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Thioredoxin-1 Redox Signaling Regulates Cell Survival in Response to Hyperoxia

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Abstract

The most common form of newborn chronic lung disease, bronchopulmonary dysplasia (BPD), is thought to be caused by oxidative disruption of lung morphogenesis which results in decreased pulmonary vasculature and alveolar simplification. Although cellular redox status is known to regulate cellular proliferation and differentiation, redox-sensitive pathways associated with these processes in developing pulmonary epithelium are unknown. Redox sensitive pathways are commonly regulated by cysteine thiol modifications. Therefore two thiol oxidoreductase systems, thioredoxin and glutathione, were chosen in order to elucidate the roles of these pathways on cell death. Studies herein indicate thiol oxidation contributes to cell death through impaired activity of glutathione dependent (GSH-dependent) and thioredoxin (Trx) systems and altered signaling through redox sensitive pathways. Free thiol content decreased by 71% with hyperoxic (95% oxygen) exposure. Increased cell death was observed during oxygen exposure when either the thioredoxin (Trx) systems or glutathione dependent were pharmacologically inhibited with aurothioglucose (ATG) or buthionine sulfoximine respectively. However, inhibition of the Trx system yielded the smallest decrease in free thiol content (1.44% with ATG treatment vs 21.33% with BSO treatment). Although, Trx1 protein levels were unchanged, Trx1 function was impaired during hyperoxic treatment as indicated by progressive cysteine oxidation. Overexpression of Trx1 in H1299 cells utilizing an inducible construct increased cell survival during hyperoxia whereas siRNA knockdown of Trx1 during oxygen treatment reduced cell viability. Overall, this indicated a comparatively small pool of proteins rely on Trx redox functions to mediate cell survival in hyperoxia, and the protective functions of Trx1 are progressively lost by its oxidative inhibition. To further elucidate the role of Trx1, potential Trx1 redox protein-protein interactions

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mediating cytoprotection and cell survival pathways were determined by utilizing a substrate trap (mass action trapping) proteomics approach. With this method, known Trx1 targets were detected including peroxiredoxin-1 as well as novel targets, including two HSP90 isoforms (HSP90AA1 and HSP90AB1). Reactive cysteines within the structure of HSP90 are known to modulate its ATPase dependent chaperone activity through disulfide formation and S-nitrosylation. While HSP90 expression is unchanged at the protein level during hyperoxic exposure, siRNA knockdown significantly increased hyperoxic cell death by 2.5-fold, indicating cellular dependence on HSP90 chaperone functions in response of hyperoxic exposure. These data support the hypothesis that hyperoxic impairment of Trx1 negatively impacts HSP90-oxidative responses critical to cell survival with potential implications on pathways implicated in lung development and the pathogenesis of BPD.

Keywords

Thioredoxin-1; hyperoxia; redox signaling; cell death

Introduction

Premature birth is associated with ~12% of births annually¹. Survival of the most premature has increased significantly since the introduction of exogenous surfactants², steroids^{3,4}, and milder ventilation techniques⁵⁻⁸. Despite advances in treatment, premature infants exposed to supplemental oxygen often develop bronchopulmonary dysplasia (BPD), a form of chronic lung disease characterized by alveolar simplification and decreased microvasculature⁹⁻¹¹. Premature infants who develop BPD demonstrate increased markers of oxidative stress when exposed to oxidative insults^{12,13} resulting from hyperoxia (>21% oxygen) and ventilation¹². Therefore it is largely accepted that BPD phenotypes are mediated by oxidative stress and arrested lung development through alterations in cell death, cellular programming, and proliferation¹⁴⁻¹⁷.

Cellular reduction-oxidation (redox) status is emerging as an important regulator of various cellular responses^{18,19}. Oxidative stress, an imbalance in pro-oxidants (intrinsic or extrinsic) and anti-oxidants, has been associated with disruption of redox circuitry, generation of free radicals, and macromolecular damage. While macromolecular damage requires the accumulation of free radicals which are easily scavenged by anti-oxidant systems in low quantities, more sensitive changes in cellular redox status are manifested through non-radical molecular modifications that alter redox-sensitive signaling pathways²⁰. Reversible oxidation of cysteine moieties has been shown to act as a redox switch to regulate protein conformation, enzyme activity, transporter activity, protein interactions, DNA binding, trafficking, and degradation^{21,22,23}. Thiol oxidation occurs as a result of reactive species generated by electron leak from mitochondrial metabolism (intrinsic) or environmental sources (extrinsic). However excessive oxidative stress (hyperoxia) may disrupt signaling through thiol oxidation of key redox sensitive proteins. Therefore maintenance of thiol redox homeostasis is critical, and it is accomplished by glutathione-dependent (GSH-dependent) and thioredoxin (Trx) oxidoreductase systems.

While GSH-dependent and Trx systems function independently, each provides similar functions. Disulfide bonds are reduced by Trx²³, either the cytosolic Trx1 or the mitochondrial Trx2, or by glutaredoxin (Grx)²⁴⁻²⁷ whereby Grx may yield fully reduced thiol groups or provide for S-glutathionylation. Both provide antioxidant functions through removal of hydrogen peroxide (H₂O₂) by peroxiredoxins (Prx)²⁸ downstream of Trx or GSH-peroxidases (GSH-Prx)^{24,29,30}. Utilization of GSH by Grx and GSH-Prxs yields oxidized GSH (GSSG) which is regenerated by GSH-reductase (GR) in a nicotinamide adenine dinucleotide phosphate (NADPH) dependent reaction^{24,31}. In a similar manner, reduction of disulfide targets and Prxs results in oxidation of Trx active site cysteines which are reduced by a corresponding Trx reductase (TrxR1 and TrxR2) and NADPH³². With respect to Trx, previous work has demonstrated a specific role for Trx1 in response to various insults, such as ischemia-reperfusion³³, UV light^{34,35}, hydrogen peroxide³⁶⁻³⁸, and hyperoxia^{39,40} (for comprehensive review⁴¹) which are relevant to many disease processes.

With respect to BPD several studies have linked impairment of the Trx1 and GSH systems with hyperoxic injury and altered lung development^{42,43}. Additional studies have focused on alveolar type II epithelial cells (AEC's) as neonatal mouse models of BPD⁴⁴ have indicated AEC's to be critical targets of oxidative induced injury as demonstrated by hindered proliferation, reduced levels of VEGF secretion, and apoptosis⁴⁵. Impairment of the Trx and GSH systems by hyperoxia appears to mediate these changes. For instance, glutathione deficiency impairs AEC growth⁴⁶, recombinant Trx1 therapy improves the survival of AEC's isolated from fetal rat lung when exposed to hyperoxia⁴⁷, and overexpression of Trx1 protects adult mice against oxygen-induced toxicity⁴⁸. While excess Trx1 offers protection, endogenous Trx1 and TrxR1 expression is altered in hyperoxic exposed neonates, and increased levels of oxidized Trx1 are detected. Together this indicates that Trx1 is overwhelmed and unable to function during oxygen induced injury⁴⁹. This may potentially impair critical redox signaling networks. Overall, these data suggest that Trx1 regulates viability during oxygen treatment and that Type II cell function is dependent on cellular redox status.

The data herein demonstrates further evidence of the contributions of hyperoxic impairment of the Trx and GSH systems to cell death, a compromised oxidative stress response, and links impairment of the Trx1 system with impairment of stress response pathways potentially mediating altered lung development during BPD pathogenesis. Inhibition of either the GSH system by BSO or the Trx system by ATG treatment contributed to increased cell death in pulmonary epithelial-like cells during hyperoxic exposure. While both systems maintain thiol redox homeostasis, the Trx system maintains the free thiol content of a select subset of thiols when compared to GSH. Focusing on the cytosolic Trx1 system during hyperoxia, redox western blotting indicated progressive oxidation of the active and non-active site cysteines which was associated with impairment of Trx1 dependent systems. Use of Trx1 overexpression constructs and siRNA knockdown indicate Trx1 redox mediated function to be critical to hyperoxic cytoprotection. This effect was ameliorated by the overexpression of a redox-null mutant Trx1 (Trx1^{C32/35S}). To better understand redox mediated interactions with Trx1, a substrate-trap proteomic approach was utilized to identify key redox targets via protein-protein interactions. Heat shock protein 90 (HSP90) was identified as a Trx1 substrate with similar cytoprotective functions during hyperoxic injury,

suggesting that the Trx1-HSP90 interaction is critical for oxygen-sensitive responses. These findings support a hypothesis that while hyperoxia impairs both the Trx and GSH systems, impairment of the Trx1 system leads to oxidative impairment of redox sensitive pathways mediating both stress response and processes critical to lung development.

Methods

Cell Culture and Treatments

Human lung adenocarcinoma H1299 cells and A549 cells (ATCC, Manassas, VA) were cultured in 5% CO₂ at 37°C in Dulbecco's modified Eagle's medium (DMEM, high glucose) with 10% fetal bovine serum, 50U/ml penicillin, 50µg/ml streptomycin (Gibco) and 20µg/ml gentamycin (Cellgro). For hyperoxic exposures, gas (95% O₂ with 5% CO₂) was flooded into a 6×10×12" plexiglass inhalation chamber at a rate of 5 L/min for 10 minutes after which the flow rate was decreased and maintained at 0.25 L/min. For treatments with aurothioglucose (ATG, Sigma-Aldrich) or buthionine sulfoximine (BSO, Sigma-Aldrich), cells were incubated in media supplemented with 100µM ATG or 100µM BSO in room air for 24 hours prior to exposure to hyperoxia.

Cell Viability

Cells were incubated with 5 ml DMEM until 50% confluency in 60mm dishes. Media was replaced, and cells were placed in the inhalation chamber for oxygen exposure. Following exposure, media, HBSS washes, and trypsinized cells were collected and centrifuged at 1250rpm for 5 minutes at room temperature. Fluid was aspirated and the pellet was resuspended in a 1:1 solution of trypan blue (Sigma-Aldrich) and HBSS. Viability was measured using the TC-10 automated cell counter (BioRad).

Western Blot and Silver Staining Analysis

Cells were lysed in 50mM Tris (pH 7.4), 150 mM NaCl, 0.2% Triton X-100, 0.3% NP-40 and 0.1mM phenylmethylsulfonyl fluoride (PMSF) supplemented with protease inhibitor cocktail (1:1000, Sigma-Aldrich). Protein concentrations were determined by the BCA method (Thermo Scientific). Cell lysates were diluted with 3× Laemmli Buffer (150mM Tris (pH 6.8), 3% β-mercaptoethanol, 6% sodium dodecyl sulfate, 0.3% bromophenol blue and 30% glycerol) and boiled for 5 minutes. Proteins were separated by SDS-PAGE, and the resulting gels were either silver stained according to the manufacturer's instructions (Pierce) or transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). The membranes were incubated with anti-Trx1 (1:500, Santa Cruz Biotechnology), anti-TrxR1 (1:500, Millipore), anti-flag (1:250, Sigma Aldrich), anti-GFP (1:5000, Clontech), β-actin (1:1000, Sigma-Aldrich), and anti-HSP90 (1:1000, Cell Signaling), epithelial growth factor receptor (EGFR, 1:1000, Cell Signaling), Akt (1:1000, Cell Signaling) antibodies overnight at 4°C in TBS-T (1× TBS with 0.01% Tween-20) with 5% nonfat milk. Membranes were then incubated at room temperature with anti-rabbit (1:5000, Southern Biotechnology) or anti-mouse (1:5000, Southern Biotechnology) HRP-conjugated secondary antibody in TBS-T with 5% nonfat milk and visualized by chemiluminescence (Amersham Biosciences).

RNA Isolation, Integrity Assessment and Reverse Transcription

RNA was isolated from intact H1299 and A549 cells following treatment using the SV Total RNA Isolation System (Promega) according to manufacturer recommendations. Genomic DNA was removed by treatment with Turbo DNA-free (Ambion/Life Technologies), and RNA with a 260nm/280nm absorbance ratio above 1.9 were acceptable (Nanodrop2000, Thermo Scientific). RNA integrity was then evaluated with a 2100 Bioanalyzer (Agilent Technologies) and RNA integrity number (RIN) values above 8.0 were considered appropriate for reverse transcription. cDNA was generated using the iScript cDNA Synthesis Kit (BioRad) according to manufacturer protocol with an RNA input of 0.195µg for each sample.

Quantitative Real-Time PCR (qPCR), Assay Design and Validation

Primers and probes for qPCR were designed for human Trx1, TrxR1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using Beacon Designer 7.91 (Premier Biosoft) and synthesized by Eurofin MWG Operon. The primer and probe sequences are as follows: Trx1 (TXN1; nm003329.3), forward 5'-ACGTGATATTCCTTGAAGTAG-3', reverse 5'-GGCATGCATTTGACTTCA-3', probe [6~FAM]ACTCTGAAGCAACATCCTGACAGT[BHQ1a~6FAM]; TrxR1 (TXNRD1; nm003330.2; forward 5'-GCTTCAGCATGTCATGTG-3', reverse 5'-CTCTGTTTCACAAACACAAC-3', probe [6~FAM]CCAATTCCGAGAGCGTTCCTTC[BHQ1a~6FAM]; GAPDH (nm002046.4), forward 5'-CATCCATGACAACCTTTGGTA-3', reverse 5'-CCATCCACAGTCTTCTGG-3', probe [6~FAM]ACCACAGTCCATGCCATCACT[BHQ1a~6FAM]. Primers were used at a final concentration of 900nM while probes were used at a final concentration of 250nM. GAPDH was selected as an appropriate internal control gene after a mass normalized experimental group of samples was evaluated in triplicate using the GAPDH assay and a difference of <0.5 cycle threshold (C_t) values were observed. The sensitivity of each assay to genomic DNA contamination was validated using an experimental group of samples treated in the absence of reverse transcriptase for each assay in triplicate. Assays were considered to be insensitive to genomic DNA when C_t values were undetermined >35 cycles. Assay efficiency was validated by combining a small fraction of each cDNA and diluting the pool across five orders of magnitude. Pooled sample dilutions were run for each assay in triplicate and it was determined that there was no more than a 10% difference between the Trx1 or TrxR1 assays and GAPDH assay efficiencies. qPCR was performed using 2× Absolute Blue master mix (Thermo Scientific) following the manufacturer protocol and used the primer and probe sets as described above. Samples were evaluated in 96-well PCR plates (Agilent Technologies) using a 7500 Real Time PCR System (Applied Biosystems). The absence of DNA contamination in the reagents was confirmed with the use of no template controls for each assay on each plate. Each of four replicate experimental sample sets was evaluated in triplicate for each assay. Fluorescence data was analyzed using 7500 System Sequence Detection Software Version 1.4 (Applied Biosystems). A cycle threshold of 0.44 was applied to all plates. To calculate fold change during hyperoxia, the average C_t ratio between Trx1 or TrxR1 and GAPDH for each time point in hyperoxia was normalized to the average C_t ratio of the group exposed to room air.

Trx1 Redox Assay

Trx1 redox status was determined based on procedures previously published⁵⁰⁻⁵². Following treatment, cells were lysed in G-lysis buffer (6M guanidine, pH 8.3, 50mM Tris-HCl, 3mM EDTA, 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Sigma-Aldrich) supplemented with 50mM iodoacetic acid (IAA). Lysates incubated at 37°C for 30 minutes, and excess IAA was removed using illustra MicroSpin G-25 columns (GE Healthcare). Protein concentration was determined using the BCA method. Samples were diluted in 5× Sample Buffer (300mM Tris-HCl, 50% glycerol, 0.05% bromophenol blue, pH 6.8) and separated by 15% non-denaturing PAGE. Proteins were transferred to PVDF membranes. Membranes were probed for Trx1 and visualized as previously mentioned.

siRNA Transfections

Cells were seeded in 12-well dishes and transfected with luciferase (GL2, ON-TARGET Plus SmartPool, Dharmacon), Trx1 (TXN, ON-TARGET Plus SmartPool; Dharmacon), or heat shock protein 90 (HSP90, ON-TARGET Plus SmartPool; Dharmacon) small interfering RNA (siRNA) oligos using Lipofectamine 2000 according to the manufacturer's instructions. After 6 hours, cells were washed and cultured in normal media and treated as described above.

Doxycycline-Inducible Cell Lines

Myc-Trx1, Myc-Trx1^{C32/35S}, Flag-Trx1^{C35S}, Flag-Trx1^{C32/35S}, and EGFP doxycycline-inducible (DOX-inducible) constructs were generated by PCR of the pcDNA3-Trx1, pcDNA3-Trx1^{C35S}, and pcDNA3-Trx1^{C32/35S} plasmids acquired from Addgene⁵³. PCR products were digested and ligated into the pBIG2i vector⁵⁴ containing the IRES2-EGFP sequence from pIRES2-EGFP (Clontech). Plasmids were purified (5 PRIME), linearized with SspI and transfected into H1299 cells using Lipofectamine 2000 (Invitrogen). Cells were grown in 200µg/mL hygromycin (Invitrogen), and stable clones were initially selected based on EGFP fluorescence following treatment with 2µg/ml doxycycline and fluorescence visualized with an Olympus IX71 inverted epifluorescent microscope (Olympus). H1299 cells with conditional transgene expression were incubated with up to 2µg/ml DOX (Sigma-Aldrich) for 24 hours prior to exposure. Transgene expression was confirmed by western blot for fusion tags (Myc or Flag) or EGFP following DOX treatment.

Free Thiol Assay

Cellular free thiols were analyzed through detection of disulfide bonds using the Measure-iT thiol assay kit (Invitrogen) according to the manufacturer's protocol. Based on Ellman's reagent chemistry, 10µg of protein lysate was incubated with the fluorescent thiol quantitation reagent for 5 minutes and fluorescence was measured using a SpectraMax M5 microplate reader (Molecular Devices; excitation/emission of 494/517nm). Free thiol concentration was determined using reduced glutathione as a thiol quantitation standard.

Immunoprecipitation

H1299 cells were treated with DOX for 36 hours followed by lysis in non-denaturing lysis buffer (0.05M Tris-HCl pH 7.4, 0.15M NaCl, 0.01M ethylenediaminetetraacetic acid) with protease (1:1000, Sigma-Aldrich) and phosphatase (1:1000, #2 and #3, Sigma-Aldrich) inhibitor cocktails and 0.1mM PMSF. Protein lysate (400ug) was rotated overnight at 4°C with EZview Red Anti-Flag M2 beads (Sigma-Aldrich). Beads were washed five times with TBS, protein was eluted with non-denaturing lysis buffer lacking Triton X-100 and supplemented with 5 mM dithiothreitol (DTT). Beads were then washed five times with TBS, and remaining protein eluted by boiling 5 minutes in 1×Laemmli buffer. Immunopurifications were confirmed by western blot comparison between input and DTT and boiled eluates.

1D LC-MSMS analysis

Proteins were reduced and alkylated by final concentration of 50mM DTT and 50mM iodoacetamide prior to digestion by trypsin at a final concentration of 25ng/μl in 50mM ammonium bicarbonate. Trypsin (Promega) was added (1:20 ratio) for overnight digestion at 37°C. After digestion, formic acid was added to the peptide solution (to 2%), followed by desalting via Microtrap (Thermo Fisher Scientific) and then on-line analysis of peptides by high-resolution, high-accuracy LC-MS/MS, consisting of a Michrom HPLC, a 15-cm Michrom Magic C18 column, a low-flow ADVANCED Michrom MS source, and a LTQ-Orbitrap XL (Thermo Fisher Scientific). A 120-min gradient of 10–30%B (0.1% formic acid, 100% acetonitrile) was used to separate the peptides. The LTQ-Orbitrap XL was set to scan precursors in the Orbitrap followed by data-dependent MS/MS of the top 10 precursors. Data were submitted to Sorcerer Enterprise v.3.5 release (Sage-N Research Inc.) with SEQUEST algorithm as the search program for peptide identification. Differential spectral count analysis and proteolytic profile mapping was done by QTools, an open source in-house developed tool for automated differential peptide/protein spectral count analysis and Gene Ontology⁵⁵.

Statistical Analyses

Values are means ± standard deviation. Group means were compared by ANOVA using Bonferroni's post hoc test or an unpaired *t*-test where applicable with GraphPad Prism 5 (GraphPad Software). *P* 0.05 was considered significant.

Results

Pharmacological inhibition of the thioredoxin and glutathione systems sensitizes cells to hyperoxic death

H1299 and A549, human immortalized lung adenocarcinoma cells, were treated with buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase, the rate-limiting step in glutathione (GSH) synthesis, or aurothioglucose (ATG), an inhibitor of cellular Trx systems through TrxR. Under normoxic conditions, viability of cells treated with BSO or ATG was similar to that of controls (Fig 1A,B) despite 74.1% and 68.4% reductions in GSH+GSSG and TrxR activity respectively (data not shown). However drug

treatment and subsequent exposure to hyperoxic culture conditions was associated with a dramatic increase in cell death with both BSO and ATG. Changes in cell morphology or confluence resulting from BSO or ATG treatment under normoxic culture conditions were not observed, but considerably fewer healthy cells were observed when cultured in high oxygen conditions (data not shown). As a similar increase in cell death was observed in response to the inhibition of the Trx and GSH systems, cellular free thiols were quantified over a 3 day time course in hyperoxia after inhibiting each system independently. Inhibition of GSH and Trx systems by BSO significantly reduced free thiol content during hyperoxic treatment of H1299 cells (Fig 1C). Interestingly, a significant decrease in free thiol content was observed with BSO treatment when compared to controls and regardless of normoxic or hyperoxic culture (Fig 1D). However no measurable loss of free thiol content was observed when cells³⁴ were treated with ATG. Similar findings were found in A549 cells (data not shown). These data support Trx interaction with select protein partners to maintain cell survival in hyperoxia.

Response of the Trx1 system during hyperoxic exposure

Trx system inhibition augmented hyperoxic cell death and Trx1 has previously been demonstrated to be responsive to various oxidative insults³³⁻⁴⁰, therefore Trx1 and TrxR1 expression was determined in response to hyperoxia. Trx1 protein expression (Fig 2A,B) and mRNA levels (Fig 2C,D) were unaffected in both cell lines throughout the three day hyperoxic time course. TrxR1 mRNA was significantly changed in the H1299 line, but this increase was not evident in A549 cells. As Trx1 redox activity requires a reduced active site, redox status of Trx1 was determined through a redox western assay technique⁵⁰⁻⁵² (Fig 3A,B). Prior to oxygen exposure, Trx1 is present in a fully reduced state or with partial oxidation of the active site cysteines. When exposed to hyperoxic conditions both H1299 and A549 demonstrated Trx1 migrational shifts consistent with increased Trx1 oxidation. In addition much larger Trx1 immunoreactive bands were detected (marked by asterisks) which may represent Trx1 complexes⁵⁶.

Trx1 Redox Activity is Required for Protection Against Hyperoxic Death

To further clarify the relevance of Trx1 specific functions with respect to cell death, H1299 cells were transfected with siRNA oligos against Trx1 and a non-mammalian control target luciferase (GL2). Trx1 protein was not detected in cell lysates after siRNA treatment while Trx2 expression was not altered, demonstrating specificity of knock down (Fig 4A). Following hyperoxic exposure, a significant increase in cell death (Fig 4B) with Trx1 siRNA but not the GL2 siRNA was observed. These data supported the previous findings obtained through pharmacological inhibition of Trx1 using ATG (Fig 1A,B). To demonstrate Trx1 redox specific functions mediate Trx1 protection, stably transfected cells utilizing a doxycycline (DOX) construct were generated for wild-type Trx1 (Myc-Trx1) or the redox-dead mutant (Myc-Trx1^{C32/35S}) (Fig 4C). H1299 cells were cultured in the absence or presence of DOX and exposed to RA or O₂ for 4 days. Overexpression of either transgene had no effect on cell viability in room air. When exposed to hyperoxic conditions, a 30% decrease in hyperoxic cell death was observed with Myc-Trx1 which is consistent with the data presented thus far (Fig 4D). When Myc-Trx1^{C32/35S} was expressed

cytoprotective functions were lost which supports Trx1 modulation of redox homeostasis provides for increased hyperoxic cell survival.

Identification of HSP90 as a Trx1 Substrate

Compared to GSH, Trx1 appears to regulate redox homeostasis of a comparatively small subset of protein thiols (Fig 1D). In addition critical interactions mediating cytoprotection are dependent on functionality of the active site cysteines (Fig 4D). To identify Trx1 target interactions, a substrate trap approach (Fig 5A) was utilized which takes advantage of the two step reduction mechanism of Trx1 whereby mutation of the second active site cysteine to a serine (Trx1^{C35S}) stabilizes target protein interactions through a disulfide bond^{57,58}. Trx1 transgene complexes were immunopurified using anti-Flag antibodies, target proteins were subsequently eluted by reduction with dithiothreitol (DTT), and the remaining protein eluted by boiling in Laemmli buffer. Over 100 proteins were identified as Trx1 redox targets within the DTT eluate of Flag-Trx1^{C35S} (Table 1, refer to Supplemental Table 1 for full list) including two known Trx1 interacting proteins peroxiredoxin-1 (Prx1)²⁸ and Trx reductase-1 (TrxR1), which validated this approach. Proteins identified were primarily localized to the cytoplasm (75.5%) or the nucleus (40.0%), and were associated with a range of molecular functions including antioxidant (9.4%), cell signaling (7.5%), and stress response/chaperone functions (19.8%) as determined by gene ontologies provided by the UniProt database. Interestingly, two isoforms of heat shock protein 90 (HSP90AA1 and HSP90AB1) were identified by mass spectrometry. Immunoblotting for HSP90 using an antibody recognizing both HSP90AA1 and HSP90AB1 was performed against input cell lysates and DTT eluates verified redox mediated interaction with Trx1 (Fig 6A) as HSP90 is readily expressed and it specifically pulled down by Trx1^{C35S} and not Trx1^{C32/35S}.

HSP90 is Regulated by and Conveys Cytoprotection During Hyperoxia

Expression of HSP90 and client proteins Akt and epithelial growth factor receptor (EGFR) were not altered during hyperoxia (Fig 6B). To better understand HSP90 associated cytoprotection, siRNA knockdown was utilized to eliminate HSP90 expression before introducing the H1299 cells to hyperoxic conditions (Fig 6C and D). While a small yet significant increase in cell death was observed in room air, knockdown of HSP90 increased hyperoxic cell death 2.5-fold. These data indicates that HSP90 chaperone functions are critical to signaling pathways involved in cell survival under hyperoxic stress.

Discussion

We have demonstrated herein that the Trx1 system may play a specific role in cytoprotective functions through regulation of key target substrates. During hyperoxic exposure, cells are subject to increased oxidative stress as a result of increased reactive oxygen species^{59,60}. Accumulation of reactive species leads to oxidative damage to proteins, including the depletion of free thiols (Fig 1C) and the formation of disulfide bonds which result in alterations to protein function^{21,61-64}. GSH and Trx systems represent endogenous antioxidant systems aimed at maintaining thiol redox homeostasis within the reduced environment of intracellular compartments. While inhibition of either the GSH-dependent system by BSO or the Trx systems by ATG increased hyperoxic cell death (Fig 1A,B), a

greater proportion of free thiols are lost over time with BSO treatment than with ATG treatment in either room air or hyperoxic conditions (Fig 1D).

As the cytosolic Trx1 system has been previously demonstrated to provide protective functions in response to a variety of oxidative insults, protein expression and redox status of Trx1 was determined. While hyperoxic exposure did not alter Trx1 or TrxR1 expression in either cell line (Fig 2A,B), a significant increase in oxidized Trx1 was observed (Fig 3A,B). Together these data indicate hyperoxic impairment of the Trx1 system which demonstrates protein expression and Trx1 oxidation consistent with a neonatal mouse model of BPD utilizing hyperoxic exposure⁴³. As Grx reduction of Trx1 is known to compensate for TrxR1 inhibition or knockdown under basal conditions^{65,66}, the outcomes observed in Figure 1 may be the result of altered TrxR function and not inhibition of Trx redox activity. Therefore Trx1 directed siRNA knockdown and overexpression constructs during hyperoxic exposure (Fig 5) provided further insight into the specific role of Trx1 redox function. Loss of Trx1 during hyperoxic resulted in cell death similar to that observed by ATG inhibition (Fig 1A and Fig 4B). Together with data from wild-type and redox-null overexpression constructs this indicates a specific role for Trx1 redox function in mediating hyperoxic cytoprotection.

Trx systems attenuate oxidative injury and play a role in cell and redox sensitive signaling systems critical to lung development as Trx1 interactions are known to regulate cellular processes involved in cell proliferation, apoptosis (apoptosis signal regulating kinase-1), regulation of nuclear thiol chemistry⁶⁷⁻⁷⁰, transcription factor activation (TTF-1, AP-1, NF- κ B, APE/Ref-1, and p53)⁷¹⁻⁸⁶. Therefore, cellular redox signaling regulated by Trx1 interaction may mediate hyperoxic cell death. While Trx1 redox targets are known, identification of novel Trx1 targets has been limited as the redox reaction between Trx1 and target disulfides is too brief for isolation through traditional immunoprecipitation approaches with results similar to redox null mutants (Trx1^{C32/35S})^{58,87}. as an alternative a substrate trap approach was utilized which has been previously utilized to identify Trx redox interactions in plants (for review of Trx networks in plants^{88,89}), but has found limited use within mammalian systems⁵⁸. Expression of the Trx1^{C35S} mutant allows a stable complex between target proteins and Trx1 to be immunopurified and redox specific interactions to be identified by elution with DTT. Identification of known Trx1 redox targets, Prx1⁹⁰ and GAPDH⁹¹ validated the approach; however the identification of two isoforms of HSP90 (HSP90AA1 and HSP90AB1) was intriguing.

HSP90 is a chaperone protein, whose client proteins are involved in various signaling pathways critical to cell survival, growth, and response to oxidative stress⁹²⁻⁹⁴; all of which are important during epithelial and vascular lung development. Chaperone functions of HSP90 are dependent on HSP90 dimer formation, and co-chaperones which regulate HSP90 function through client protein interaction, and ATPase activity^{92,94}. Compounds inhibiting HSP90 activity often bind to the N-terminal or C-terminal domains. Some also induce oxidative species which potentially further inhibit HSP90 functions. Several studies have indicated a role for oxidative modification of HSP90 activities through reactive cysteines⁹⁵⁻⁹⁷ and even a potential role in regulation of cellular redox homeostasis⁹⁸. Additionally, studies examining the effects of various oxidative stresses on cytosolic

disulfide bond formation have indicated oxidative stress may induce disulfide bond formation within HSP90⁹⁹⁻¹⁰¹. For example, Tubocapsenolide A was shown to promote ROS production which was associated with a concomitant increase in HSP90 disulfide formation and decrease in chaperone refolding activity¹⁰⁰. Concomitant use of N-acetyl cysteine was shown to attenuate disulfide formation.

While oxidative stress has been shown to inhibit HSP90 activity, little is known about HSP90 activity during hyperoxic induced stress. Hyperoxic exposure does not alter HSP90 expression or expression of Akt and EGFR, two client proteins (Fig 6B). Nevertheless a protective role for HSP90 was established through siRNA knockdown. While HSP90 knockdown increased cell death observed in room a small but significant amount, knockdown of HSP90 expression in hyperoxic conditions more than doubled cell death (Fig 6D). Together these data indicate that HSP90 activity plays an important response to oxidative stress imposed by hyperoxic exposure. As an interaction between Trx1 and HSP90 was determined by the substrate trap IP approach, it may be that HSP90 activity may be maintained by reduction of a disulfide bond formed by hyperoxic oxidation.

One caveat to the substrate trap system is the potential to isolate Trx1-mediated denitrosylation targets¹⁰²⁻¹⁰⁵, with potential implications to Trx1-HSP90 interactions. Previous work demonstrated that overexpression of Trx1^{C32/35S} mutant was associated with increased HSP90 nitrosylation, indicating Trx1 may mediate HSP90 denitrosylation^{106,107}. However, direct regulation of HSP90 nitrosylation through Trx1 interaction was not demonstrated. As S-nitrosylation of HSP90 was previously demonstrated to attenuate HSP90 ATPase activity and thus chaperone functions, Trx1 may serve to regulate and maintain HSP90 functions related to S-nitrosylation.

These data are intriguing in light of studies linking HSP90 and functions of Trx-related proteins to mediators of angiogenesis, as proper vascular development is a critical component to normal lung development which is altered in BPD¹⁰⁸⁻¹¹¹. HSP90 provides chaperone functions to proteins known to promote angiogenesis through stabilization of HIF-1 α ¹¹², and regulation of nitric oxide synthase activity¹¹³⁻¹¹⁵ in addition to activities involved in cell survival through client proteins Akt¹¹⁶ and survivin¹¹⁷. From the Trx standpoint, Trx1 promotes cell survival and cell proliferation^{73,118}. However, enzymes modulating Trx1 function have been implicated in modulation of pathways critical to vascular development. Vascular endothelial growth factor (VEGF) is a critical factor mediating vascular growth and development, and within the lungs, VEGF is produced by type II cells¹¹⁹. Studies by the Tipple lab¹²⁰ have indicated an inverse relationship between thioredoxin-interacting protein (Txnip) and VEGF expression as decreasing levels of Txnip during the first days of life was associated with increased VEGF, and attenuation of Txnip expression was delayed in mice exposed to hyperoxia. As Txnip inhibits Trx1 function through interaction with the Trx1 active site¹²¹, these data provide a link between modulation of Trx1 systems and vascular development.

Data presented here represents a functional link between the Trx1 and the HSP90 systems. Both the Trx1 and the HSP90 systems play critical roles in hyperoxic cytoprotection whereby loss of function of either system yields significant increases in cell death. Heat

shock proteins, including HSP90 are known to be sensitive to oxidation and regulated by oxidative modifications, which result in decreased chaperone function. As Trx1 is a major thiol oxidoreductase mediating disulfide reduction in the cell, Trx1-HSP90 interaction may mediate recovery of HSP90 function and restoration of pro-survival signaling to oxygen-sensitive pathways critical to cell survival in culture as presented here and with respect to epithelial cells impacted by oxygen induced oxidative stress and injury during pulmonary development. Cancer cell lines such as those used herein are known to have augmented antioxidant capacity through Trx and GSH-dependent systems and are known to have an increased sensitivity to ROS induced cell death¹²²⁻¹²⁴. While this may be the case the Trx1 system was impaired by hyperoxic exposure similar to previous findings in a murine model of BPD⁴³. Future studies aimed at understanding the role of HSP90 and its interaction with Trx1 in pulmonary development and hyperoxic injury in mouse models are necessary and may represent targets of potential therapeutics aimed at preventing hyperoxic alterations to redox sensitive proteins and signaling pathways critical to lung development through protection and maintenance of pulmonary epithelial and endothelial development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation List

ATG	aurothioglucose
BPD	bronchopulmonary dysplasia
BSO	buthionine sulfoximine
DOX	doxycycline
EGFP	enhanced green fluorescent protein
GFP	green fluorescent protein
HSP90	heat shock protein 90
IAA	iodoacetic acid
IRES	internal ribosomal entry site
TRX1	thioredoxin-1
TRXR1	thioredoxin reductase-1
TXNIP	thioredoxin interacting protein

Highlights

- Hyperoxic alterations in thiol/disulfide redox circuitry
- Trx1 oxidoreductase activity is cytoprotective against hyperoxic death
- Proteomic identification of novel redox-dependent Trx1 substrates

Figure 1. Thioredoxin Inhibition Sensitizes Cells to Hyperoxic Death

Viability of (A) H1299 and (B) A549 cells treated with 100μM BSO or 100μM ATG during two days hyperoxic treatment was determined by trypan blue dye exclusion. Free thiol content of H1299 during a (C) hyperoxic time course and (D) following BSO or ATG treatment in room air or hyperoxia (n=3; *p<0.0001, #p<0.01, ^p<0.05 by ANOVA).

Figure 2. Trx1 and TrxR1 Expression in Response to Hyperoxia

Protein expression of Trx1 and TrxR1 was determined by immunoblot in protein lysates isolated from (A) H1299 and (B) A549 after cells were exposed to hyperoxia for 0, 1, 2, and 3 days. (β-actin was used as a loading control. Under the same treatment conditions, (C) mRNA expression was determined by qPCR in lysates from both cell lines and normalized to GAPDH (n=4; *p<0.05 by *t*-test).

Figure 3. Hyperoxic Oxidation of Trx1

Following treatment in hyperoxia for 0, 1, 2, and 3 days, lysates from (A) H1299 and (B) A549 cells were collected with G-lysis buffer containing 50mM iodoacetic acid (IAA), and membranes were incubated were probed for Trx1.

Figure 4. Trx1 Redox Activity is Required for Protection Against Hyperoxic Death

H1299 cells were transfected with 10, 25, or 50nM siRNA oligos targeting luciferase (GL2) or Trx1 for 24 hours, then cultured in room air or hyperoxia for 2 days. (A) Total protein lysates isolated from cells cultured in room air were immunoblotted for Trx1, Trx2, and β -actin. (B) Cell death was measured following treatment by trypan blue dye exclusion. (C) H1299 cells with inducible expression of Myc-Trx1 and Myc-Trx1^{C32/35S} were cultured in increasing concentrations of doxycycline (DOX) for 24 hours, total protein was isolated from H1299+Trx1 and H1299+Trx1^{C32/35S} expressing cells and lysates were analyzed by Western blot against Trx1, EGFP, and β -actin. Asterisks indicate endogenous Trx1 while arrows specify Myc-Trx1 or Myc-Trx1^{C32/35S}. (D) Viability of Myc-Trx1 and Myc-Trx1^{C32/35S} inducible cells in an absence (-) or presence (+) of DOX for 24 hours of treatment prior to 96 hours of treatment in room air or hyperoxia was measured by trypan blue dye exclusion (n = 3; *p<0.001, #p=0.0125 by *t*-test).

Figure 5. Identification of Trx1 Substrates

(A) Schematic demonstrating stable substrate interaction with Flag-Trx1^{C35S}, immunoprecipitation, and elution of redox-dependent and redox-independent Trx1 protein interactions. (B) Total lysate from H1299+EGFP, H1299+Trx1^{C32S}, and H1299+Trx1^{C32/35S} cells was subjected to immunoprecipitation for Flag and proteins were eluted with DTT and Laemmli boil, separated by SDS-PAGE and analyzed by either immunoblot or (C) silver stain.

Figure 6. HSP90 is Targeted by Trx1 and is Cytoprotective during Hyperoxic Injury

(A) Immunoblot for Trx1 and HSP90 with input and Trx1-immunopurified DTT-sensitive eluate from H1299+EGFP and H1299+Trx1^{C32S} cell lysates separated by SDS-PAGE. (B) Protein expression for HSP90, Akt, EGFR, and β-actin was determined by immunoblot of total protein lysates isolated from H1299 cells exposed to hyperoxia for 0, 1, 2, and 3 days. (C) H1299 cells were transfected with 50nM siRNA oligos targeting luciferase (GL2) or HSP90 for 24 hours, then cultured in room air or hyperoxia for 2 days. Total protein lysates isolated from cells cultured in room air were immunoblotted for HSP90 and β-actin. (D) Cell death after 2 days hyperoxia was measured following treatment by trypan blue dye exclusion (n=4; *p=0.023, #p=0.0022 by *t*-test).

Table 1

Protein Name	Gene ID	UniProt Entry	Protein MW(Da)	Amino Acid Length	# Peptides Identified	% Coverage
Tubulin beta chain	TUBB	Q5JP53	47767	426	22	74.2
Peroxioredoxin-1	PRDX1	Q06830	22110	199	13	70.9
Cofilin-1	CFL1	P23528	18502	166	15	59.6
Tubulin beta-2A chain	TUBB2A	Q13885	49907	445	17	54.4
Isoform 1 of sequestosome-1	SQSTM1	Q13501	47687	440	15	54.1
Peroxioredoxin-5	PRDX5	P30044	22086	214	7	48.1
Isoform M2 of Pyruvate Kinase Isozyme M1/M2	PKM2	P14618	57937	531	18	47.3
Serpin B6	SERPINF6	P35237	46370	376	14	46.8
Cystatin-B	CSTB	P04080	11140	98	3	45.9
Inorganic pyrophosphatase	PPA1	Q15181	32660	289	10	45.3
Peroxioredoxin-4	PRDX4	Q13162	30540	271	8	44.6
Low molecular weight phosphotyrosine protein phosphatase isoform d	ACP1	P24666	12230	158	4	42
Heat shock protein beta	HSP90AB1	P08238	83264	724	26	41.7
Isoform 1 of OBG-like ATPase1	OLA1	Q9NTK5	44744	396	15	40.4
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	P04406	36053	335	10	46.2
Heat shock protein 90 alpha	HSP90AA1	P07900	98161	732	19	33.1
Cofilin-2	CFL2	Q9Y281	18737	166	1	30.7
Isoform 1 of low molecular weight phosphotyrosine protein phosphatase	ACP1	P24666	18042	158	1	30.7
Isoform long of 14-3-3 protein beta/alpha	YWHAB	P31946	28082	246	2	20.9
Melanocyte-stimulating hormone receptor	MC1R	Q01726	34706	317	18	20.2