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Oxidation of nuclear thioredoxin during oxidative stress

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Abstract Thioredoxin 1 (Trx1) is a key redox control system within the nucleus, yet little is known about the sensitivity of nuclear Trx1 to oxidative stress. The present study compared oxidant-induced changes in the redox states of nuclear Trx1, cytoplasmic Trx1, and cellular glutathione (GSH). Nuclear Trx1 was more reducing than cytoplasmic Trx1 and cellular GSH in proliferating cells. *tert*-Butylhydroperoxide caused an increase in the total amount of nuclear Trx1, but this was accompanied by a 60 mV oxidation. Thus, the increase in nuclear Trx1 levels did not correspond to an increase in the overall reducing capacity of Trx1 in the nucleus.

Key words: Thioredoxin; Redox; Oxidative stress; Nucleus; Protein oxidation; Glutathione

1. Introduction

The function of many proteins is affected by oxidation of critical cysteine (Cys) residues [1]. Aside from those participating in structural disulfides, Cys residues of intracellular proteins are normally maintained in the reduced (thiol) form, but can be oxidized by peroxides and other oxidants [2]. Thioredoxin 1 (Trx1) and glutathione (GSH) are components of systems that maintain the thiol/disulfide redox state of redox-sensitive proteins and protect cells from oxidant-mediated damage. Trx1 reduces protein disulfides directly and serves as a reductant for the peroxiredoxins [3]. GSH protects cells through direct scavenging of peroxides and by serving as a reductant for GSH peroxidase [4]. The reduced forms of Trx1 and GSH are regenerated by Trx1 reductase and GSH reductase, respectively, using electrons from reduced nicotinamide adenine dinucleotide phosphate (NADPH) [4,5].

Oxidative stress causes an increase in the expression of protective genes under the control of redox-sensitive transcription factors such as NF-κB and AP1. The DNA-binding activity of these transcription factors is inhibited by oxidation and enhanced by reduction of Cys residues within their DNA-binding domains [6,7]. In vitro DNA-binding assays suggest that GSH is a poor reductant for oxidized transcription factors, and that vicinal dithiol-containing proteins like Trx1 and Ref1 are much more efficient activators of DNA-binding activity [8]. The reduced form of Trx1 restores DNA-binding activity to oxidized transcription factors, and redox-inactive mutants do not serve this function [9].

Reducing conditions in the cytoplasm, including increased expression of Trx1, inhibit the release of NF-κB from its inhibitor protein IκB in the cytoplasm. However, reducing conditions in the nucleus would be expected to enhance the DNA-binding activity of NF-κB [9]. This suggests that the redox state of nuclear Trx1 is regulated independently of the redox state of cytoplasmic Trx1. Distinct pools of GSH exist within the cell [10]. The redox states of GSH in the cytoplasm, mitochondria, and endoplasmic reticulum have been determined through subcellular fractionation and subsequent measurement of GSH and its oxidized form, glutathione disulfide (GSSG) [11]. The determination of the redox environment within the nucleus has been more difficult. Nuclear membranes contain a nuclear pore complex that allows diffusion of small compounds like GSH and GSSG during standard subcellular fractionation protocols [10,12]. Studies using non-aqueous fractionation [13,14] and GSH-specific fluorescent probes in intact cells [15] suggest that the GSH concentration in the nucleus is similar to that in the cytoplasm, but that a distinct pool of GSH may exist within the nucleus [16]. Unfortunately, these studies focused on GSH and provide no information on the nuclear redox state.

In this report, we used a Western blot technique which separates reduced and oxidized Trx1 in nuclear fractions of THP1 cells to determine nuclear Trx1 redox state in proliferating cells and following oxidative stress. Results showed that the redox state of nuclear Trx1 was more reduced than that of cytoplasmic Trx1 or cellular GSH. Following exposure to a toxic concentration of *tert*-butylhydroperoxide (tBH), both nuclear and cytoplasmic Trx1 were oxidized in parallel with cellular GSH, but the extent of oxidation of both was considerably less than that of GSH. Thus, the results show that nuclear Trx1 is normally more reduced than the cytoplasmic pool but both are relatively protected against oxidation compared to the cellular GSH pool.

2. Materials and methods

2.1. Cell culture conditions

THP1 human monocyte cells were obtained from American Type Culture Collection (Rockville, MD,USA) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

2.2. Subcellular fractionation

Nuclei were isolated based on the procedure of Janssen and Sen [17], with modifications to allow redox measurements of Trx1 via the Redox Western blot. Cells were pelleted by centrifugation and lysed in

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Abbreviations: Trx1, human thioredoxin; GSH, glutathione; GSSG, glutathione disulfide; tBH, *tert*-butylhydroperoxide; IAA, iodoacetic acid; PAGE, polyacrylamide gel electrophoresis

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hypotonic lysis buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.2 mM NaF, 0.2 mM $Na_3VO_4{\cdot}6H_2O$) with freshly added protease inhibitors (leupeptin, aprotinin, pepstatin, phenylmethylsulfonyl fluoride). For Redox Western blots, 50 mM iodoacetic acid (IAA) was added to the lysis buffer and the pH was adjusted to pH 7.8. The suspensions were incubated on ice for 5 min, and Nonidet P40 was added to a final concentration of 0.6%. Following centrifugation at 16 000 × *g*, the pellet (nuclei) and the supernatant (cytosol) were separated and analyzed by Western blot or Redox Western blot analysis, as described below.

2.3. Derivatization of protein thiols with IAA

To allow separation of Trx1 according to redox state, extracts were treated with IAA, which introduces a negative charge for each thiol. Nuclear and cytosolic proteins were suspended in guanidine–Tris solution (6 M guanidine–HCl, 50 mM Tris, pH 8.3, 3 mM EDTA, 0.5% Triton X100) supplemented with 50 mM IAA and incubated at 37°C for 30 min. Excess IAA was removed by Sephadex chromatography (MicroSpin G-25 columns, Amersham-Pharmacia, Piscataway, NJ, USA).

2.4. Redox Western blot analysis

The redox state of Trx1 was determined by a Western blot method in which the electrophoresis was performed under native conditions to facilitate separation of the differently charged forms of Trx1 [18,19]. Derivatized proteins (see above) were separated by native polyacrylamide gel electrophoresis (PAGE). Gels were electroblotted to polyvinylidene difluoride (PVDF) membranes (Amersham) and probed with an antibody to Trx1 (American Diagnostica, Greenwich, CT, USA) and a secondary antibody conjugated to horseradish peroxidase (HRP; anti-goat IgG–HRP conjugate, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands corresponding to Trx1 were visualized using chemiluminescent detection (ECL, Amersham) of HRP by exposure of the membranes to X-ray film (Kodak, Rochester, NY, USA). Control experiments showed that the antibody provided nearly equivalent detection of the different redox forms of Trx1.

2.5. Western blot analysis

Nuclear and cytosolic proteins were separated by discontinuous sodium dodecyl sulfate (SDS)–PAGE, transferred to PVDF membranes and probed with antibodies specific for Trx1 (American Diagnostica), nucleolin (MBL, Nagoya, Japan), and IkB-α (Research Antibodies, Santa Cruz, CA, USA). HRP-conjugated secondary antibodies were detected by chemiluminescence.

2.6. Measurement of GSH and GSSG by high-performance liquid chromatography (HPLC)

GSH and GSSG were assayed by HPLC as *S*-carboxymethyl, *N*-dansyl derivatives using γ-glutamylglutamate as an internal standard [11].

2.7. Calculation of E_h values

The Nernst equation was used to calculate the redox states of GSH and Trx1. To convert total GSH and GSSG to intracellular concentrations, the cell volumes were assumed to be constant, and a conversion factor of 5 μl cell volume per mg cell protein was used [20]. For both Trx1 and GSH redox calculations, the intracellular pH was assumed to be 7.4 and to be unchanged by tBH treatment. In these calculations, the E_0 for the GSH/GSSG couple was −264 mV at pH 7.4 [21,22], and the E_0 for the active site dithiol/disulfide of Trx1 was −254 mV at pH 7.4.

3. Results

3.1. Oxidation of the GSH/GSSG pool by tBH

Under normal culture conditions, the GSH pool was predominantly in the reduced (GSH) form, with only about 2% in the oxidized (GSSG) form. Upon exposure to the peroxide tBH, there was a rapid oxidation of GSH to GSSG followed by a gradual return toward baseline redox conditions (Fig. 1). Maximal oxidation occurred within 2 min, at which time the GSH pool was 50% oxidized. The overall pool size remained constant over the time course of these experiments, indicating that there was no extensive loss of GSH due to export or formation of mixed disulfides with other cellular thiols.

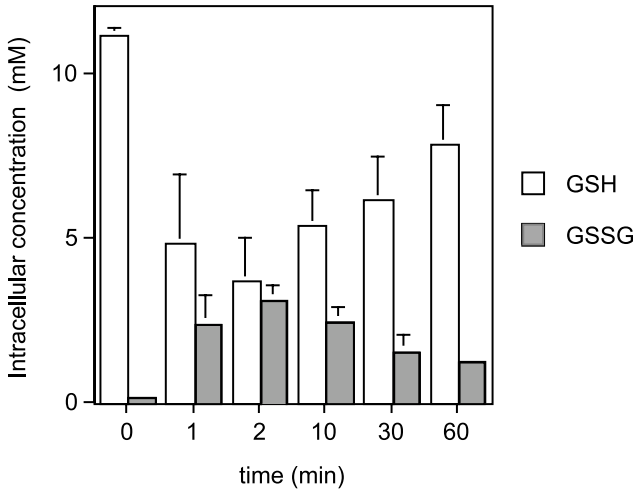

Fig. 1. GSH is oxidized to GSSG in cells treated with tBH. Cells were exposed to 1 mM tBH for the indicated times, and cellular GSH and GSSG was analyzed as described in Section 2. Values are the mean ± S.E.M. of three separate experiments.

3.2. Determination of the redox states of nuclear and cytosolic Trx1

In untreated cells, both the nuclear and the cytosolic pools of Trx1 were predominantly in the fully reduced form; nuclear Trx1 was 95% reduced, and cytosolic Trx1 was 85% reduced (Fig. 2). tBH caused a rapid and transient oxidation of both pools of Trx1, with maximal oxidation occurring within 2 min. Redox Western blot analysis of tBH-treated cells revealed the presence of a band with lower electrophoretic mobility than those observed in extracts of untreated cells. This band represents a two-disulfide form of Trx1 that contains a non-active site disulfide in a region proximal to the active site [23]. This fully oxidized form was undetectable in control cells, but became 16% and 5% of the total in nuclei and cytosol, respectively, after 2 min exposure to tBH. Both the nuclear and the cytosolic pools of Trx1 were oxidized under these conditions, and both pools became more reduced over the next 2 h.

3.3. Comparison of the redox potentials for Trx1 and GSH/GSSG

The E_h value, calculated from the Nernst equation, provides a quantitative measure of the redox state of a redox-active couple and allows convenient comparison of different reducing systems in cells. In untreated cells, the redox state of Trx1 was −280 ± 10 mV in the cytosol and −300 ± 10 mV in the nucleus (Fig. 3). It should be noted that a 20 mV difference in E_h values corresponds to a 4.6-fold change in the ratio of oxidized to reduced forms of Trx1. The active site of nuclear Trx1 was oxidized by 60 mV within 2 min of tBH exposure, then returned to a level that was 30 mV more oxidized than baseline within 1 h. Cytosolic Trx1 was oxidized by only 30 mV, but remained more than 20 mV oxidized throughout the course of the experiment (Fig. 3).

The cellular GSH/GSSG E_h was −260 ± 3 mV in untreated cells, and was oxidized by 70 mV (to −190 ± 18 mV) after 2 min tBH exposure (Fig. 3). Even 2 h after tBH treatment,

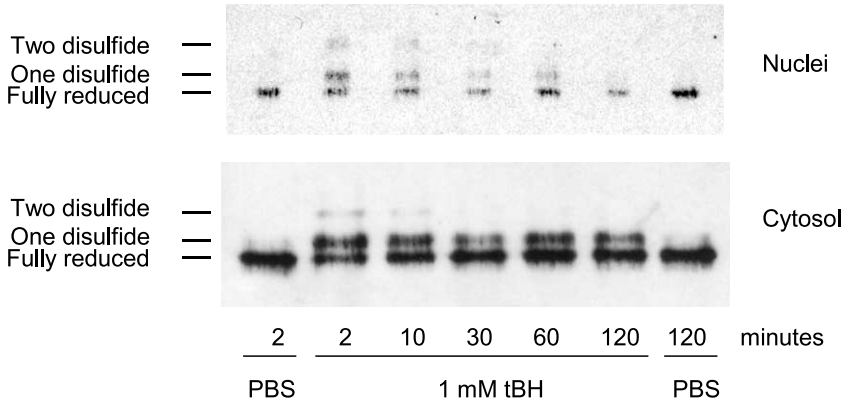

Fig. 2. Time course of oxidation of Trx1 from nuclear and cytosolic fractions. Cells were treated with 1 mM tBH for the indicated times, then nuclear and cytosolic proteins were isolated and analyzed by Redox Western blot as described in Section 2. Shown is a representative blot.

the GSH/GSSG couple was still oxidized by 35 mV. Although the extent of oxidation of the GSH pool was greater, the oxidation and subsequent reduction of all three redox pools (i.e. nuclear Trx1, cytosolic Trx1, and cellular GSH/GSSG) followed similar time courses.

3.4. Trx1 redistributes to the nucleus following tBH exposure

Trx1 redistributes to the nucleus in response to many stimuli, including UV irradiation, phorbol esters, TNF, and hydrogen peroxide [9,24]. Because the Redox Western data contained multiple bands, these data did not clearly reveal such an increase in response to tBH. Therefore, we performed Western blot analyses to determine whether tBH also caused an increase in Trx1 in the nucleus. The results showed that the amount of Trx1 in the nucleus doubled 2 min after tBH, and was gradually lost over the next 2 h (Fig. 4). Thus, the results show that oxidation of Trx1 is associated with an accumulation of Trx1 in the nucleus.

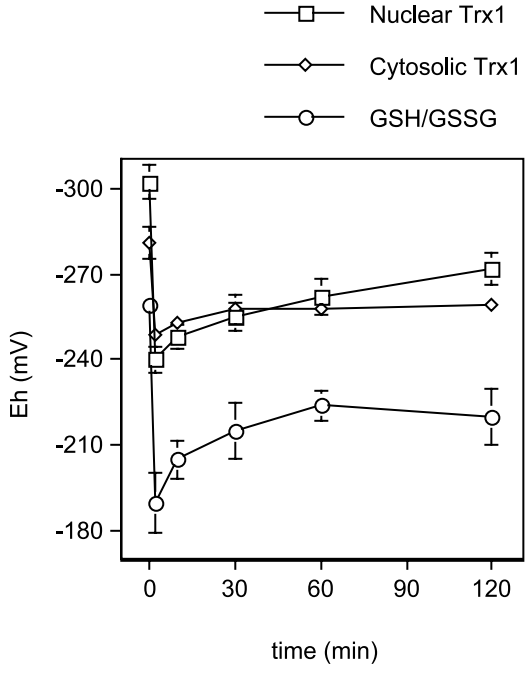

Fig. 3. Comparison of the redox states (E_h values) of cellular GSH, nuclear Trx1, and cytosolic Trx1 in tBH-treated cells. Cells were treated with 1 mM tBH for the indicated times, and the redox states of nuclear Trx1, cytosolic Trx1, and cellular GSH were determined as described in Section 2. Values represent the mean ± S.E.M. of three separate experiments.

4. Discussion

The intracellular environment is normally maintained in a relatively reduced state [2]. In the present report we show that the GSH/GSSG couple, the predominant redox buffering system, is −260 mV in THP1 cells under normal culture conditions. This value is very similar to those reported for other cell lines under proliferating conditions [20,25,26]. In the present report, tBH treatment resulted in a 70 mV oxidation of the GSH/GSSG couple (see Fig. 3).

Escherichia coli Trx has been estimated to be about 40% oxidized [18] to 70% oxidized [27]. In bovine endothelial cells, Trx1 was completely reduced, and was only slightly oxidized upon exposure of the cells to 8 mM H_2O_2 [19]. The structure of Trx is highly conserved among diverse species, but mammalian Trx1 contains three Cys residues in addition to the two-active-site Cys [28]. We found that Trx1 from human monocytes was about 90% reduced, and that 1 mM tBH caused a substantial oxidation of Trx1. The different response to peroxides noted between bovine endothelial cells [19] and

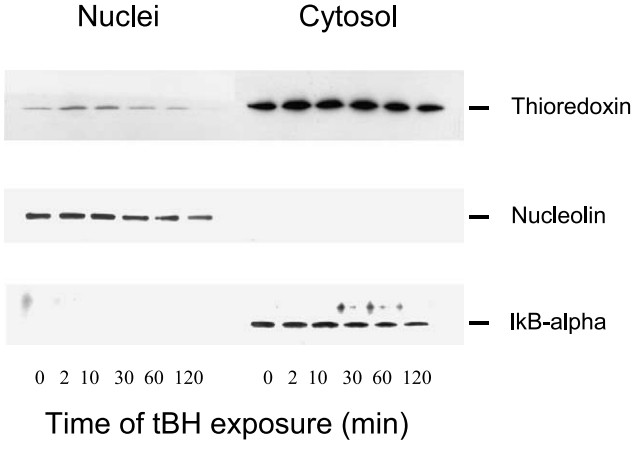

Fig. 4. Nuclear Trx1 is transiently increased following exposure to tBH. Cells were treated with 1 mM tBH for the indicated times, then nuclear and cytosolic proteins were isolated and analyzed by Western blot as described in Section 2.

human monocytes (Fig. 1) may be due to differences in cell type or assay conditions, or differences between the responses to tBH and H_2O_2. Recently, we reported that the redox state of the total cellular pool of Trx1 in Caco-2 cells [25] was 90–95% reduced, corresponding to an E_h value of about −280 mV. Thus, the redox states of nuclear and cytosolic Trx1 in proliferating control cells are comparable to those reported for Trx1 and GSH in other mammalian systems, and are considerably more reduced than those reported for bacterial Trx.

A transient increase in the amount of Trx1 occurred in the nucleus following tBH exposure (Fig. 4). Nuclear translocation of Trx1 also occurs in response to H_2O_2, TNF, phorbol esters, and UV light [9,24]. In principle, either stimulation of import or inhibition of export from the nucleus can result in the accumulation of a protein in the nucleus. There are no recognizable nuclear localization or nuclear export sequences in Trx1 [5], so it is unclear how the subcellular distribution of Trx1 is maintained.

Accumulation of Trx1 in the nucleus in response to oxidative stress could provide the necessary reducing equivalents to allow maximal expression of stress response genes under the control of redox-sensitive transcription factors like NF-κB and AP1. In the experiments described here, a decrease in amount of reduced Trx1 in the nucleus occurred immediately following tBH exposure (compare lanes 1 and 2 in Fig. 2). This corresponded to a 60 mV decrease in the overall reducing capacity of the Trx1 in the nucleus (i.e. a 100-fold increase in the ratio of the oxidized to reduced forms of Trx1). Thus, despite the observed increase in the total amount of Trx1 in the nucleus following tBH (see Fig. 4), there was actually a decrease in the reducing capacity of the nucleus, suggesting that redox-sensitive transcription factors were not maximally active under these conditions. NF-κB mediates the expression of genes that afford protection against oxidative stress [29], but a toxic concentration of tBH (1 mM) was used in these studies; under these conditions, one would not expect to see a maximal protective effect.

Trx1 and GSH were oxidized to similar extents and over similar time courses following treatment with 1 mM tBH (Fig. 3). This response to oxidative stress differs from redox changes induced by changes in cell growth. GSH became more oxidized in Caco-2 colon carcinoma cells undergoing spontaneous differentiation, but the redox state of Trx1 did not change in this model [25]. Thus, although the redox states of GSH and Trx1 are regulated independently under physiologic conditions, both of these thiol/disulfide pools respond similarly to an acute oxidative stress. The observed changes in GSH and Trx1 redox may reflect their role in the detoxification of the peroxide, or may simply reflect the change in the overall redox environment within the cell. In either case, the redox state of Trx1 seems to be, like GSH redox, a useful marker of oxidative stress. Furthermore, the ability to isolate the nuclear pool of Trx1 provides a means to specifically assess oxidative stress in the nuclear compartment.

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