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Research Paper

Resolution of oxidative stress by thioredoxin reductase: Cysteine versus selenocysteine

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ABSTRACT

Thioredoxin reductase (TR) catalyzes the reduction of thioredoxin (TRX), which in turn reduces mammalian typical 2-Cys peroxiredoxins (PRXs 1–4), thiol peroxidases implicated in redox homeostasis and cell signaling. Typical 2-Cys PRXs are inactivated by hyperoxidation of the peroxidatic cysteine to cysteine-sulfinic acid, and regenerated in a two-step process involving retro-reduction by sulfiredoxin (SRX) and reduction by TRX. Here transient exposure to menadione and glucose oxidase was used to examine the dynamics of oxidative inactivation and reactivation of PRXs in mouse C10 cells expressing various isoforms of TR, including wild type cytoplasmic TR1 (Sec-TR1) and mitochondrial TR2 (Sec-TR2) that encode selenocysteine, as well as mutants of TR1 and TR2 in which the selenocysteine codon was changed to encode cysteine (Cys-TR1 or Cys-TR2). In C10 cells endogenous TR activity was insensitive to levels of hydrogen peroxide that hyperoxidize PRXs. Expression of Sec-TR1 increased TR activity, reduced the basal cytoplasmic redox state, and increased the rate of reduction of a redox-responsive cytoplasmic GFP probe (roGFP), but did not influence either the rate of inactivation or the rate of retro-reduction of PRXs. In comparison to roGFP, which was reduced within minutes once oxidants were removed reduction of 2-Cys PRXs occurred over many hours. Expression of wild type Sec-TR1 or Sec-TR2, but not Cys-TR1 or TR2, increased the rate of reduction of PRXs and improved cell survival after menadione exposure. These results indicate that expression levels of TR do not reduce the severity of initial oxidative insults, but rather govern the rate of reduction of cellular factors required for cell viability. Because Sec-TR is completely insensitive to cytotoxic levels of hydrogen peroxide, we suggest TR functions at the top of a redox pyramid that governs the oxidation state of peroxiredoxins and other protein factors, thereby dictating a hierarchy of phenotypic responses to oxidative insults.

Introduction

Cellular oxidants modulate the strength and duration of signaling through redox-responsive pathways that govern cell proliferation and survival [1,2]. In many disease states management of endogenous and/or exogenous oxidants may be compromised, leading to oxidative stress, damage to macromolecules and loss of cell viability. Phenotypic adaptation to chronic oxidative stress, which occurs in many cancers, often involves up-regulation of stress response proteins and antioxidant enzymes, leading to improved cell survival [3]. Among the families of antioxidant enzymes that are often over-expressed in cancer and other disease states are thioredoxin reductases (TRs) and peroxiredoxins (PRXs), both of which are involved in the metabolism of hydrogen peroxide [4]. Mammalian TRs are a family of selenocysteine-containing oxidoreductases that act as the primary reductases for thioredoxin (TRX) in a NADPH dependent reaction [4,5]. Recent studies show that selenocysteine in the C-terminal redox center renders mitochondrial TR insensitive to inactivation from oxidants [6],

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suggesting TR and other selenoenzymes may mediate resistance to oxidative stress.

Malignant mesotheliomas express elevated levels of TR1 [7,8] and mesothelioma cells propagated in vitro have higher levels of TR activity than untransformed mesothelial cells do [9]. Given that this phenotype has been observed in many other tumor types, TR has emerged as an attractive therapeutic target in human malignancies [10,11]. Because of the limited number of cellular proteins that contain selenocysteine, one approach is to use compounds (such as aurothioglucose and auranofin) that selectively target selenocysteine [12–14]. However, the effects of inhibition or over-expression of TR on cell growth and survival are not straightforward, as expression of TR in which the activity of selenium is compromised may induce cell death due to pro-oxidant activity [15]. TR and TRX have been shown to influence the activity of transcription factors, kinases and other factors in cell signaling circuits, and in some contexts the pro-proliferative role of its primary substrate thioredoxin (TRX) has been ascribed to the regulation of the ERK pathway leading to the expression of cyclin D1 [16].

Peroxiredoxins are a family of thiol peroxidases that are ubiquitously expressed throughout eukaryotes. In mammals there are six family members, with peroxiredoxins 1–4 representing typical 2-Cys PRXs; these enzymes function as head-to-tail homodimers with two reaction sites, each consisting of a peroxidatic cysteine in one monomer and a resolving cysteine in the opposing subunit [17]. During the metabolism of hydrogen peroxide the initial step in the peroxiredoxin reaction cycle is oxidation of the peroxidatic cysteine to sulfenic acid (–SOH), which then spontaneously reacts with the resolving cysteine in the opposing subunit to produce an intermolecular disulfide bond. The intermolecular disulfide bond then is reduced by thioredoxin, which in turn is reduced by TR, using reducing equivalents from NADPH. The peroxidatic cysteines of 2-Cys PRXs are among the most reactive cysteines in the cell [18], in part due to the stabilization of the thiolate in the active site and structural interactions positioning peroxide for attack [19–21]. Curiously, an evolutionarily conserved carboxy-terminal extension on PRXs 1–4 stabilizes the peroxidatic −SOH intermediate, making it susceptible to further attack by hydrogen peroxide, thereby leading to the formation of sulfinic acid (–SOOH). This process, which has been termed “over-oxidation” or “hyperoxidation”, precludes disulfide bond formation and would be expected to permanently inactivate the enzyme, for sulfinic acid residues in proteins generally are not repaired. PRXs are a notable exception, for with the aid of ATP the reductase sulfiredoxin (SRX) is capable of “retro-reduction” of PRX-SOOH and regeneration of enzyme activity [22,23]. In addition to homodimers, the oxidation state of PRXs influences their relative distribution in dimers, decamers and higher order oligomers that have chaperone and other functions [24]. The unusual features of PRX catalysis and oligomerization, as well as their interaction with many cellular regulatory factors, have led to proposals that PRXs act as peroxide sensors in which structural transitions and/or changes in protein–protein interactions with cellular regulators modulate responses to oxidative stress [25,26]. In yeast, the TR system is devoted to metabolism of hydrogen peroxide [27], and regulation of hierarchical responses to oxidative insults by thiol peroxidase Tsa1 has been elegantly dissected [28,29]. However, detailed descriptions of similar hierarchical pathways in mammalian cells have yet to be reported.

Both TR and PRXs may be over-expressed in cancer, and studies in which TR has been down-regulated by RNA interference strongly support the hypothesis that elevated expression of TR promotes tumor cell proliferation in culture and tumor progression in animal models [30,31]. To mimic the situation often encountered in malignancies, we have expressed cytoplasmic TR1 and mitochondrial TR2 in mouse lung epithelial C10 cells that are immortalized but not tumorigenic. Previously we described the relationship between the oxidation state of PRX2 and C10 cell cycle progression in response to fluxes of hydrogen peroxide, and showed that expression of cyclin D1 and cell cycle progression was not recovered after oxidative insult until PRX2 was completely reduced [32]. However, in contrast to PRXs, mammalian TRs with selenocysteine in the C-terminal catalytic site are remarkably resistant to inactivation by hydrogen peroxide and other oxidants in vitro [6], raising the possibility that TR sits at the top of a redox pyramid that includes peroxiredoxins as exquisitely sensitive detectors of cellular peroxide.

To test this possibility, we compared the ability of TRs encoding selenocysteine in the C-terminal redox center to TRs in which the codon for selenocysteine had been changed to that for cysteine to mediate recovery from oxidative insults. Our data suggest that TR levels do not directly influence the extent of the initial insult, but rather dictate the rate of recovery and cell viability, restoring proliferative capacity over time. Moreover, only wild type TRs had the capacity to improve cell survival, indicating that the insensitivity of selenocysteine to inactivation by hydrogen peroxide is an important feature of mammalian TRs.

Materials and methods

Cell culture

C10 mouse lung epithelial cells were maintained in CMRL cell culture media (Corning Cellgro, Manassas, VA), supplemented with 10% fetal bovine serum (FBS), 200 mM glutamine, and 0.5% penicillin streptomycin and propagated in a humidified incubator at 37 °C and 5% CO_2. Cells were trypsinized and re-plated to obtain 75% confluence on the following day for all subsequent experiments.

Molecular cloning

Full length human TR1 was amplified from pCMV6-XL4 vector by PCR using specific forward (TR1: 5′-GAAAGTCGAGGAGACAGTTAAGCATG-3′) and reverse (TR1: 5′-CACAAGGAAAGGTCATGCTAAAACTG-3′) primers and subsequently cloned into pcDNA 3.1 mammalian expression vector (Promega, Madison WI). Full length cDNA for human TR2 was recovered from pCMV6-XL4 by digestion with XbaI and EcoRI, purified by gel electrophoresis, and ligated into linear pcDNA 3.1 using T4 DNA Ligase. Insertion of wild type Sec-TR1 and Sec-TR2 full-length cDNAs, including the 3′ SECIS elements, into pcDNA 3.1 was confirmed by sequencing using the appropriate forward and reverse primer sets (T7 Forward: BGH Reverse). PCR-based mutagenesis was performed by Mutagenex (Piscataway, NJ) to replace the Sec codon with Cys and was confirmed by sequencing. Expression vectors for human TRX1 and TRX 2 were purchased from Open Biosystems (Thermo Scientific, Waltham, MA). Expression vectors for cytosolic and mitochondrial targeted roGFPs were a kind gift from J.A. Melendez (College of Nanoscale Science and Engineering, Albany, NY).

Plasmid transfection and siRNA silencing

Plasmid expression vectors were introduced into C10 cells by transfection or co-transfection using Lipofectamine 2000™ following the manufacturer's guidelines (Invitrogen, Grand Island, NY). Pilot experiments with GFP and RFP expression vectors showed co-transfection efficiency with Lipofectamine 2000™ in C10 cells was 90% or better (data not shown). Specific siRNA to TR1 (si-TR1, 5′-CCAUAGAGGGCGAAUUUAAUU-3′) was introduced into C10