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Original article

Inhibitory nitrosylation of mammalian thioredoxin reductase 1: Molecular characterization and evidence for its functional role in cellular nitroso-redox imbalance

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ABSTRACT

Mammalian thioredoxin 1 (Trx1) and the selenoprotein Trx reductase 1 (TrxR1) are key cellular enzymes that function coordinately in thiol-based redox regulation and signaling. Recent studies have revealed that the Trx1/TrxR1 system has an S-nitrosothiol reductase (denitrosylase) activity through which it can regulate nitric oxide-related cellular processes. In this study we revealed that TrxR1 is itself susceptible to nitrosylation, characterized the underlying mechanism, and explored its functional significance. We found that nitrosothiol or nitric oxide donating agents rapidly and effectively inhibited the activity of recombinant or endogenous TrxR1. In particular, the NADPH-reduced TrxR1 was partially and reversibly inhibited upon exposure to low concentrations ($<10\ \mu M$) of S-nitrosocysteine (CysNO) and markedly and continuously inhibited at higher doses. Concurrently, TrxR1 very efficiently reduced low, but not high, levels of CysNO. Biochemical and mass spectrometric analyses indicated that its active site selenocysteine residue renders TrxR1 highly susceptible to nitrosylation-mediated inhibition, and revealed both thiol and selenol modifications at the two redox active centers of the enzyme. Studies in HeLa cancer cells demonstrated that endogenous TrxR1 is sensitive to nitrosylation-dependent inactivation and pointed to an important role for glutathione in reversing or preventing this process. Notably, depletion of cellular glutathione with L-buthionine-sulfoximine synergized with nitrosating agents in promoting sustained nitrosylation and inactivation of TrxR1, events that were accompanied by significant oxidation of Trx1 and extensive cell death. Collectively, these findings expand our knowledge of the role and regulation of the mammalian Trx system in relation to cellular nitroso-redox imbalance. The observations raise the possibility of exploiting the nitrosylation susceptibility of TrxR1 for killing tumor cells.

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1. Introduction

A growing body of research has demonstrated that nitric oxide (NO)-induced signaling and cytotoxic responses are substantially mediated by S-nitrosylation of protein cysteine residues, to form S-nitrosothiols (SNOs) [1,2]. S-nitrosylation has indeed been shown to regulate various cellular signaling pathways and thereby affect diverse cellular processes, including metabolism, gene activation and intracellular trafficking [1,2]. In addition, there is evidence to suggest that dysregulated S-nitrosylation is involved in cellular dysfunction and pathogenesis of human diseases, including cancer, neurodegenerative and metabolic disorders [3,4]. In particular, hyper S-nitrosylation of proteins, which is due to increased generation of SNOs and/or their decreased breakdown, is frequently associated with cellular stress and death, consistent with the notion that nitrosylation/denitrosylation balance is important for maintaining cellular function and viability [3–6].

Recent research has established that endogenous protein

Abbreviations: BIAM, biotin-conjugated iodoacetamide; BSO, L-buthionine-sulfoximine; CysNO, S-nitrosocysteine; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GSH, glutathione; GSNO, S-nitrosoglutathione; JS-K, O^2-(2,4-dinitrophenyl) 1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate; MS, mass spectrometry; NADPH, nicotinamide adenine dinucleotide phosphate, reduced; NEM, N-ethyl maleimide; NO, nitric oxide; PAPA/NO, 1-[*N*-(3-ammoniopropyl)-*N*-(*n*-propyl) amino]diazen-1-ium-1,2-diolate; SNO, S-nitrosothiol; PBS, phosphate-buffered saline; Prx, peroxiredoxin; Trx, thioredoxin; TrxR, thioredoxin reductase

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denitrosylation, i.e. catabolism of SNOs, is mediated in large part by glutathione (GSH), thioredoxin (Trx), and their associated redox systems [7]. Indeed, it is now recognized that the GSH and Trx redox systems play a widespread role in cellular SNO reduction beyond their well-established roles in disulfide reduction. In particular, the Trx system, which consists of Trx, Trx reductase (TrxR) and NADPH, has emerged as a major denitrosylating system in different cell types and organisms [7,8]. By denitrosylating diverse substrates, Trx/TrxR plays an important role in regulating multiple NO/SNO-mediated signaling processes as well as in alleviating SNO-related cellular stress [7–9]. The alternative TrxR substrate thioredoxin-related protein of 14 kDa (TRP14) was also found to have potent denitrosylase activity [10]. Of note, recent studies have demonstrated that targeting the Trx system sensitizes cancer cells to nitrosylating agents, underscoring the importance of Trx/TrxR in protecting cancer cells from SNO-related stress and damage [11–13]. In addition, recent intriguing findings suggest that the Trx system itself could be regulated by SNO; however, the mechanisms and consequences of such regulation remain poorly understood [8].

Mammalian TrxR1, a selenocysteine (Sec)-containing flavoenzyme, is the main reductant of oxidized Trx1 and is therefore regarded as the "engine" of the Trx system [14]. Through its redox activity, TrxR1 plays important roles in a wide range of cellular processes, such as DNA synthesis, transcriptional control and metabolism of reactive oxygen species. Therefore, proper regulation of TrxR1 activity is important for maintaining cellular redox balance, fitness and survival. Recently, while studying the effects of S-nitrosylation on the 2-Cys peroxiredoxin, Prx1, we found that nitrosylating agents, such as S-nitrosocysteine (CysNO), potently inhibited the regeneration of oxidized Prx1, an effect that was attributed mainly to inactivation of TrxR1 [15]. These findings, together with early observations by Nikitovic and Holmgren [16], suggested the possibility of SNO-dependent regulation of TrxR1. In the present study, we extend these previous findings by showing that TrxR1 is subject to inhibitory nitrosylation in vitro and in HeLa cancer cells. Our observations indicate that nitrosylation targets reactive thiol(s) as well as the selenol-containing active site of TrxR1. Importantly, we present evidence that depletion of cellular GSH augments nitrosylation and inactivation of TrxR1, leading to a marked Trx1 oxidation and extensive cell death. Collectively, these findings expand our understanding of the crosstalk between SNO, Trx/TrxR and GSH, and the importance of this crosstalk in cellular redox homeostasis and cell death.

2. Materials and methods

2.1. Materials

N-ethyl maleimide (NEM) was obtained from Thermo Scientific. Sephadex G-25 (fine) was from GE healthcare. NADPH was from Calbiochem. PAPA/NO was from Cayman Chemical. CysNO or GSNO were synthesized by combining an equimolar concentration of L-cysteine or GSH with sodium nitrite in 0.2 N HC1, and used within 1 h. The recombinant proteins human Prx1, human Trx1, rat TrxR1, yeast Trx (yTrx) and yeast TrxR (yTrxR) were expressed and purified as previously described [15]. TRFS-green was kindly provided by Dr. Jianguo Fang, (Lanzhou University, China). Other materials were obtained from Sigma unless otherwise indicated.

2.2. Cell culture

HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C under 5% CO_2. Tissue culture media and reagents were from Biological Industries (Beit HaEmek, Israel).

2.3. Determination of the activity of recombinant and cellular TrxR

TrxR1 activity in vitro was measured using the direct 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) reduction assay as previously described [15]. In brief, TrxR1 (specific activity $\approx$15 U/mg) was diluted in assay buffer (25 mM potassium phosphate, 1 mM EDTA, pH 7.0) to 15 nM and incubated with 250 μM NADPH and 2.5 mM DTNB. The rate of DTNB reduction was monitored following the increase in absorbance at 412 nm. For juglone reduction, 15 nM TrxR1 was incubated with 250 μM NADPH and 50 μM juglone and the rate of NADPH oxidation was monitored at 340 nm. In experiments involving both yeast and rat TrxR proteins (Fig. 2C) enzymatic activity was determined using yeast Trx and mammalian Prx proteins, as follows. Yeast or rat TrxR1 (0.5 μM) were preincubated with CysNO (0–1000 μM) and NADPH (800 μM) in assay buffer (25 mM potassium phosphate, 1 mM EDTA, pH 7.0) for 10 min at 37 °C. The reaction mixture was then diluted 1:4 in 50 mM Hepes and 1 mM EDTA (pH 7.5) and combined with untreated yeast Trx (3 μM) and human Prx1 (5 μM). Finally, 100 μM H_2O_2 was added and NADPH oxidation was monitored by following the decrease of absorbance at 340 nm.

TrxR activity in cell lysates was measured in 96-well microtiter plates using an end-point insulin assay as detailed before [15]. In brief, 25 μg of protein cell lysate was incubated in a final volume of 50 μl containing 0.3 mM insulin, 660 μM NADPH, 2.5 mM EDTA, and 5 μM human Trx1 in 85 mM Hepes (pH 7.5) for 20 min at room temperature. Control reactions excluding Trx1 were used for background subtraction. Then, 250 μl of 1 mM DTNB, 240 μM NADPH, and 200 mM Tris–HCl, pH 8, in 6 M guanidine hydrochloride was added and the absorbance was determined at 412 nm.

The activity of TrxR in living cells was probed using TRFS-green fluorescent probe [17]. For this, 25,000 cells were seeded in a 96-well plate and allowed to adhere for 24 h. Following different treatments in full media, 10 μM of TRFS-green was added to the cells and the fluorescence at 438/538 was measured every 10 min for 1 h at 37 °C. TrxR activity was obtained from plots of fluorescence versus time.

2.4. Alkylation of TrxR with biotin-conjugated iodoacetamide (BIAM)

TrxR1 (0.1 μM) was incubated with 450 μM NADPH and different concentrations of CysNO in assay buffer (25 mM potassium phosphate, 1 mM EDTA, pH 7.0) for 10 min at 37 °C. After incubation, the treated protein (0.045 μM) was mixed with 95 μM BIAM (Molecular Probes, catalog no. B1591) in 0.2 M Tris-HCl and 1 mM EDTA, with the pH of the buffer set to either 6.5 or 8.5, and incubated at 30 °C for another 30 min to alkylate the remaining free -SeH and -SH groups. Thereafter, 50 ng of the BIAM-modified enzyme were subjected to SDS-PAGE and detected by Western blotting with 5-FAM-streptavidin conjugate (AnaSpec) using the Odyssey Infrared Imaging System (LI-COR Biosciences).

2.5. Assessment of nitrosylation of TrxR1 under cell-free conditions

TrxR1 (0.5 μM) was incubated with 450 μM NADPH and NO/SNO donors (as detailed in the legend of Fig. 3) in HEN buffer (250 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine, pH 7.5). Part of the samples were then subjected to alkylation with 10 mM NEM for 10 min. Excess CysNO and NEM were removed by repeated cycles of filtration through a 10-kDa-cutoff filter and washing with HEN buffer. The samples as well as GSNO standards were assayed for SNO content by chemical reductive chemiluminescence (CuCl/

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