the reduced state (Nordberg and Arner, 2001; Powis and Montfort, 2001; Watson et al., 2003) which is critical for their function.

The thioredoxins have multiple roles, including: (a) the reduction of cysteines in a variety of proteins and peptides, e.g. ribonucleotide reductase, protein disulfide isomerase, peroxiredoxins and others; (b) attenuation of reactive oxygen species (ROS) effects by reducing ROS-generated disulfides, and by contributing to the degradation of some ROS, e.g. through the regeneration of peroxiredoxins; and (c) enhancing the binding of certain transcription factors to DNA (Arner and Holmgren, 2000; Nordberg and Arner, 2001; Powis and Montfort, 2001). Knockout mice lacking either Trx1 or Trx2 do not survive (Powis and Montfort, 2001; Nonn et al., 2003). Inhibition or genetic suppression of Trx results in increased ROS and apoptosis (Hansen et al., 2006a) and increased sensitivity of cells to oxidants (Chen et al., 2006). Conversely, cells that overexpress Trx2 are more resistant to oxidant-induced apoptosis (Chen et al., 2006; Hansen et al., 2006a). Trx1 stimulates cell growth and inhibits apoptosis (Arner and Holmgren, 2000; Powis and Montfort, 2001). Therefore, factors which enhance the oxidation of Trx could decrease cell survival.

This paper describes the effects of acrolein on the redox status of the thioredoxins and elucidates the ability of low micromolar concentrations of acrolein to differentially oxidize Trx1 and Trx2 in human endothelial cells. It describes the partial recovery of the redox status of Trx1 following removal of the acrolein, and the differential requirement for protein synthesis for redox recovery depending on the acrolein concentration. The ability of NAC to protect thioredoxins had not been previously explored, but we noted that NAC pretreatment protected Trx1, but not Trx2, from acrolein oxidation. The concentrations of acrolein that oxidize the thioredoxins correspond to those that cause cytotoxicity, and NAC pretreatment significantly diminished the cytotoxic effects of acrolein.

2. Materials and methods

2.1 Reagents

The following were purchased from Invitrogen Corp. (Carlsbad, CA): MCDB 131 medium (Cat. no. 10372-019), TrypLE Express, Hank's Balanced Salt Solution (HBSS), epidermal growth factor, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonate (AMS), and pre-cast gels

(12% Bis-Tris, 16% Tris-Glycine) and matching electrophoresis and loading buffers. Phenylmethylsulfonyl fluoride (PMSF) and Tris were from Research Organics (Cleveland, OH). EDTA, guanidine-HCl, and trichloroacetic acid (TCA) were obtained from Fisher Scientific (Hampton, NH). The following antibodies were obtained from Abcam (Cambridge, MA): rabbit anti-human Trx 1, rabbit anti-human Trx 2, and rabbit anti-human GAPDH. The Supersignal West Pico Chemiluminescent substrate was obtained from Pierce (Rockford, IL). All other chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO) or from sources indicated below. Acrolein is volatile, potentially toxic, and has a low flash point and should be stored and handled with proper ventilation to prevent inhalational exposure.

2.2 Cell culture

Human microvascular endothelial cells (HMEC-1) were provided by the Centers for Disease Control and Prevention (CDC, Atlanta, Georgia). Cells were grown at 37°C in humidified air containing 5% CO₂ in MCDB131 medium with 2 mM glutamine, 10% fetal bovine serum (FBS Optima, Atlanta Biologicals), hydrocortisone (1 µg/ml), epidermal growth factor (10 ng/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells were passaged every 3–4 days using TrypLE Express according to manufacturer's instructions. Cells were typically plated at a density of 6,000 cells/cm².

2.3 Trx1 redox blots

The redox status of Trx1 was determined as adapted from Halvey et al. (Halvey et al., 2005). Cells were grown to 85–95% of confluence (typically in T-75 or T-25 flasks), washed once in pre-warmed HBSS, and treated with acrolein in HBSS at 37°C for the indicated times. Following acrolein treatment, the cells were washed once in HBSS and scraped into chilled 0.25 ml of 6 M guanidine HCl, 100 mM Tris-HCl pH 8.3, 3 mM EDTA, 0.5 % Triton X-100, 50 mM iodoacetic acid (Halvey et al., 2005). After brief sonication on ice to shear the DNA, guanidine and unreacted iodoacetate were removed using Microspin G25 columns (GE Healthcare, Piscataway NJ). The eluates were mixed with an equal volume of loading buffer, separated by native PAGE (16% Tris-Glycine), transferred to nitrocellulose, blocked with 5% bovine serum albumin (BSA) and probed with anti-Trx1 and HRP-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA).

In some cases where indicated, cells were pretreated for 2 hr with 2 mM *N*-acetylcysteine (NAC) in cell culture medium with FBS and supplements. The cells were then washed twice with HBSS and treated with acrolein.

2.4 Trx2 redox blots

The redox status of Trx2 was determined according to Chen et al. (Chen et al., 2006). Cells (typically in T-75 or T-150 flasks) were washed and treated with acrolein as for Trx1, but were then scraped into 0.5 ml of 10% TCA. Following incubation on ice for ≥ 30 min, they were briefly sonicated on ice, and centrifuged for 10 min at 4°C (12000 × *g*). The pellets were resuspended in ice-cold 100% acetone, iced for 30 min, and centrifuged for 15 min. The pellets were air-dried and then resuspended in 80 mM Tris-HCl pH 7, 2% SDS, 1 mM PMSF. AMS was added to a final concentration of 25 mM, and the samples were incubated on ice for 15 min and then for 10 min at 37°C (Chen et al., 2006). Following centrifugation for 5 min, LDS loading buffer was added and the samples were separated by SDS-PAGE (12% Bis-Tris) under non-reducing conditions, and transferred to nitrocellulose. The blots were blocked with 5% milk (Carnation nonfat) and probed with anti-Trx2 and HRP-conjugated goat anti-rabbit IgG.

2.5 Isolation of mitochondria for Trx2 controls

Cells were harvested by scraping and centrifugation, and the pellet was washed in cold HBSS, cold STE (0.25 M sucrose, 10 mM Tris pH 7.2, 2 mM EDTA), and resuspended in a small volume of cold STE. Cells were lysed using a Teflon-on-glass Potter-Elvehjem homogenizer. The suspension was periodically examined and homogenization was considered complete when >90% of the cells were lysed. The lysate was centrifuged for 10 min at 4°C at $735 \times g$ to pellet nuclei, large debris and unbroken cells. The supernatant fraction was transferred to a new tube and centrifuged for 10 min at 4°C ($12000 \times g$) to pellet mitochondria. The mitochondria were washed and resuspended in STE, and stored at -20°C until use. Western blots demonstrated a high specific content of the mitochondrial markers VDAC-1 and cytochrome *c* in the isolated mitochondria; these markers were absent in the post- $12000 \times g$ supernatant (not shown). Trx2 becomes oxidized in vitro, so the mitochondria were used directly as a reference for oxidized Trx2. To prepare a reference for reduced Trx2, the mitochondria were incubated with 20 mM dithiothreitol (DTT) for 10 min at room temperature. Both reduced and oxidized mitochondria were then mixed with TCA and processed through the AMS labeling procedure described above.

2.6 Miscellaneous

Differences between multiple groups were assessed using one-way ANOVA and the Tukey-Kramer post test (Instat software, Graphpad). Significance was assumed at $p < 0.05$.

3. Results

3.1 Acrolein oxidizes Trx1 and Trx2

The effects of acrolein on the redox status of Trx1 in cells were analyzed by redox western blot. Iodoacetate covalently labels the $-\text{SH}$ groups of reduced Trx1; the resulting negative charge increases migration in native PAGE relative to oxidized Trx1. Reduced Trx1 refers to the form in which both dithiols are reduced and represents the active form. The active site dithiol is the more easily oxidized, so partially oxidized Trx1 largely represents oxidation of the active site C32/C35, whereas both dithiols (C32/C35, C62/C69) are oxidized in the fully oxidized form (Watson et al., 2003). In untreated cells, 83% of Trx1 was fully reduced, with 17% partially oxidized (Fig. 1). Acrolein oxidized Trx1 in a dose-dependent manner. At 1 μM acrolein, 45% of Trx1 was partially oxidized, with the remainder reduced. At 2.5 μM acrolein, 62% of Trx1 was partially oxidized and 9% fully oxidized. At 5 μM acrolein and higher none of the Trx1 was in the reduced state, with all of it being in the partially and fully oxidized states. At 12.5 μM acrolein and above, 83 to 100% of the Trx1 was fully oxidized (Fig. 1). At 100 and 250 μM acrolein, the total amount of Trx1 appeared diminished and mostly ran as a smear migrating slower than fully oxidized Trx1. The reason for this smearing is unknown but one possibility is that Trx1 is somehow aggregated or bound to other cell components.

The redox status of the mitochondrial form (Trx2) was also examined by redox western blot. In contrast to Trx1, Trx2 only has one dithiol. Reduced Trx2 migrates slower because AMS bonds to the two $-\text{SH}$ groups of the active site thereby increasing the mass of Trx2 (Hansen et al., 2006a). In untreated cells and those treated with 1 or 2.5 μM acrolein, all of the Trx2 was reduced ($-\text{SH}_2$), i.e. it migrated coincident with Trx2 in DTT-treated mitochondria (Fig. 2A). In cells treated with 12.5 μM or more acrolein, all of the Trx 2 was oxidized ($-\text{S}-\text{S}-$), i.e. it migrated coincident with Trx2 in air-oxidized mitochondria (Fig. 2A,B). In cells treated with 5 μM acrolein, Trx2 migrated faster than reduced Trx2, but lagged slightly behind the fully oxidized Trx2 band in the cells treated with 12.5 μM acrolein. This lag was seen in five replicate experiments, and is better visualized in Fig. 2C which compares the 5 and 12.5 μM samples side-by-side relative to the DTT-treated mitochondria. The 5 μM samples therefore suggest

some Trx2 oxidation. Densitometric scans of each lane were consistent with a single band, i.e. they did not suggest two species within a fused band. It is possible that one of the two –SH groups of Trx2 in the 5 μ M samples remains reduced, i.e. it binds one AMS rather than the two AMS bound by fully reduced Trx2; this might account for the migration between the fully reduced and fully oxidized forms. An intermediate (partially oxidized) Trx band in which AMS apparently reacted with only one cysteine of yeast Trx has been reported (Kuge et al., 2001). Overall, the results are consistent with some Trx2 oxidation in cells exposed to 5 μ M acrolein and complete Trx2 oxidation in cells treated with ≥ 12.5 μ M acrolein.

3.2 Recovery of Trx redox status after acrolein removal

The experiments above illustrate the redox status of the thioredoxins immediately following a 30-minute acrolein exposure. Additional experiments were conducted to determine if the Trx redox status could recover from acrolein-mediated oxidation. After exposure to different concentrations of acrolein for 30 min, the acrolein was removed and cells were incubated in acrolein-free complete medium for 1, 2, or 4 hr (Fig. 3). For cells treated with 2.5, 5, and 12.5 μ M acrolein, the percentage of total Trx1 in the reduced state increased significantly following a 4 hr recovery (Fig. 3A); some recovery was noted after just 2 hr for cells exposed to 2.5 or 5 μ M acrolein. For all recovery times, a significant decrease in the fully oxidized Trx1 was observed for cells treated with 5, 12.5 or 25 μ M acrolein (Fig. 3C). Significant increases in partially oxidized Trx1 were also noted during recovery of cells treated with 12.5 or 25 μ M acrolein (Fig. 3B), and these largely corresponded to the declines in the fully oxidized form (Fig. 3C). With 5 μ M acrolein, significant changes in the percent of partially oxidized Trx1 were not observed during the recovery (Fig. 3B) because declines in the fully oxidized form were offset by increases in the reduced form. Overall, there is a partial recovery of the redox status of Trx1 once acrolein is removed, with partial restoration of reduced (active) Trx1 and a significant decline in the fully oxidized form.

In initial experiments, changes in the redox state of Trx2 were not observed during one or two hour acrolein-free recovery periods (not shown).

The partial restoration of reduced Trx1 during the acrolein-free recovery could be due to reduction of the oxidized thiols of existing Trx1 and/or to the synthesis of new Trx1. To distinguish between these possibilities, the redox state of Trx1 following a 4-hr recovery was determined in the presence or absence of the protein synthesis inhibitor geneticin. The reduced form of Trx1 showed significant recovery following exposure to 2.5 and 5 μ M acrolein (Fig. 3A), and this recovery was not affected by geneticin (Fig. 4A) suggesting that protein synthesis was not required. However, for the 12.5 μ M acrolein treatment, the recovery of the reduced form was completely blocked by geneticin (Fig. 4A), with a large percent of the Trx1 in the partially oxidized state (Fig. 4B). These data suggest that synthesis of new Trx1 was required for the reappearance of reduced Trx1 following treatment with 12.5 μ M acrolein. Geneticin did not affect the percent of Trx1 that remained fully oxidized after the 4 hr recovery (Fig. 4C). Overall, the data show that protein synthesis is not required for the Trx1 redox recovery observed following exposure to lower acrolein concentrations (2.5 and 5 μ M) whereas protein synthesis was required for the recovery of reduced Trx1 following treatment with 12.5 μ M acrolein.

3.3 Protection provided by N-acetylcysteine

Since acrolein can react with thiols, the potential for the thiol NAC to protect Trx1 from oxidation by acrolein was examined. In order to prevent acrolein from being scavenged extracellularly by excess NAC, the cells were pretreated for 2 hr with NAC (2 mM) and then washed twice in HBSS prior to acrolein exposure. Since significant redox recovery occurs without NAC during acrolein-free recovery periods, the effects of NAC were assessed

immediately after acrolein exposure (without an acrolein-free recovery period, as in Figs. 1 and 2). The NAC-pretreated cells showed less acrolein-mediated oxidation of Trx1 (Fig. 5). NAC-pretreated cells had significantly more of their Trx1 in the reduced state following exposure to 2.5 or 5 μM acrolein (Fig. 5B). However, whether or not cells were pretreated with NAC, there was no reduced Trx1 in cells immediately following exposure to higher concentrations of acrolein (12.5 or 25 μM) (Fig. 5B). NAC pretreatment did, however, provide for significant decreases in the percent of Trx1 that was fully oxidized in cells that were treated with the higher acrolein concentrations (5, 12.5, and 25 μM) (Fig. 5D), i.e. some of the fully oxidized form was shifted to the partially oxidized state (Fig. 5A,C). Thus, when considering both the reduced and fully oxidized forms, NAC pretreatment afforded some protection against Trx1 oxidation at all concentrations tested. The shifts in the percentages of the reduced and fully oxidized forms resulted in some changes in partially oxidized Trx1 (Fig. 5C). Because the cells were washed twice between NAC pretreatment and acrolein exposure, the effects of NAC are associated with the cells themselves and do not represent scavenging of acrolein by extracellular NAC.

In limited experiments with Trx2, NAC pretreatment did not cause a noticeable alteration in the oxidation of Trx2 for the acrolein concentrations tested (0–12.5 μM) (not shown).

3.4 Effects on cell morphology

The effects of acrolein treatment on cell morphology were also examined as an indicator of cell stress. Stressed cells were defined as those that had become rounded and/or contracted and had therefore lost normal cell morphology. Immediately after the 30 min acrolein treatment, nearly all cells looked normal (not shown). After a subsequent 14- to 16-hr acrolein-free period, however, those treated with higher concentrations of acrolein had significantly larger percentages of stressed cells (Fig. 6, 7). In cells not pretreated with NAC, a small but significant increase in cell stress was observed with 2.5 μM acrolein, whereas nearly all cells were adversely affected by 12.5 and 25 μM (Fig. 7). NAC pretreatment significantly decreased the percentage of stressed cells following subsequent exposure to 5 or 12.5 μM acrolein, but did not afford protection against 25 μM acrolein (Fig. 7).

4. Discussion

Trx1 and Trx2 are not in redox equilibrium with each other, so they have been used to differentially assess the impacts of other oxidants on the thiol redox status of the cytosolic and mitochondrial compartments (Halvey et al., 2005; Hansen et al., 2006a). For the acrolein results reported here, Trx1 is more sensitive to oxidation by acrolein than is Trx2. The partially oxidized form of Trx1 represents oxidation of the active site thiols (C32/C35) and therefore an inactive form of Trx1. At 2.5 μM acrolein, 60% of the Trx1 is already oxidized, whereas Trx2 maintains a reduced state (Figs. 1, 2). With 5 μM acrolein, essentially all Trx1 is oxidized (i.e. none of the reduced form remains), whereas 12.5 μM is the point at which Trx2 is clearly fully oxidized. This is in contrast to *t*-butyl hydroperoxide or diamide which preferentially oxidize Trx2 relative to Trx1 (Chen et al., 2006; Hansen et al., 2006a). However, Trx2 is not significantly oxidized by epidermal growth factor-induced reactive oxygen species (ROS) (Halvey et al., 2005; Hansen et al., 2006a). The effects of acrolein on the redox status of the two thioredoxins are therefore different from those of other oxidants.

The oxidation of Trx1 and Trx2 by acrolein likely results from a direct chemical oxidation of their sulfhydryls (Uchida et al., 1998). Since acrolein can stimulate ROS generation (Nardini et al., 2002; Jaimes et al., 2004), ROS conceivably could be responsible for the oxidation of thioredoxins. However, Trx oxidation in our studies was not likely mediated by common ROS such as H_2O_2 or superoxide. Treatment of the HMEC-1 cells with high levels of H_2O_2 (1 or 5 mM) for 15 min failed to significantly increase the oxidation of either Trx (not shown). 25 mM

H₂O₂ did not cause an oxidation of Trx1, but a decline in the total amount of Trx1 was apparent (not shown). Relative to acrolein, H₂O₂ may have less impact on the redox state of the thioredoxins in HMEC-1. Alternatively, the thioredoxins may have been protected from H₂O₂ by cellular peroxidases (e.g. glutathione peroxidase), or the reduced state of the thioredoxins was already restored when the cells were harvested. The lack of impact of H₂O₂ in these studies agrees with a report in which 0.6 to 12 mM H₂O₂ for 5 min did not cause significant oxidation of Trx1 in bovine aortic endothelial cells (Fernando et al., 1992). Also, treatment of HMEC-1 cells for 10 min with 0.5 or 1 mM sodium chromate, which is known to generate ROS, did not cause detectable oxidation of Trx1 (not shown).

Acrolein-mediated Trx oxidation in HMEC-1 cells was not likely due to superoxide (O₂^{•-}) because the levels of acrolein that caused Trx oxidation were well below those that stimulate O₂^{•-} generation. 12.5 μM acrolein caused extensive oxidation of both thioredoxins (Figs. 1, 2), well below the 30 μM acrolein that is required to cause a statistically significant increase in O₂^{•-} in bovine pulmonary artery endothelial cells (Jaimes et al., 2004). In bovine aortic endothelial cells, 100 μM acrolein is needed to induce significant increases in O₂^{•-} and H₂O₂ (Wu et al., 2006).

In A549 cells (human lung adenocarcinoma, hypotriploid), ≥ 25 μM acrolein resulted in a >90% inhibition of in vitro Trx activity in cell lysates, as measured by NADPH oxidation with the addition of thioredoxin reductase and insulin (Yang et al., 2004). Approximately 50% inhibition was observed with 10 μM acrolein (Yang et al., 2004). The A549 studies did not determine which of the thioredoxins were affected, their redox status, or cytotoxicity. With respect to the effects on Trx, the HMEC-1 are more sensitive than the A549 cells with 5 μM and 12.5 μM completely eliminating the reduced forms of Trx1 and Trx2, respectively. With HMEC-1, cytotoxicity commenced at 2.5 μM acrolein for 30 min and was essentially maximal at 12.5 μM and higher (Fig. 6, 7). In contrast, A549 cells are much less sensitive, with 200 μM for 48 hr reported as toxic, whereas 100 μM was not (Hoshino et al., 2001). Other studies noted that A549 cells are relatively resistant to oxidant stresses including cadmium and H₂O₂ (Watjen and Beyersmann, 2004). A549 cells are very distinct from normal cells in a number of ways: they have known chromosomal abnormalities and are highly tumorigenic (Giard et al., 1973), and they consist of at least four different cell types which can be distinguished both morphologically and biochemically (Croce et al., 1999).

Our results suggest that the two dithiols of Trx1 have differential susceptibility to the oxidant effects of acrolein. In studies with the thiol oxidant diamide, the active site dithiol (C32/C35) was more sensitive to oxidation, i.e. the partially oxidized form of Trx1 largely consisted of protein with an oxidized active site (C32/C35) and a reduced C62/C69 dithiol (Watson et al., 2003). It is therefore likely that acrolein similarly oxidizes the active site dithiol first, so that the partially oxidized form has C32/C35 in the disulfide state. Partially oxidized Trx1 was observed at lower acrolein concentrations whereas the fully oxidized form of Trx1 predominated at higher (≥12.5 μM) concentrations (Fig. 1). In the fully oxidized form, both C32/C35 and C62/C69 are disulfides (Watson et al., 2003). While the C32/C35 disulfide is a substrate for reduction by thioredoxin reductase, the C62/C69 site is not (Watson et al., 2003). Restoration of fully reduced Trx1 from the partially oxidized form (C32/C35 disulfide) is therefore likely mediated by thioredoxin reductase. At lower concentrations of acrolein (2.5 or 5 μM) restoration of the reduced form was observed during the acrolein-free recovery period (Fig. 3) and this restoration did not require protein synthesis (Fig. 4) which would be consistent with reduction of existing Trx1. In cells exposed to 12.5 μM acrolein, the vast majority of Trx1 was fully oxidized (83%) (Fig. 1); while there was some recovery of the reduced form after a 4-hr acrolein free period (Fig. 3), this recovery was completely dependent on protein synthesis (Fig. 4). Redox recovery of fully oxidized Trx1 is likely more difficult. Not only does oxidation of C62/C69 delay the reduction of the C32/C35 active site by thioredoxin reductase, the C62/

C69 site itself is not reducible by thioredoxin reductase but is instead reduced by active Trx1 (Watson et al., 2003). It is interesting that the limited recovery of reduced Trx1 following 12.5 μ M acrolein required new protein synthesis, but that this redox recovery was not sufficient to prevent irreversible cytotoxicity. In contrast, Trx1 recovery following lesser concentrations of acrolein did not require protein synthesis, and these concentrations were much less toxic to cells. It is possible that the duration over which Trx is oxidized contributes to the ultimate effects on cells. Recovery of Trx1 redox status by thioredoxin reductase would be expected to be quicker than replacement by protein synthesis.

Acrolein is toxic to a number of cell types, although direct comparisons are confounded by differences in the method and duration of acrolein exposure and the cytotoxic endpoints. There may also be differences in the susceptibility of different cell types to the effects of acrolein. The HMEC-1 cells used here were sensitive to lower concentrations of acrolein than some other cell types. In HMEC-1 cells exposed to 5 μ M acrolein (10.7 fmol per cell) for 30 min followed by a 16 hour acrolein-free period, $29 \pm 3\%$ of the cells were stressed, whereas nearly all HMEC-1 were rounded 16 hours following a 30 min treatment with 12.5 or 25 μ M acrolein (Fig. 6, 7). These concentrations are well below the 150 μ M acrolein needed for initiation of toxicity in human proximal tubule cells (Schwerdt et al., 2006). Furthermore, the tubule cells were serum-deprived for 24 hr before acrolein treatment (Schwerdt et al., 2006) whereas the HMEC-1 cells in our studies were not. In CHO cells, one hour or longer exposure to 30–50 fmol acrolein per cell is required to cause a significant increase in toxicity (Tanel and Averill-Bates, 2005; Tanel and Averill-Bates, 2007). A 48-hr exposure to 200 μ M acrolein was damaging to human A549 cells, whereas 100 μ M was not (Hoshino et al., 2001). A 4-hr exposure to 50 μ M acrolein was not toxic to human alveolar macrophages, whereas 25 μ M for 24 hr was toxic (Li et al., 1997). These were far longer treatments than the 30 min used here for the HMEC-1 cells. The acrolein sensitivity of HMEC-1 cells is more similar to that reported for murine lymphocytes for which 10 μ M (10 fmol per cell) acrolein for 30 min caused toxicity (Kern and Kehrer, 2002). Human bronchial epithelial cells (HBE1) exposed to 10 μ M acrolein for 30 min followed by a 24 hr recovery period showed 74% of the cells normal, whereas only 33% were normal after 25 μ M acrolein (Nardini et al., 2002). Unlike the HMEC-1 cells, the HBE1 cells were deprived of growth factors for 12 hr before acrolein exposure (Nardini et al., 2002). Longer treatment (24 hr) with 10 μ M acrolein is needed to cause cytotoxicity in rat PC12 cells (Luo et al., 2005).

To our knowledge, the potential for NAC to protect the redox status of Trx had not yet been explored. The studies reported here demonstrate that pretreatment of cells for 2 hr with 2 mM NAC affords some protection from acrolein. NAC pre-treated cells had significant increases in reduced Trx1 and significant decreases in fully oxidized Trx1 (Fig. 5). NAC therefore clearly affords some protection of Trx1 from acrolein. NAC also provided significant protection against the cytotoxic effects of 5 and 12.5 μ M acrolein (Fig. 7). A recent report noted the ability of NAC (1 mM for 24 hr) to increase clonogenic survival in CHO cells exposed to acrolein and to diminish acrolein-induced markers of apoptosis in these cells (Tanel and Averill-Bates, 2007). Either co-treatment, or 6-hr pre-treatment, of cells with 0.3 mM NAC protected the SN56 mouse cholinergic neuronal cell line from 100 μ M acrolein (Wood et al., 2007). However, the impact of NAC on thioredoxin was not explored in the CHO or SN56 cells. NAC can diminish the cytotoxicity of cigarette smoke extract (CSE), a known source of acrolein as well as several other cytotoxic agents. As examples: (1) NAC protected endothelial cells from cell detachment induced by CSE (Nagy et al., 1997); (2) NAC reduced mitochondrial depolarization and morphological changes in CSE-treated human lung fibroblasts, (Baglolle et al., 2006); (3) NAC attenuated CSE-induced stress protein induction and apoptosis in transfected human bone marrow endothelial cells (Vayssier-Taussat et al., 2001); (4) NAC protected against DNA damage induced by CSE and acrolein in human lymphoid cells (Yang et al., 1999); and (5) co-incubation of A549 cells with NAC decreased the cytotoxicity of CSE

(Hoshino et al., 2001). Co-incubation with NAC has also been noted to protect cells from other insults including the cytotoxic effects of organic extracts of diesel exhaust particles on rat heart microvessel endothelial cells (Hirano et al., 2003). In some of these reports, NAC was in the medium during the chemical insult and may therefore have chemically quenched the oxidants extracellularly. In other cases, it was not clear if the NAC was completely removed from the medium prior to oxidant exposure. In the studies reported here as well as those in the CHO cells (Tanel and Averill-Bates, 2007), the cells were washed free of NAC prior to acrolein treatment so extracellular oxidant quenching was not a factor. One study reported that 0.02 mM NAC largely diminished CSE-induced mitochondrial depolarization in human monocytes (Banzet et al., 1999), but it was not clear if the NAC was washed away before the CSE treatment. We did not observe any protection from acrolein-mediated Trx1 oxidation in HMEC-1 cells pre-incubated with 0.02, 0.05, or 0.1 mM NAC, nor did 0.1 mM NAC protect the cells from the cytotoxic effects of acrolein (not shown).

Since both thioredoxins are critical to cell survival, the oxidation of either (or both) of them by acrolein could negatively impact cell survival. Mitochondrial thiols are recognized as important targets of oxidant-induced cytotoxicity (Hansen et al., 2006a). When the redox status was examined immediately following exposure to 12.5 μ M acrolein (without an acrolein-free recovery period), Trx2 was fully oxidized (Fig. 2); this corresponds to the treatment that resulted in subsequent stress to nearly all cells. In contrast, the reduced form of Trx1 was eliminated by 5 μ M acrolein which caused subsequent stress to 29% of the cells. However, the duration over which each thioredoxin is oxidized could also be an important determinant of the ultimate implications for survival. Some redox recovery of Trx1 was noted over a 4-hr acrolein-free period, and recovery occurred earlier and was more robust following acrolein treatments that subsequently resulted in lesser cytotoxicity. The ability of NAC to partially protect Trx1 from oxidation might contribute to enhanced cell survival. The impact of acrolein on each of the thioredoxins and the implications for cell health and survival warrant future study.

The studies reported here examined endothelial cells in which the thioredoxin system has a prominent antioxidant role and is a major regulator of redox balance (Miller et al., 2001; Haendeler et al., 2004). Endothelial cells contain more thioredoxin reductase than other human cells, and they have more thioredoxin reductase than glutathione peroxidases (Anema et al., 1999). It is therefore possible that the thioredoxin system may be more critical for cell survival in human endothelium than in other cell types. Under normal conditions, cells maintain Trx1 and Trx2 largely in the reduced state (Nordberg and Arner, 2001; Powis and Montfort, 2001; Watson et al., 2003) which is critical for their function. Therefore, the ability of acrolein to oxidize thioredoxins could contribute to the cytotoxic effects of acrolein, and could have important implications for cell survival and the ability of the cells to tolerate other oxidant insults.

Acrolein can be carried through the blood as protein-carbonyl adducts (Uchida et al., 1998) and released at distant sites in the circulation (Barnoya and Glantz, 2005). Immunoreactive acrolein adducts in human urine are in the range of 0.37 to 1.24 mM, and those in serum are 10- to 20-fold lower (Satoh et al., 1999) (i.e. approx. 18 to 124 μ M). These levels are greater than those needed to mediate thioredoxin oxidation in the HMEC-1 endothelial cells reported here. Significant amounts of acrolein can be generated during lipid peroxidation (Uchida et al., 1998) which can occur with various vascular insults and pathologies. The thioredoxin system in endothelial cells could also therefore be impacted by this endogenously generated acrolein.

In summary, this report describes the effects of acrolein on the redox status of the thioredoxins, demonstrating that low micromolar concentrations of acrolein differentially oxidize Trx1 and Trx2 in human endothelial cells. Following removal of acrolein, partial recovery of the redox

status of Trx1 was observed for cells treated with lower concentrations of acrolein. Redox recovery from $\leq 5 \mu\text{M}$ acrolein did not require protein synthesis and only a limited percentage of the cells were adversely affected by these concentrations. In contrast, redox recovery from $12.5 \mu\text{M}$ acrolein did require protein synthesis, and nearly all cells were significantly stressed by this concentration of acrolein. It was noted that pretreatment protected Trx1, but not Trx2, from acrolein oxidation, and NAC pretreatment significantly diminished acrolein cytotoxicity. The concentrations of acrolein that oxidized the thioredoxins are similar to those that caused cytotoxicity. These effects of acrolein on the thioredoxin system should therefore be taken into account when considering the potential implications for cell health and survival.

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Fig. 1.

A: Redox western blot of Trx1 in HMEC-1 cells treated with the indicated concentrations of acrolein for 30 min, washed once in HBSS and immediately processed for Trx1 redox status. The 5 μ M acrolein is equivalent to 10.7 fmol acrolein per cell. Iodoacetate covalently labels the -SH groups of reduced Trx1, and the resulting negative charge increases migration in native PAGE relative to oxidized Trx1. In partially oxidized Trx1 the active site C32/C35 is oxidized, whereas both dithiols (C32/C35, C62/C69) are oxidized in the fully oxidized form (Watson et al., 2003). A western blot of SDS-PAGE for GAPDH (loading control) for the same samples is shown. B: The relative distribution of the three redox states of Trx1 as determined by densitometry of redox blots. Points and error bars represent the mean $\pm$ S.D. (n = 3 independent experiments). For some, the error bars are smaller than the points as shown. Densitometry was not done on the 100 and 250 μ M acrolein lanes because the bulk of material runs as a slower migrating smear rather than as distinct bands (see panel A).

Fig. 2.

Redox western blots of Trx2. A,B: HMEC-1 cells were either untreated or treated with the indicated concentrations of acrolein for 30 min, washed once in HBSS and immediately processed for Trx2 redox status. Standards for the migration of oxidized and reduced Trx2 are included, and consist of isolated mitochondria that had either been stored frozen (“mito, oxid.”) (Trx2 oxidizes in storage), or had been pre-treated with DTT which reduces Trx2 (“mito, DTT”). The AMS bonds to the –SH groups of the active site of reduced Trx2, increasing its mass. Reduced (AMS-adducted) Trx2 therefore migrates slower (Hansen et al., 2006a). Oxidized Trx2 migrated at 12 kDa consistent with its known mass. The blots were stripped and reprobed for GAPDH (36 kDa; loading control for cell samples). Relative to the cell lysates, only small amounts of the purified mitochondria were loaded so that the Trx2 band intensity better matched that of cell lysates; these lower mitochondrial protein loads are reflected in the weak GAPDH bands. The blots shown are representative of at least five independent

experiments. C: Enlarged view of a Trx2 redox blot showing the 5 and 12.5 μ M acrolein-treated cells.

Fig. 3.

Redox status of Trx1 during acrolein-free recovery. HMEC-1 cells were treated with the indicated concentrations of acrolein for 30 min, washed in HBSS and incubated for the indicated times in MCDB131 medium with 10% FBS and supplements. At the end of the recovery time, the cells were washed once in HBSS and immediately processed for Trx1 redox status. The data (mean $\pm$ S.D. for 3 independent experiments) show the percentage of the total Trx1 that is in each of the three possible redox states: reduced (A), partially oxidized (B), and fully oxidized (C). * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$) relative to the corresponding “No Recovery” samples for each acrolein concentration.

Fig. 4.

The effect of geneticin on the redox status of Trx1 during a 4 hr acrolein-free recovery period. HMEC-1 cells were treated with the indicated concentrations of acrolein for 30 min, washed in HBSS and incubated for 4 hr in MCDB131 medium with 10% FBS and supplements, either with or without the protein synthesis inhibitor geneticin (800 μg per ml). At the end of the 4 hr period, the cells were washed once in HBSS and immediately processed for Trx1 redox status. The data (mean $\pm$ S.D. for 3 independent experiments) show the percentage of total Trx1 that is in each of the three possible redox states: reduced (A), partially oxidized (B), and fully oxidized (C). * ($p < 0.05$) or *** ($p < 0.001$) relative to the corresponding 4 hr recovery samples without geneticin.

Fig. 5.

The impact of NAC pretreatment on the redox status of Trx1. **A:** Redox western blot of Trx1 in HMEC-1 cells treated with the indicated concentrations of acrolein for 30 min. Prior to acrolein exposure, cells were either untreated (left) or pre-treated with 2 mM NAC for 2 hr in cell culture medium with FBS and supplements. The cells were then washed twice with HBSS and then treated with acrolein. Note that NAC-pretreated cells were not tested at 1 μ M acrolein. **B, C, D:** The percentage of total Trx1 that is in the reduced (**B**), partially oxidized (**C**), or fully oxidized (**D**) form in cells with or without NAC pretreatment, as determined by densitometry of the redox blots (mean $\pm$ S.D. for 3 independent experiments). ** ($p < 0.01$) or *** ($p < 0.001$) relative to the corresponding “No NAC” samples.

Fig. 6. Effect of acrolein on HMEC-1 cell morphology. Cells with or without NAC pretreatment (2 mM for 2 hr) were treated with the indicated concentrations of acrolein for 30 min, washed, and incubated in fresh medium for 16 hr. The phase contrast images shown are representative of triplicate experiments.

Fig. 7.

Quantification of the cytotoxic effects of acrolein on HMEC-1 cells and the partial protection afforded by NAC pretreatment. Cells with or without NAC pretreatment (2 mM for 2 hr) were exposed to the indicated concentrations of acrolein for 30 min, washed, and incubated in fresh medium without acrolein for 16 hr. From recorded phase contrast images (examples in Fig. 6), the percentage of cells that displayed visual signs of stress (e.g. rounded, condensed) were determined (mean $\pm$ S.D., $n = 3$). ** ($p < 0.01$) or *** ($p < 0.001$) relative to the corresponding “No NAC” samples. # ($p < 0.05$) and ## ($p < 0.001$) relative to the 0 μM acrolein for “no NAC”.