Oxidation of thioredoxin reductase in HeLa cells stimulated with tumor necrosis factor-α

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Abstract Stimulation of cells with tumor necrosis factor-α (TNF-α) results in the increase in generation of H_2O_2 in mitochondria that leads to apoptosis. The effect of H_2O_2 produced by TNF-α on the redox status of selenocysteine (SeCys) residue essential for mitochondrial thioredoxin reductase (TrxR2) was investigated in HeLa cells. TNF-α caused accumulation of oxidized TrxR2 with a thioselenide bond. The conditional induction of SeCys-deficient TrxR2 resulted in the increased production of H_2O_2 and apoptosis. These results suggest that the SeCys residue of TrxR2 plays a critical role in cell survival by serving as an electron donor for Trx-II and subsequent peroxiredoxin-III, which is a primary line of defense against H_2O_2 in mitochondria.

Keywords: Thioredoxin reductase; Selenocysteine; Reactive oxygen species; Apoptosis

1. Introduction

Treatment of cells with the cytokine, tumor necrosis factor-α (TNF-α) induces the increase in intracellular concentration of reactive oxygen species (ROS) such as H_2O_2 and O_2^-. ROS are generated at multiple sites within the cells by mitochondria in various cell lines [1–4]. In addition, ROS generation by NADPH oxidase in endothelial cells [5] and by 5-lipoxygenase in rat-2 fibroblasts [6] has been reported. Induction or expression of enzymes that can scavenge ROS such as mitochondrial manganous superoxide dismutase [7,8], phospholipid hydroperoxide glutathione peroxidase [9], catalase [10] and peroxiredoxin (Prx) [11] render the cells resistant to cell death, indicating that ROS are required for the cytotoxicity of TNF-α.

Recent evidences indicate that ROS play as second messengers regulating or participating in the signaling pathway leading to cell death. ROS-mediated signaling is thought to be possible by reversible oxidation of thiolate anion of reactive cysteine (Cys) residues or selenolate anion of selenocysteine (SeCys) residues in various proteins [12–14]. The redox modification of reactive Cys residues of ROS target proteins can be regulated by cellular redox systems such as thioredoxin (Trx) system composed of Trx, Trx reductase (TrxR), NADPH; glutathione system composed of glutathione, glutaredoxin, glutathione reductase and NADPH [15]. Reduced Trx, which catalyzes the reduction of disulfides of proteins, exists in distinct isoforms, cytosolic Trx-I and mitochondrial Trx-II [16]. The decrease in the concentration of reduced Trx-I was previously suggested to serve as a signal to trigger apoptosis by derepressing the activity of apoptosis signal-regulating kinase 1 (ASK1) [17,18]. Mitochondrial Trx-II are synthesized with a mitochondrial leader sequence (MLS) and localized to mitochondria. Overexpression of Trx-II renders human cells resistant to oxidant-induced apoptosis [16,19], and the deletion of Trx-II causes massive apoptosis and early embryonic lethality in mice [20]. These results show that both Trx-I and Trx-II play essential roles in cell-survival and protecting cells from ROS mediated apoptosis.

Oxidized Trx is reduced by NADPH through the catalytic activity of TrxR [21]. Three isozymes of mammalian TrxR are localized in cytoplasm (TrxR1), mitochondria (TrxR2) and microsomes (TGR), respectively [22–24]. TrxRs are homodimeric flavoproteins that contain a penultimate SeCys residue at the COOH-terminus, which is a critical redox center for enzyme activity [25–28]. The selenol of the SeCys residue is expected to be ionized to selenolate anion at physiological pH because the pK_a value of the selenol is 5.4 [29]. The selenolate anion is very susceptible to oxidation by H_2O_2. Therefore, the SeCys residue can act as a cellular redox sensor sensing H_2O_2 produced in cells which are exposed to the ROS-inducing

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Abbreviations: Trx, thioredoxin; TrxR, Trx reductase; Prx, peroxiredoxin; Cys, cysteine; SeCys, selenocysteine; IAM, iodoacetamide; BIAM, biotin-conjugated IAM; CAM, carboxamidomethyl; (B)CAM, biotinyl CAM; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; LC-Mass, mass spectrometry coupled with HPLC; mu, mass unit; TNF-α, tumor necrosis factor-α; ROS, reactive oxygen species

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factors such as epidermal growth factor [13,30]. Recently, it was shown that incorporation of SeCys residue deficient TrxR1 into human cells rapidly induced cell death [31]. From these observations, it is expected that the selenolate anion of SeCys residue of TrxRs is the primary target of ROS produced by TNF-α and that oxidation of the SeCys residue subsequently accelerates ROS mediated apoptosis.

Here, using cytosolic TrxR1 purified from rat liver, we have unambiguously shown that the SeCys residue forms a thioselenide with an adjacent Cys residue upon oxidation by H_2O_2. We have also shown that a significant portion of TrxR1 and TrxR2 accumulated in the oxidized form in HeLa cells when treated with TNF-α. We also observed that induction of SeCys residue-deficient TrxR2 dominant negative mutant induced the increase in intracellular concentration of H_2O_2 and the increase of cell death in TNF-α treated cells.

2. Materials and methods

2.1. Materials

TrxR1 was purified from rat liver as described [23,32]. Rabbit antisera to TrxR1 and TrxR2 were produced by standard procedures. The monospecifc anti-TrxR1 antibodies were prepared from the serum with the use of purified TrxR1 absorbed to a nitrocellulose membrane, and anti-TrxR2 antibodies were isolated from the serum by protein G–agarose column chromatography. Horseradish peroxidase (HRP)-conjugated antibody to rabbit IgG was obtained from Amersham Pharmacia and biotin-conjugated iodoacetamide (BIAM) was from Molecular Probes Inc., Eugene, OR.

2.2. Determination of protein concentration

The concentration of TrxR1 subunit was determined spectrophotometrically with ε_{463} of 11 300 M^{-1} cm^{-1} subunit as described [33].

2.3. Preparation of reduced TrxR (EH_4)

Purified TrxR1 was incubated for 30 min in 1 × phosphate-buffered saline (PBS) (pH 7.4) containing 1 mM EDTA and 200 μM NADPH. The reduced TrxR1 (EH_4) was then dialyzed against the same buffer devoid of NADPH in an anaerobic chamber.

2.4. High performance liquid chromatography (HPLC)/mass spectrometry (LC/Mass)

Electrospray mass spectroscopy was performed on a Hewlett–Packard model G1946A instrument interfaced to a Hewlett–Packard model 1100 HPLC system equipped with a Vydac 218TP5205 narrowbore C_{18} column (Vydac, Hesperia, CA). Peptide mixtures were fractionated into both spectrophotometer and mass spectrometer using a linear gradient (0–60%, v/v) of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.2 ml/min over 60 min. The effluent from the spectrophotometric detector was mixed in a T-shaped device with 100 μl/min acetic acid pumped by another model 1100 pump, and the mixture was introduced into the mass spectrometer [34]. The capillary voltage was 4500 V, and the fragmentor was programmed to ramp from 50 V at 50 mass units (mu), 80 V at 1500 mu, and 140 V at 2500 mu. Data were collected from 550 to 2000 mu.

2.5. Immunoprecipitation of BIAM-labeled TrxR 1 and TrxR2 in HeLa cell extracts

HeLa cells, cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 units/ml), and 0.5 μM sodium selenite, were allowed to reach 60–70% confluence in 100-mm dishes. The cells were deprived of serum for 16 h, and then cultured in DMEM supplemented with 1% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 units/ml) and 0.5 M sodium selenite. After treatment with cycloheximide (5 μg/ml) for 1 h, the cells were stimulated with TNF-α (25 ng/ml). The cells were rinsed and then exposed to 1 ml of oxygen-free lysis buffer [50 mM Bis-Tris–HCl (pH 6.5), 0.5% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, leupeptin (1 μg/ml), aprotinin (1 μg/ml), and 0.1 mM AEBSF] containing 20 μM BIAM. As a control, cells that were not stimulated with TNF-α were likewise lysed and labeled. After incubation for 10 min at 37 °C in the dark, the labeling reaction was stopped by adding IAM to 5 mM. TrxR1 and TrxR2 in the reaction mixtures were precipitated with the use of rabbit antibodies to TrxR1 (7 μg per mixture), TrxR2 (10 μg per mixture) and Protein G–Sepharose (15 μl of 50% slurry per mixture).

2.6. Cell viability assay

KL1 and KL2 stable HeLa cell lines expressing a dominant negative form of TrxR2 (TrxR2DN) under the control of the tetracyclin-off (TO) system were generated as described [35]. HeLa-TO/TrxR2DN cells were plated at 1×10^5 cells/well in 6-well culture plates and cultured for 60 h in the presence or absence of 1 μg/ml doxicycline in the DMEM containing 10% Tet system approved FBS, 1% penicillin, and 1% streptomycin. The cells were deprived of serum for 16 h, and then cultured in DMEM supplemented with 1% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 units/ml) and 0.5 μM sodium selenite. After treatment with cycloheximide (5 μg/ml) for 1 h, the cells were stimulated with TNF-α (25 ng/ml). After incubation for 8 h, cell viability was determined by flow cytometric analysis with propidium iodide as described [36].

2.7. Detection of intracellular H_2O_2 by flow cytometric analysis

Intracellular levels of H_2O_2 were analyzed by flow cytometry using dihydrorhodamine 123 (Molecular Probes, Eugene, OR) as a specific fluorescent dye probe [37,38]. Dihydrorhodamine 123 is oxidized to membrane-impermeable, fluorescent rhodamine 123 in the presence of H_2O_2.

After serum deprivation, TrxR2DN-induced or -uninduced HeLa cells were incubated in DMEM containing 10 μM dihydrorhodamine 123 for 30 min and washed two times with DMEM. Cells were treated with 10 ng/ml TNF-α for 30 min followed by 2 μg/ml catalase to remove the extracellular H_2O_2. After harvesting by trypsin–EDTA treatment, cells were fixed in 1% paraformaldehyde. The intracellular rhodamine 123 fluorescence intensity of 10 000 cells was measured for each sample using a Becton–Dickinson FACS Caliber flow cytometer.

2.8. Cytoplasmic DNA preparation

TNF-α, UV or etoposide treated cells were harvested and lysed in 200 μl of DNA isolation buffer (5 mM Tris–HCl, pH 7.4, 20 mM EDTA, 0.5% Triton X-100 and 1 mM PMSF). After centrifugation at 12 000 × *g* for 20 min at 4 °C, the supernatants were transferred to new tubes. Protein concentration was measured using a BCA protein assay kit. Solutions containing equal amount of proteins were extracted with equal volumes of tris-saturated phenol, phenol/chloroform and chloroform, and then DNAs were precipitated with the equal volume of isopropanol. Precipitated DNAs were dissolved in 10 μl TE (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA) containing RNAse (1 mg/ml) and separated in a 2% agarose gel containing ethidium bromide.

3. Results

3.1. Selective alkylation and identification of SeCys as a redox sensitive site

Cytosolic TrxR (also called TrxR1 to distinguish it from the mitochondrial TrxR2 and microsomal TGR) purified from rat liver was treated with excess amounts of NADPH and then dialyzed in an anaerobic chamber to remove NADPH. As reported previously, the NADPH-reduced enzyme exhibited a long wavelength absorbance band at 540 nm that is attributable to the charge transfer complex between FAD and the thiolate anion of $Cys^{64}S^-$ [33]. Upon incubation with H_2O_2, the absorbance at 540 nm decreased in a time dependent manner. Furthermore, the absorbance at 540 nm reappeared when NADPH was added to the H_2O_2-oxidized enzyme. As shown below, the NADPH-reduced enzyme contains the FAD-thiolate charge transfer complex, the reduced active site Cys pair of $Cys^{59}SH$ and $Cys^{64}SH$, and the reduced COOH-terminal pair of $Cys^{497}SH$ and $SeCys^{498}SeH$. Thus, the

NADPH-reduced enzyme is denoted as $E(FAD)(SH)_2(SH–SeH)$, or EH_4. Likewise, the fully reduced enzyme is denoted as $E(FADH_2)(SH)_2(SH–SeH)$, or EH_6, and the fully oxidized enzyme is denoted as $E(FAD)(S_2)(S–Se)$, or E_0. Previously, we have shown that the SeCys residue of TrxR2 can be alkylated selectively with a BIAM [23]. BIAM also specifically modifies the SeCys residue in the reduced TrxR1 at pH 7.4 as described [25]. The selective BIAM labeling procedure was used as a probe to monitor the oxidation state of SeCys in TrxR. EH_4 was oxidized with H_2O_2 ($E–H_2O_2$) and then reduced with NADPH ($E–H_2O_2–NADPH$). The resulting enzymes were then labeled with BIAM, separated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose membrane, and detected by streptavidin blot with HRP-conjugated streptavidin and ECL. Control EH_4 was labeled strongly, whereas no biotinylation was detectable with $E–H_2O_2$. Importantly, the labeling intensities of $E–H_2O_2–NADPH$ were similar to that of control EH_4 (Fig. 1). These results suggest that the SeCys residue in the H_2O_2-treated enzyme could not be labeled because it was oxidized and that the residue could be labeled again when reduced by NADPH.

3.2. Identification of a thioselenide in oxidized TrxR1

To characterize the chemical nature of the oxidized SeCys residue, $E–H_2O_2$ and $E–H_2O_2–NADPH$ were prepared from EH_4 as described in Fig. 1. The resulting enzymes were then labeled with BIAM followed by IAM and cleaved with Lys-C, and finally, the digested peptide mixtures were analyzed by LC/MS. The total ion chromatogram included peaks with a mass of 1297.3 Da (retention time, 20.5 min) and of 1682.3 Da (20.9 min), which are essentially identical, respectively, to the 1297.3 Da expected for the COOH-terminal fragment R^{487}SGGDILQSGCUG^{499} containing Cys^{497} and $SeCys^{498}$ (denoted by one-letter codon "U") bridged by a thioselenide bond (S–Se peptide), and to the 1682.4 Da expected for the same COOH-terminal fragment containing IAM-Cys^{497} and BIAM-$SeCys^{498}$ (SIAM/SeBIAM peptide) (Fig. 2A). Edman degradation of the 20.5-min peptide yielded a complete sequence of RSGGDILQSG. Together with the mass data, the sequencing result conclusively proves that the 20.5-min peptide is indeed the S–Se peptide. As described in the previous section, the 20.9-min peptide was further purified on a Neutravidin affinity column and sequenced to confirm its identity as the SIAM/SeBIAM peptide. The total ion chromatograms also included peaks of 3128.8 Da (retention time, 37.0 min) and 3244.9 Da (36.0 min), which are identical, respectively, to the mass predicted for the Lys-C fragment of residues 38–67, V^{38}MVLDFVTPTPLGTRWGLGGTCVNVGCIPK^{67}, which contain Cys^{59} and Cys^{64} bridged by a disulfide (S–S peptide), and to that predicted for the same 30-residue fragment containing IAM-Cys^{59} and IAM-Cys^{64} (SIAM/SIAM peptide) (Fig. 2B). Edman degradation of the 36-min peptide yielded a complete sequence of V^{38}MVLDFVTPTPLGTRWGLGGT-CVNVGCIPK^{67}. Sequencing of the 37.0-min peptide yielded an 18 residue sequence of VMVLDFVTPTPLGTRWGL.

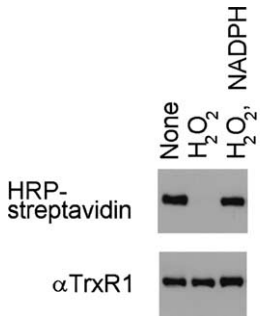

Fig. 1. Effect of exposure of NADPH-reduced TrxR1 to H_2O_2 on labeling with BIAM. Purified rat liver TrxR1 was reduced by NADPH and then dialyzed to remove NADPH and $NADP^+$ in an anaerobic chamber. The reduced TrxR1 was incubated for 10 min at room temperature with 200 μM H_2O_2 to produce H_2O_2-oxidized enzyme. The oxidation reaction by H_2O_2 was stopped by the addition of 1 μg of catalase (65 units/μg). One half of the oxidized enzyme sample was incubated for 20 min at room temperature with 250 μM NADPH to produce H_2O_2–NADPH-reduced enzyme. An aliquot (5 μg) of each sample was incubated for 10 min with 50 μM BIAM. The labeling reaction with BIAM was stopped by the addition of 10 mM IAM. The samples were subjected to SDS–PAGE on a 10% gel and then transferred to a nitrocellulose membrane. The biotinyl carboxamidomethyl [(B)CAM] labeled proteins were detected by streptavidin blotting with HRP-conjugated streptavidin and enhanced chemiluminence detection. Equal application of protein among gel lanes was confirmed by immunoblot analysis with antibodies to TrxR1.

The mass chromatograms of the S–Se peptide and the SIAM/SeBIAM peptide are shown in Fig. 2A, and those for the S–S peptide and the SIAM/SIAM peptide are shown in Fig. 2B. Peaks for the S–Se and S–S peptides were observed with oxidized enzymes, $E–H_2O_2$, whereas peaks for the SIAM/SeBIAM and SIAM/SIAM peptides were observed with NADPH-treated enzymes, $E–H_2O_2–NADPH$. These results suggest that upon oxidation with H_2O_2, the Cys^{59} and Cys^{64} pair and the COOH-terminal Cys^{497} and $SeCys^{498}$ pair form a disulfide and a thioselenide linkage, respectively, and that both of these linkages are readily reduced by NADPH.

To more accurately evaluate the ratio of oxidized to reduced Se-containing peptide, we assayed for Se by atomic absorption spectrometry in Lys-C fragments after separation by HPLC. Because the S–Se and SIAM/SeBIAM peptides were not well separated by HPLC, we labeled $E–H_2O_2$ and $E–H_2O_2–NADPH$ directly with IAM, omitting the BIAM step before digestion with Lys-C, and analyzed with LC/MS. As expected, the S–Se peptide came out as a peak eluted at 20.5 min with a mass value of 1297.4, whereas no Se-containing peak was detected at 20.9 min (Fig. 2C). Instead, another Se-containing peak eluted at 17.5 min, which was identified as a COOH-terminal fragment containing IAM-Cys and IAM-SeCys (SIAM/SeIAM peptide) based on amino acid sequencing and mass analysis (observed mass, 1413.8; calculated mass, 1413.4) results. Furthermore, when the 20.5-min peptide was treated with dithiothreitol (DTT), labeled with IAM, and then subjected to HPLC analysis, the 20.5-min peak was completely converted to the 17.5-min peak (Fig. 2D). These results further support the notion that the 20.5-min peak represents the S–Se peptide and that the 17.5-min peak represents the SIAM/SeIAM peptide. Importantly, the 17.5- and 20.5-min fractions are the only fractions containing a detectable amount of Se.

3.3. TNF-α-induced oxidation of TrxR 1 and TrxR2 in HeLa cells

A variety of stress signals including TNF-α, Fas ligand, UV light, heat shock, and serum withdrawal induce apoptosis, with ROS serving as known mediators of the death signaling [12,14]. A characteristic of oxidatively stressed, apoptosing cells is a decrease in cellular reductants, including $Trx(SH)_2$. Recently, the decrease in the concentration of $Trx(SH)_2$ was

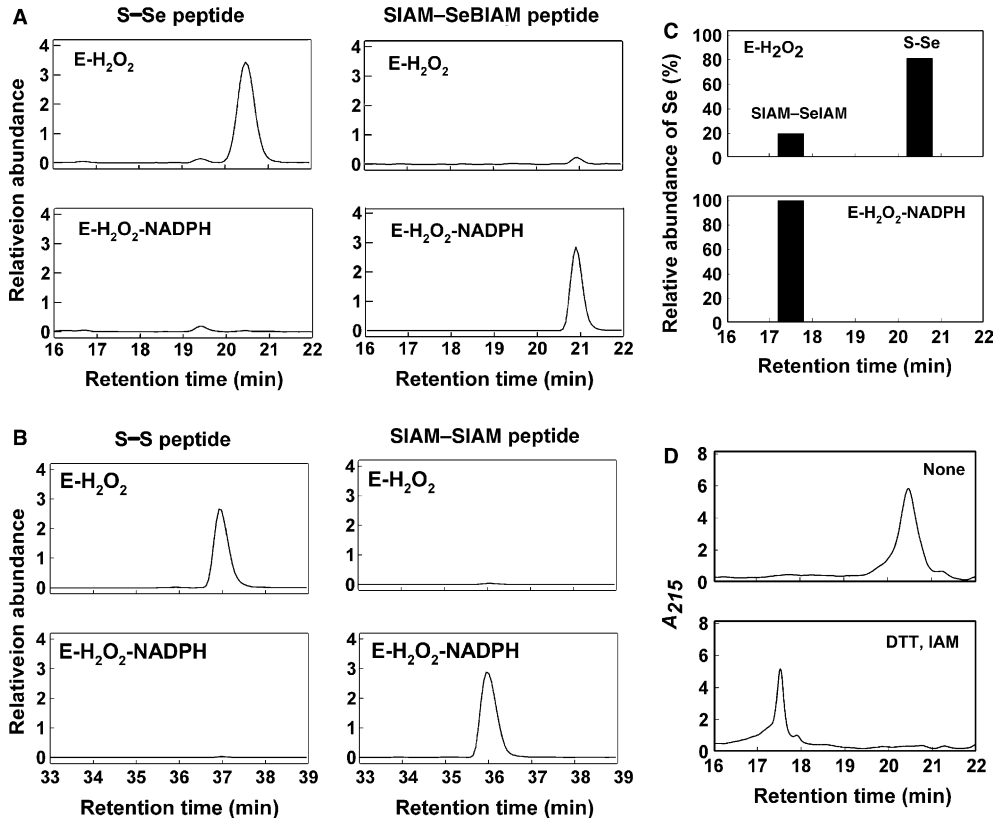

Fig. 2. Identification of the Cys^{497}/$SeCys^{498}$ thioselenide and the Cys^{59} and Cys^{64} disulfide in the H_2O_2-oxidized TrxR1 and H_2O_2–NADPH-reduced TrxR1. A and B, H_2O_2-oxidized and H_2O_2–NADPH-reduced enzyme samples were prepared from reduced enzyme as described in Fig. 1. The resulting enzymes (10 μg) were labeled with 50 μM BIAM for 20 min in oxygen-free PBS buffer (pH 7.2) containing 1 mM EDTA and then with 1 mM IAM for 1 min in 50 mM Tris–HCl buffer (pH 8.8) containing 6 M guanidine–HCl. After adjustment to pH 5.2 by the addition of 3 M sodium acetate buffer, the enzyme samples were dialyzed for 4 h against 10 mM sodium acetate buffer (pH 5.2) and then for 2 h against 20 mM Tris–HCl buffer (pH 8.0). The dialyzed samples were added to 10% acetonitrile (vol/vol) and then incubated with endoproteinase Lys-C at 37 °C. The resulting peptide mixtures were analyzed by HPLC-MS. (A) Extracted ion chromatograms for $m/z = 649.6$ (1297.3 mu) of the Cys^{497}/$SeCys^{498}$ thioselenide-containing peptide (left) and for $m/z = 842.2$ (1682.3 mu) of the CAM-Cys^{497}/(B)CAM-$SeCys^{498}$-containing peptide (right). (B) Extracted ion chromatograms for $m/z = 1043.9$ (3128.8 mu) of the Cys^{59}/Cys^{64} disulfide-containing peptide (left) and for $m/z = 1082.6$ (3244.9 mu) of the CAM-Cys^{59}/CAM-Cys^{64}-containing peptide (right). (C) Lys-C peptides were fractionated by HPLC on a C_{18} column as in panel A and fractions (0.2 ml per fraction) were collected manually. Each fraction was analyzed for Se with a Perkin–Elmer model 4100 ZL atomic absorption spectrometer as described [25]. (D) The 20.5-min peptide was reinjected to the same HPLC column (upper panel) with or without treatment with DTT followed by labeling with IAM (lower panel).

suggested to serve as a signal to trigger apoptosis by derepressing the activity of apoptosis signal-regulating kinase 1 (ASK1). This was based on the observation that $Trx(SH)_2$, but not $Trx(S)_2$, is a potent inhibitor of ASK1. Activation of ASK1 and the subsequent activation of the JNK and p38 MAP kinase pathways are required for TNF-α induced apoptosis [17,18]. As such, a compromised conversion of $Trx(S)_2$ to $Trx(SH)_2$ due to the accumulation of oxidized TrxR can be a cause of apoptosis. We investigated whether ROS generated by TNF-α are sufficient to cause the accumulation of oxidized TrxR1 and TrxR2. HeLa cells were pretreated with cyclohexamide, a protein synthesis inhibitor, and then incubated for various times with TNF-α. The cells were lysed in the buffer containing BIAM at pH 6.5. TrxR1 and TrxR2 were precipitated from the cell lysate with antibodies specific to TrxR1 and TrxR2 and then subjected to streptavidin blot analysis as in Fig. 1. BIAM-labeled TrxR1 decreased with time of incubation of the cells with TNF-α. The extent of the decrease in the labeling intensity after stimulation of cells for 10 min, 30 min, 1 h, 6 h and 12 h was 3%, 2%, 10%, 39%, and 40%, respectively (Fig. 3A). BIAM-labeled TrxR2 more rapidly decreased with time of incubation of the cells with TNF-α compared to BIAM-labeled TrxR1. The extent of the decrease in the labeling intensity of TrxR2 was 12%, 19%, 27%, 50%, and 72%, respectively (Fig. 4A). The oxidation of TrxR1 and TrxR2 was reversible, as TrxR1 and TrxR2 derived from TNF-α-treated cells can be fully labeled by BIAM after incubation of the cell lysates with NADPH (Figs. 3B and 4B).

3.4. Effect of TrxR2DN expression on the intracellular concentration of H_2O_2 in TNF-α-stimulated cells

Stimulation of HeLa cells with TNF-α results in a transient increase in the intracellular concentration of H_2O_2 generated from mitochondria. Mitochondrial Prx-III and mitochondrial glutathione peroxidase (mGPx) are responsible for removing of H_2O_2. Prx-III and mGPx can be provided of necessary electrons from NADPH through mitochondrial TrxR2. Therefore, the oxidation of TrxR2 by H_2O_2 might enhance the accumulation of H_2O_2 within the cells when treated with TNF-α. We investigated the effect of oxidation of TrxR2 on the production of H_2O_2 by expressing a dominant negative form of TrxR2 that has the C-terminal SeCys and Gly residues

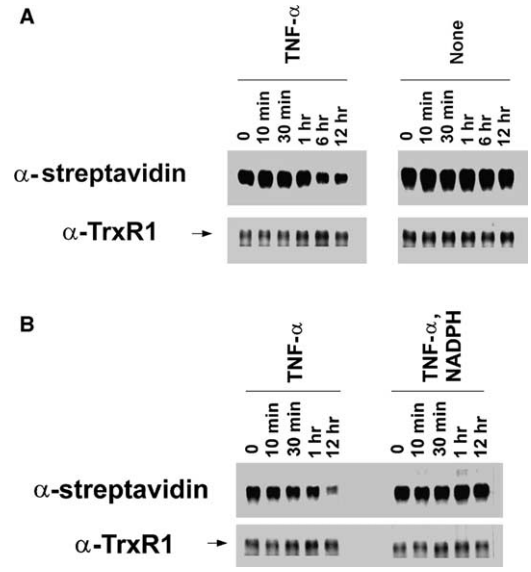

Fig. 3. TNF-α induced oxidation of TrxR1 in HeLa cells. (A) TNF-α induced oxidation of TrxR1. HeLa cells were incubated with or without treatment with TNF-α (20 ng/ml) for the indicated times. The cells were lysed in a buffer containing 20 μM BIAM. The lysates were incubated for 20 min and then treated with 1 mM IAM. (B) Reduction of oxidized TrxR1 by NADPH. Two sets of cells were treated with TNF-α (20 ng/ml) for the indicated times. One set of cells treated with TNF-α was lysed in a buffer containing 20 μM BIAM. The lysate was incubated for 20 min and then treated with 1 mM IAM. The other set of cells treated with TNF-α was lysed in a buffer containing 200 μM NADPH. The lysate was incubated for 10 min and then incubated with 20 μM BIAM for 20 min and then treated with 1 mM IAM. TrxR1 was precipitated from the cell lysate with polyclonal antibodies and subjected to SDS–PAGE on a 10% gel. The separated proteins were transferred to a nitrocellulose membrane. The menbrane was subjected to blot analysis with HRP-conjugated streptavidin (upper panel) or with antibodies to TrxR1 (lower panel). Representative data from three independent experiments are shown.

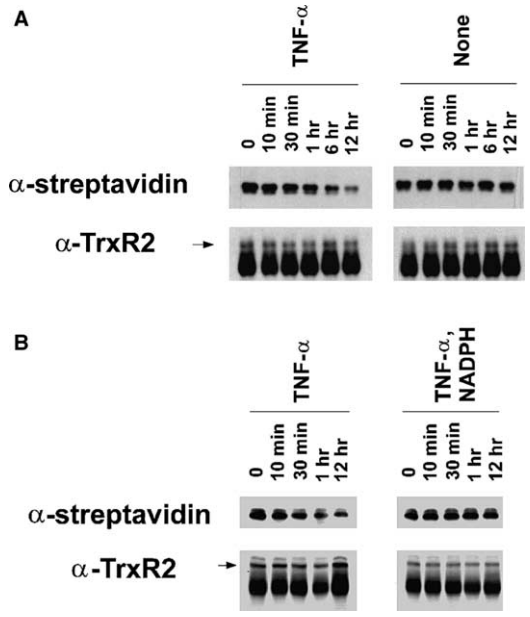

Fig. 4. TNF-α induced oxidation of TrxR2 in HeLa cells. (A) TNF-α induced oxidation of TrxR2. HeLa cells were incubated with or without treatment with TNF-α (20 ng/ml) for the indicated times. The cells were lysed in a buffer containing 20 μM BIAM. The lysates were incubated for 20 min and then treated with 1 mM IAM. (B) Reduction of oxidized TrxR2 by NADPH. Two sets of cells were treated with TNF-α (20 ng/ml) for the indicated times. One set of cells treated with TNF-α was lysed in a buffer containing 20 μM BIAM. The lysate was incubated for 20 min and then treated with 1 mM IAM. The other set of cells treated with TNF-α was lysed in a buffer containing 200 μM NADPH. The lysate was incubated for 10 min and then incubated with 20 μM BIAM for 20 min and then treated with 1 mM IAM. TrxR2 was precipitated from the cell lysate with polyclonal antibodies and subjected to SDS–PAGE on a 10% gel. The separated proteins were transferred to a nitrocellulose membrane. The menbrane was subjected to blot analysis with HRP-conjugated streptavidin (upper panel) or with antibodies to TrxR2 (lower panel). Representative data from three independent experiments are shown.

truncated as described [35]. We monitored the intracellular H_2O_2 level using an oxidation-sensitive fluorescence probe dihydrorhodamine 123 and flow cytometry. As shown in Fig. 5A, exposure of both TrxR2DN-induced and -uninduced cells to TNF-α resulted in an increase in relative fluorescent intensity. The relative fluorescent intensity of induced cells was significantly higher than that of uninduced cells ($P < 0.01$, Fig. 5B). These results suggest that the interference of TrxR2 function by the expression of TrxR2DN might cause accumulation of H_2O_2 by compromising the activity of the mitochondrial Trx2-dependent peroxidase Prx-III.

3.5. Effect of TrxR2DN induction on apoptosis

Recently, it was shown that introduction of the SeCys-deficient inactive TrxR1 into A549 human lung carcinoma cells induces rapid cell death [31]. To determine whether or not the TrxR2DN induction also affects cell death, TrxR2DN-induced and -uninduced cells were treated with TNF-α, etoposide, or UV irradiation and then cytoplasmic DNA fragmentation was examined. TrxR2DN-induced cells showed a higher degree of cytoplasmic DNA fragmentation by treatment of TNF-α, etoposide, and UV irradiation compared to the uninduced cells (Fig. 6). After incubation of TrxR2DN-induced or -uninduced cells with TNF-α, cell death was also monitored by FACS analysis. As expected, the induction of TrxR2DN increased TNF-α-induced cell death (Fig. 7).

4. Discussion

In this study, we have provided evidence that upon oxidation with H_2O_2, NADPH-reduced TrxR1 [termed E(FAD)$(SH)_2$(SH–SeH or EH4)] forms a thioselenide linkage between the COOH-terminal Cys^{497} and $SeCys^{498}$ pair, in addition to the disulfide between the Cys^{59} and Cys^{64} pair. The evidences include mass spectral analysis of the COOH-terminal S–Se peptide containing the thioselenide between Cys^{497} and $SeCys^{498}$, the amino acid sequence of the S–Se peptide, and conversion of the S–Se peptide to a peptide containing Cys–SH and SeCys–SeH by DTT. We have also demonstrated the reversible oxidation of the SeCys residue in vitro and in vivo using selective labeling sagent, BIAM as a probe (see Fig. 8).

A growing body of evidence indicates that redox state of cellular thiols modulates various aspects of cellular function, including proliferation and cell death [12,14]. Treatment of HeLa cells with TNF-α induces production of ROS, which are believed to mediate cell death when new protein synthesis is inhibited. Typical apoptotic signs such as chromatin condensation and membrane blebbing are obvious 12 h after treatment of HeLa cells with TNF-α in the presence of cycloheximide. One of the consequences of enhanced oxidative

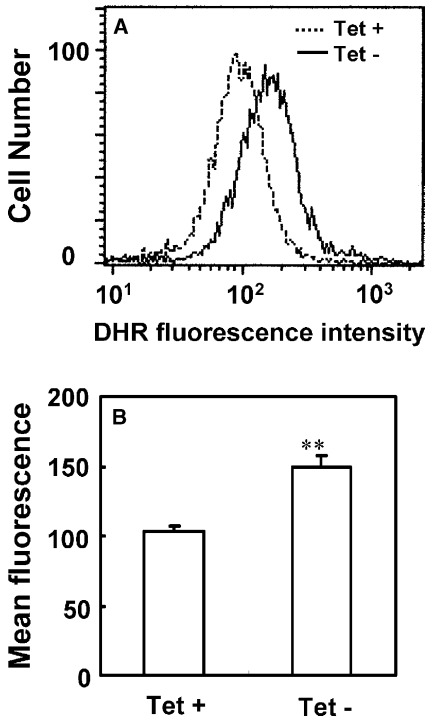

Fig. 5. Intracellular H_2O_2 in TrxR2DN-induced and -uninduced cells treated with TNF-α. KL1 HeLa-TO/TrxR2DN cells were incubated in the presence (+) or absence (−) of tetracycline (1 μg/ml) for 3 days and then starved for 2 days in serum. TrxR2DN-induced or -uninduced and serum-deprived HeLa cells were incubated in DMEM containing 10 μM dihydrorhodamine 123 for 30 min and washed two times with DMEM. Cells were treated with 10 ng/ml TNF-α for 30 min followed by 2 μg/ml catalase to remove the extracellular H_2O_2. After harvesting by trypsin-EDTA treatment, cells were fixed in 1% paraformaldehyde. The intracellular rhodamine 123 fluorescence intensity of 10 000 cells was measured for each sample using a Becton–Dickinson FACS Caliber flow cytometer. (A) The intracellular rhodamine 123 fluorescence intensity in cells; representative data from duplicates of two independent experiments using two different cell clones. (B) Mean fluorescence of the cells. Values are means ± S.D. of duplicates of two independent experiments using two different cell clones. Statistic analysis (students *t*-test, $^{**}P < 0.01$)

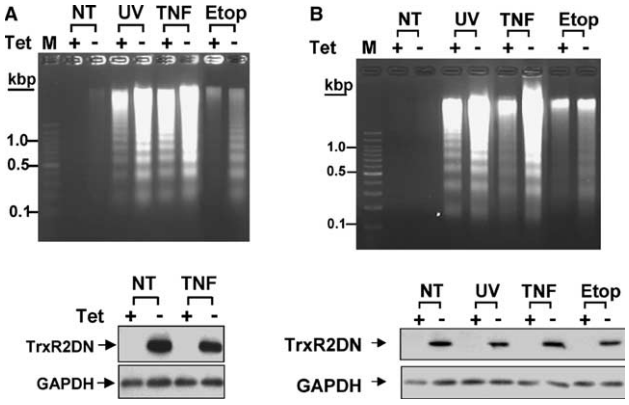

Fig. 6. Effect of TrxR2DN induction on cytoplasmic DNA fragmentation. (A) TrxR2DN-induced and -uninduced KL1 (A) and KL2 (B) cells were starved for 24 h and then treated with UV irradiation (3000 μJ/cm^2), 4 ng/ml TNF-α and 10 μg/ml cycloheximide, or 25 μM etoposide. Cells were harvested at the indicated times, cytoplasmic DNAs were prepared and analyzed by agarose gel electrophoresis. M, 100 bp ladder. Tetracycline-repressible TrxR2DN induction was confirmed by immunoblot analysis with a TrxR2 antibody. TrxR2DN induction and equal loading of proteins were analyzed with TrxR2 and GAPDH antibodies. Representative data from three independent experiments are shown.

stress is the depletion of $Trx(SH)_2$ and a concomitant accumulation of $TrxS_2$, which is directly linked to the activation of ASK1 [17,18], indicating that the shift in redox balance of Trx to $TrxS_2$ in TNF-α-treated cells may be attributed to inefficient TrxR.

Mammalian TrxR exhibits a broad substrate specificity, reducing not only $TrxS_2$ but also many other disulfide-containing proteins as well as smaller compounds like 5,5′-dithiobis(2-nitro benzoic acid) and lipoic acid in the presence of NADPH [39]. The COOH-terminal region of reduced TrxR appears to be well exposed to solution as it can be readily cleaved by proteases and modified by various sulfhydryl reagents [22,25,40,41]. Based on these observations, it was speculated that the Cys–SH/SeCys–SeH pair is the primary site of oxidation by H_2O_2 in the cells. We have shown that the essential SeCys residue of cytosol TrxR1 as well as mitochondrial TrxR2 was apparently oxidized by H_2O_2 generated by TNF-α in HeLa cells.

The oxidation of the SeCys residue might result in inhibition of various antioxidant and anti-apoptotic functions of TrxRs that are required for directly removing H_2O_2 and lipid peroxides [42] and indirectly through Trx peroxidase [43] and glutathione peroxidase [44], reducing disulfide bonds in a variety of proteins and transcription factors [45–47], inhibiting ASK cascade through reduced Trx [17,18], activating antiapoptotic activity of NF-κB through Trx [48], and regulating opening of mitochondrial permeability transition pore [49]. In addition, oxidation of the SeCys residue can change the enzymatic property of TrxR as modification of the Cys^{497} and $SeCys^{498}$ residues with an alkylating agent, dinitrohalobenzene, induces NADPH oxidase activity which produces superoxide anion in the presence of NADPH [50]. Therefore, the negative regulation of TrxRs by H_2O_2 would culminate in the shifting of the balance between ROS generation and detoxification to the oxidative stress and finally in the acceleration of apoptosis (Fig. 8).

Oxidized TrxR1 might act as a death-inducing factor based on the recent report that introduction of the SeCys residue-deficient or SeCys residue-compromised TrxR1 into cells induces rapid cell death [31]. In this study, conditional induction of the SeCys residue-deficient TrxR2 dominant negative mutant induced the increase in the intracellular concentration of H_2O_2 and subsequent cell death following TNF-α treatment. This result indicates that a significant accumulation of oxidized TrxR2 accelerates the TNF-α-induced apoptosis. The mitochondria are the major sources of intracellular superoxide anions, which are readily converted to H_2O_2 by the action of MnSOD when treated with TNF-α [7,8]. Mitochondrial Prx-III and glutathione peroxidase might be primarily responsibe for removing H_2O_2. TNF-α-induced oxidation of TrxR2 is likely to inhibit the peroxidase activity of Prx-III by blocking the transfer of electrons from NADPH to Prx-III through Trx-II, which culminates in increases of H_2O_2.

Recently, it was reported that the sulfenic acid of active site Cys residue in the oxidized Prx-III is subsequently further oxidized to sulfinic acid by H_2O_2 in the cells when treated with TNF-α [51–53]. It appears then that tardy reduction of Trx-II and Prx-III by oxidation of TrxR2 results in the accumulation

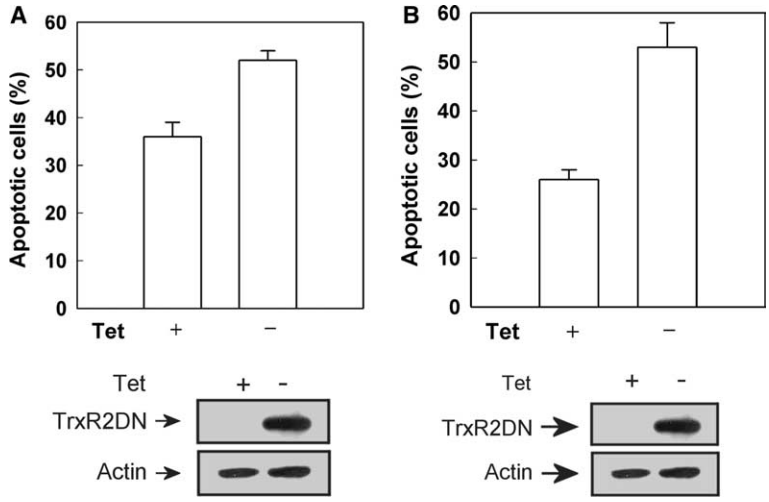

Fig. 7. Effect of TrxR2DN induction on TNF-α-induced cell death. TrxR2DN-induced and -uninduced KL1 (A) and KL2 (B) cells were incubated for 8 h with TNF-α (25 ng/ml). The cells were harvested, stained with propidium iodide and then analyzed with a flowcytometer. TrxR2DN induction and equal loading of proteins were confirmed by western blot analysis with TrxR2 and actin antibodies. Values are mean ± S.D. of triplicates of two independent experiments.

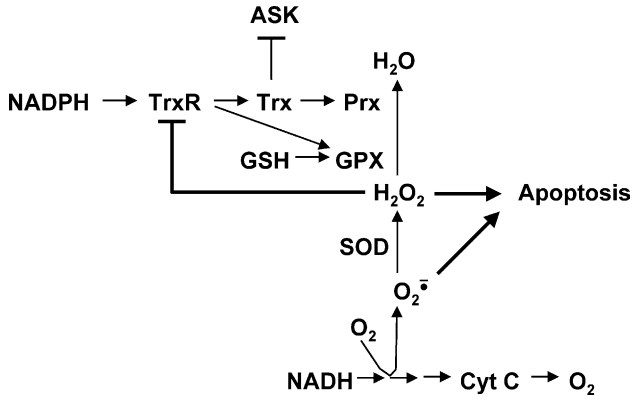

Fig. 8. Roles of reversible oxidation of TrxR by H_2O_2 and NADPH in ROS-mediated apoptosis.

of H_2O_2 and subsequently increases cell death. Consistently, deletion of the mitochondrial TrxR and Trx induces susceptibility to H_2O_2-induced cell death in *S. cerevisiae* [54]. These results suggest that TrxR2 together with Trx-II and Prx-III play a critical role in cell survival by serving as a primary defense line against H_2O_2 produced in mitochondria.

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