Redox-Sensing Release of Human Thioredoxin from T Lymphocytes with Negative Feedback Loops

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Redox-Sensing Release of Human Thioredoxin from T Lymphocytes with Negative Feedback Loops

Norihiko Kondo,‡‡ Yasuuki Ishii,†† Yong-Won Kwon,† Masaki Tanito,† Hiroyuki Horita,† Yumiko Nishinaka,† Hajime Nakamura,‡ and Junji Yodoi*†‡

Thioredoxin (TRX) is released from various types of mammalian cells despite no typical secretory signal sequence. We show here that a redox-active site in TRX is essential for its release from T lymphocytes in response to H2O2 and extracellular TRX regulates its own H2O2-induced release. Human T cell leukemia virus type I-transformed T lymphocytes constitutively release a large amount of TRX. The level of TRX release is augmented upon the addition of H2O2, but suppressed upon the addition of N-acetylcysteine. In the culture supernatant of a Jurkat transfectant expressing the tagged TRX-wild type (WT), the tagged TRX protein is rapidly released and kept at a constant level until 6 h after the addition of H2O2. In contrast, another type of transfectant expressing the tagged TRX mutant (C32S/C35S; CS) fails to release the protein. H2O2-induced release of TRX from the transfectant is inhibited by the presence of rTRX-WT in a dose-dependent manner. Preincubation of the transfectant with rTRX-WT for 1 h at 37°C, but not 0°C, results in a significant suppression of the TRX release, reactive oxygen species, and caspase-3 activity induced by H2O2, respectively. Confocal microscopy and Western blot analysis show that extracellular rTRX-WT added to the culture does not obviously enter T lymphocytes until 24 h. These results collectively suggest that the oxidative stress-induced TRX release from T lymphocytes depends on a redox-sensitive event and may be regulated by negative feedback loops using reactive oxygen species-mediated signal transductions. The Journal of Immunology, 2004, 172: 442-448.

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1 This work was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Abbreviations used in this paper: TRX, thioredoxin; HTLV-I, human T cell leukemia virus type I; NAC, N-acetylcysteine; WT, wild type; ROS, reactive oxygen species; LDH, lactate dehydrogenase; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; PI, propidium iodide.

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a final concentration of 1 mM isopropyl-β-D-thiogalactopyranoside (N-acetyl
Tosque), in the case of the TRX-WT or TRX-CS/p3xFLAG-CMV-10
vector (Sigma-Aldrich), DNA fragments were amplified by PCR using two
kinds of primers, a forward primer (primer 1, 5’Nol) and a reverse primer
(primer 2, 3’BanHi) and were inserted.

**Purification of rTRX-WT or TRX-CS**

6x Histidine-tagged rTRXs were purified from E. coli, which was trans-
fected with pQE80L vectors containing TRX-WT and TRX-CS genes by Ni2+-
chelating column chromatography according to the instruction manual of
the QiAexpress system (Qiagen). Approximately 2.7 mg of purified rTRXs
was obtained per milligram (wt) of cells after DTT treatment at
37°C for 30 min. After PD-10 column chromatography (Amersham Bio-
science, Piscataway, NJ), the purity of the rTRXs was confirmed by SDS-
PAGE. The purity of the acquired rTRXs was over 98%. Alexa fluores-
cence-labeled (Alexa-) rTRX-WT or rTRX-CS was prepared using an
Alexa Fluor 488 Protein Labeling kit (Molecular Probes, Eugene, OR)
according to the instruction manual provided by the manufacturer. Briefly,
2 mg/ml of purified rTRXs was mixed and incubated in the reaction buffer
including the fluorescent compounds for 1 h. The labeled rTRXs were
subjected to gel filtration chromatography on a PD-10 column. The purity
of the acquired Alexa-rTRXs was confirmed using SDS-PAGE and a
Typhoon 9410 Variable Mode Imager (Molecular Dynamics, Sunnyvale, CA).

**Stable transfectants for TRXs**

For stable expression of the TRX genes and proteins, Jurkat cells were
transfected with 10 μg of plasmid DNA: TRX-WT and TRX-CS/
p3xFLAG-CMV-10 using a Gene Pulser electroporation system (Bio-Rad,
Hercules, CA) with a capacitance of 960 μF and an electrical field of 320
V. The transfectants were cultured in RPMI 1640 medium containing 400
μg/ml G418 to screen stable transfectants. The drug-resistant bulk popu-
lation of the cells was cloned by limiting dilution. Expression of TRX
protein in the cells was confirmed by Western blot analysis. To avoid
variation among clones, three randomly selected clones were tested.

**Specific sandwich ELISA for TRX**

The amount of TRX in the culture supernatant of cells was measured with a
TRX ELISA kit (Redox Bioscience, Kyoto, Japan) as previously de-
scribed with minor modification (22). 3’,3’,5,5’,5’-tetramethylbenzidine was
used as a substrate for peroxidase and 30 min later stopping solution (2 N
H2SO4) was added. The absorption at A450 was measured with an ELISA
plate reader (Molecular Devices, Sunnyvale, CA).

**Immunoprecipitation assay**

Briefly, transfectant cells were exposed to 50 μM H2O2 and the supernatant
of these cells was collected. The released FLAG-tagged TRXs in these
supernatants or cell lysates was measured by a TRX ELISA system.

**Lactate dehydrogenase (LDH) activity assay**

The LDH activity in culture supernatants was measured, as previously de-
scribed (23), after the cells were treated with 50 μM H2O2 for 1, 3, or 6 h.

**Assay for apoptosis**

To detect the percent DNA contents of the sub-G1 population, the cells
were fixed in 70% ethanol on ice overnight after the stimulations. Then the
cells were treated with 50 μg/ml RNase for 1 h at 37°C and then incubated
with propidium iodide (PI; Calbiochem, La Jolla, CA) staining solution for
1 h (24). The stained cells were analyzed by a flow cytometer (FACScalib-
ur; BD Biosciences, Mountain View, CA) using CellQuest software.

**Measurement of caspase-3 activity**

After the cells were stimulated with H2O2 for 24 h, the cell lysate was
prepared with lysis buffer containing 0.5% Nonidet P-40, 50 mM Tris-HCl
(pH 7.5), and protease inhibitors (Roche, Basel, Switzerland) from treated
cells. The caspase-3 activity in this lysate was measured using a fluorom-
eter (Spectra Fluor) as previously described (25).

**Measurement of intracellular ROS**

To measure the amount of intracellular ROS generated by the cells, 2’,7’-
dichlorofluorescein diacetate (DCFH-DA; Molecular Probes) was used.
This compound is a nonfluorescent, cell-diffusible dye. Intracellular exter-
ases cleave the acetyl groups from the molecule to produce nonfluorescent
DCFH. This is trapped inside the cell and in the presence of ROS, DCFH
is subsequently modified to fluorescent DCFH, which can be detected by
flow cytometry. After the cells were incubated with 10 μg/ml rTRX-WT for
1 h, they were preloaded with 5 μM DCFH-DA at 37°C for 30 min. Each
sample was analyzed using a FACScalibur. The data are expressed as the
mean fluorescence intensity.

**Preparation of cytosolic fraction**

The cytosolic fraction was prepared by caviation and centrifugation (26).
The treated cells were collected and suspended with hypotonic buffer after
washing out with excess PBS buffer. Next, caviation was performed using
nitrogen gas at 1500 pounds per square inch on ice. The suspension was
subjected to a series of sequential centrifugations: 1,500 and 50,000 rpm.
The supernatant acquired from 50,000 rpm spin was used as the cytosolic
fraction.

**Confocal imaging**

Imaging analysis was performed with a Bio-Rad RTS 2000 confocal laser
microscope, a modified version of the Nikon RCM 8000 video-rate con-
focal instrument (Nikon, Melville, NY) (27). This system uses a krypton/
argon laser of the American Laser Cooperation (Salt Lake City, UT). Data
after capturing images were transferred directly from a real-time image-
capturing subsystem to a hard disk developed specifically for this system
by Nikon. These operations were all managed under Windows NT. For Fig.
4, images were taken at 2.1 s frame-1 after 0, 24, 48, or 72 h. The culture
medium was replaced with HEPES-buffed Krebs-Ringer solution before
capturing the images by confocal laser.

**Results**

**TRX release from HTLV-I-infected T lymphocytes is regulated by redox state**

Since viral infections induce the production of ROS such as H2O2 in
various cell types, we considered that oxidative stress may in-
duce the release of TRX from HTLV-I-transformed T lymphocytes,
ATL2 cells. The cells were cultured for 24 h with 10 or 50
μM H2O2, and TRX in the culture supernatants and the cell lysates
was measured using the ELISA system. As shown in Table I, the
addition of H2O2 increased the amount of TRX protein in both
culture supernatant and cell lysate in a dose-dependent manner.
Moreover, a reducing reagent, N-acetylcysteine (NAC) decreased
the amount of TRX in the supernatant but not that in cell lysate
(Table I). These results suggest that the release of TRX from
HTLV-I-transformed T lymphocytes is regulated by redox status.

**Redox-active site in TRX is indispensable for its release by H2O2**

To test whether the redox-active site of TRX is involved in the
release of TRX in response to H2O2, we generated stable transfect-
ants of Jurkat cells transfected with vector DNA, the FLAG-
tagged TRX-WT gene or the FLAG-tagged TRX-CS gene in which
both cysteine residues of the active site of TRX were converted
into serine. In the absence of H2O2, FLAG-tagged TRX was not
detected in the culture supernatants of any transfectants by West-
ern blot analysis using anti-FLAG mAb after the immunoprecipita-
tion by anti-TRX mAb (Fig. 1A, left panel). In contrast, the

### Table I. Release of TRX in response to H2O2 and NAC

<table>
<thead>
<tr>
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<th>H2O2</th>
<th>NAC</th>
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<tr>
<td></td>
<td>None</td>
<td>10 μM</td>
</tr>
<tr>
<td>Supematant</td>
<td>635</td>
<td>784</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>1210</td>
<td>1930</td>
</tr>
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*4 A total of 1.2 × 105 cells of ATL2 cells was cultured in the absence or presence of H2O2 or NAC for 24 h, and the total weight (nanograms) of TRX in the culture supernatants or cell lysates was measured by a TRX ELISA system.*
tagged TRX was rapidly released from the Jurkat transfectant expressing TRX-WT at 1 h after the addition of 50 μM H₂O₂ to the culture, but the amount of tagged TRX in the culture supernatant did not significantly increase for the next 5 h (Fig. 1A, right panel). However, the tagged TRX was not detectable in the culture supernatants of the transfectant expressing TRX-CS within 6 h after the addition of H₂O₂ (Fig. 1A, right panel). To exclude the possibility that H₂O₂ might have induced necrosis of the transfectant expressing the tagged TRX-WT, the LDH activity in the culture supernatant was determined until 6 h after the addition of 50 μM H₂O₂ in the culture. As shown in Fig. 1B, LDH activity in the culture supernatant of all transfectants at 1, 3, or 6 h after the start of culture was comparable to that at 0 h, indicating that necrosis had not begun within 6 h. Then, the transfectants were assessed for oxidative stress-induced apoptosis. As a preliminary experiment to test whether the concentration of H₂O₂ (50 μM) used in this report induces apoptosis of the cells transfected with the control vector, TRX-WT or TRX-CS, caspase-3 activity in the transfecants was measured after a 24-h culture in the presence of H₂O₂ (Fig. 1C). The caspase-3 activity of the transfectant expressing TRX-WT was significantly lower than that of the control vector. In contrast, that of the transfectant expressing TRX-CS was significantly higher than that of the control. Furthermore, the rate of apoptosis was determined as percent DNA content of sub-G₁ using flow cytometric analysis with PI staining. In the absence of H₂O₂, the percent DNA content of sub-G₁ was 3.3% in cells transfected with control vector, 3.6% in cells with TRX-WT, and 6.8% in cells with TRX-CS. After the addition of H₂O₂, the cells transfected with TRX-WT and control vector showed 9.4 and 15.5% apoptosis, respectively. In contrast, 31.6% of TRX-CS transfecants underwent apoptosis in the presence of H₂O₂. The results clearly show that the caspase-3 activity of the transfectant expressing TRX-WT was weaker than that of the control, while conversely, the activity of caspase-3 in the transfectant expressing TRX-CS was much stronger than that of the control. These results indicate that the expression of TRX-WT in Jurkat cells protects against apoptosis, whereas the expression of the mutant TRX-CS enhances apoptosis. Thus, it is suggested that the intact sequence of a redox-active site (–CPGC–) in

FIGURE 1. Release of tagged TRX from Jurkat transfectants in response to H₂O₂. A, FLAG-tagged TRX-WT or FLAG-tagged TRX-CS was immunoprecipitated by using ADF11 mAb from the culture supernatants of each Jurkat transfectant in the absence (–H₂O₂) or presence of 50 μM H₂O₂ (+H₂O₂) for 0, 1, 3, or 6 h. B, LDH activity in each culture supernatant of the transfectant exposed to 50 μM H₂O₂ for 1, 3, or 6 h. Vec, Cells transfected with vector. C, Caspase-3 activities of transfectants were measured after stimulation with 50 μM H₂O₂ for 24 h. *, p < 0.01, when compared with control. D, The percent DNA content of sub-G₁ transfecants was measured after stimulation with 50 μM H₂O₂ for 48 h.

FIGURE 2. Inhibition of TRX release by the addition of extracellular TRX in a dose-dependent manner. Both 50 μM H₂O₂ and a concentration of 0, 0.01, 0.1, 1, or 10 μg/ml rTRX-WT were simultaneously added to the culture medium of the transfectant expressing TRX-WT. Each culture supernatant was recovered 3 h after the addition of H₂O₂, and analyzed by the same method used in Fig. 1A. rec, Recombinant.
TRX protein is indispensable for its active release from T lymphocytes by H$_2$O$_2$ and that TRX is rapidly released within 1 h, irrespective of the cellular sensitivity to H$_2$O$_2$-induced apoptosis.

**TRX release is regulated by extracellular TRX**

Since the amount of tagged TRX in the culture supernatant of the Jurkat transfectants was constant for 6 h, it is possible that the TRX released from the transfectants might regulate further its own release. To test this possibility, various doses of rTRX-WT (0.01–10 µg/ml) were added to the culture of the Jurkat transfectant expressing FLAG-tagged TRX-WT along with 50 µM H$_2$O$_2$. After 3 h, the tagged TRX in the culture supernatants was immunoprecipitated using anti-TRX mAb (anti-TRX mAb) and protein G-Sepharose beads. The samples were analyzed by SDS-PAGE, followed by Western blotting using anti-FLAG mAb. As shown in Fig. 2, the tagged TRX in the supernatants decreased in inverse proportion to the amount of rTRX-WT added to the culture and was undetectable at 10 µg/ml (0.8 µM) rTRX-WT. The expression level of the intracellular tagged TRX was not affected by the concentration of rTRX-WT added (data not shown). These results

FIGURE 3. Suppression of H$_2$O$_2$-induced cell events by extracellular rTRX. A. After the pretreatment of the transfectant expressing TRX-WT with 10 µg/ml rTRX-WT for 1 h at 0 or 37°C, 50 µM H$_2$O$_2$ was added to the culture. Each culture supernatant was recovered 3 h after the addition of H$_2$O$_2$, and analyzed by the same method used in Fig. 1A. B. The transfectant expressing TRX-WT was preincubated with 10 µg/ml rTRX-WT at 0 or 37°C for 1 h. After being washed with medium, the cells were incubated with DCFH-DA for 30 min, and these treated cells were analyzed by flow cytometer. C. The transfectant expressing TRX-CS was cultured with 50 µM H$_2$O$_2$ for 24 h after treatment with rTRX for 1 h at 0 or 37°C. Caspase-3 activity in the supernatant of these treated cells was measured. *, p < 0.01 vs 1; #, p < 0.01 vs 2; †, p < 0.01 vs 3. Values of p were calculated using one-way ANOVA followed by Sheffe’s post hoc tests. D. The transfectant expressing TRX-CS was cultured with 50 µM H$_2$O$_2$ in the absence or presence of rTRX in a dose-dependent manner. After the 48-h stimulation, the cells were stained by PI and measured by the FACS system (micrograms per milliliter). rec, Recombinant.
indicate that H$_2$O$_2$-induced TRX release from the cells is inhibited by a suitable dose of TRX-WT in the culture supernatant.

**Pretreatment with rTRX-WT suppresses H$_2$O$_2$-induced release of TRX and apoptosis**

To clarify the roles of exogenous TRX-WT in the regulatory mechanism of TRX release, the transfectant expressing TRX-WT was stimulated by the addition of H$_2$O$_2$ after pretreatment with rTRX-WT. The cells were preincubated with 10 µg/ml (0.8 µM) rTRX-WT for 1 h at 0 or 37°C. After a wash with the culture medium, the cells were subsequently cultured in the presence of 50 µM H$_2$O$_2$ for 3 h. The release of tagged TRX was not fully inhibited by preincubation with rTRX-WT at 0°C (Fig. 3A). However, the preincubation at 37°C remarkably suppressed the release of tagged TRX from the cells (Fig. 3A). It appeared that the interaction of rTRX-WT with the transfectant expressing the tagged TRX resulted in prevention of the H$_2$O$_2$-induced release of TRX from the cells via the cellular active process. To investigate the cellular active processes involved in the regulation of TRX release, the intracellular ROS production after the treatment of the transfectant with rTRX-WT was analyzed using a flow cytometer. The Jurkat transfectant expressing the tagged TRX-WT was incubated with 10 µg/ml rTRX-WT for 1 h at 0 or 37°C, followed by 5 µM DCFH-DA for 30 min. Fig. 3B shows that ROS production after the incubation with rTRX-WT at 0 and 37°C was reduced 36 and 48% compared with that without rTRX-WT, respectively. As possible signal transduction mediated by ROS, apoptotic signaling was subsequently analyzed. It has been shown that H$_2$O$_2$ induces apoptosis of Jurkat cells (28). Since the caspase-3 activity of the transfectant expressing TRX-CS was significantly higher than that of the control (Fig. 1C), the effect of the preincubation with rTRX-WT was examined using the transfectant. Fig. 3C clearly showed that the caspase-3 activity after the addition of H$_2$O$_2$ in the culture preincubated with rTRX-WT was significantly inhibited at 37°C, but not at 0°C. Moreover, after the pretreatment of rTRX-WT at concentrations of 0, 1, 10, and 100 µg/ml, the percent DNA content of sub-G1 cells was 27.5, 24.1, 23.9, and 19.8%, indicating a dose-dependent suppression of apoptosis by rTRX-WT (Fig. 3D). Hence, the results collectively suggest that extracellular rTRX-WT regulates ROS production and its mediated independent signal transductions responsible for the H$_2$O$_2$-induced release of TRX and apoptosis.

**Extracellular TRX enters cells**

As a mechanism for extracellular TRX to regulate cellular signal transduction, the possibility that the extracellular TRX enters the cells was examined. Alexa fluorescence-labeled (Alexa-) rTRX-WT or TRX-CS was prepared and added at a concentration of 100 ng/ml into culture medium of ATL2 cells. Confocal microscopy revealed that some cells cultured with Alexa-rTRX-WT emitted fluorescence within 24 h and almost all did after 72 h (Fig. 4A). In contrast, the cells cultured with Alexa-rTRX-CS failed to emit fluorescence after 72 h (data not shown). To confirm the cellular localization of Alexa-rTRX-WT, each subcellular fraction of the cells cultured with the His-tagged rTRX-WT for 0, 24, 48, or 72 h was prepared and analyzed by Western blotting using anti-His mAb. Fig. 4B clearly shows that the tagged TRX-WT accumulated in the cytosolic fraction in a time-dependent manner. Almost the same result was observed in Western blot analysis of particulate fractions containing plasma membranes (data not shown).
shown). The data clearly indicate that extracellular TRX-WT enters cultured cells on a regular basis within 24 h.

**Discussion**

It has been reported that HTLV-I-transformed T lymphocytes secrete a large amount of TRX protein in the culture supernatant (3, 29). Although the data of Table I and Fig. 1A indicate that two cysteines at the redox-active site of TRX might regulate the oxidative stress-induced release of TRX, it remains unclear whether the reducing enzymatic activity of TRX is required for its release. A recent report showed that the release of TRX-C35S containing a serine instead of a cysteine at residue 35 is not altered by the redox status of the cells (30). We obtained the same result in that TRX-C35S could be released from Jurkat cells under oxidative stress (data not shown). Hence, it is suggested that Cys32 at the redox-active site of TRX is indispensable for the oxidative stress-induced TRX release.

Another question regarding the release of TRX is related to the nature of the mechanism by which the redox-sensitive release occurs. In an E. coli system, Imaba and Ito (31) have discovered a thiol-mediated protein folding system on the periplasmic membrane of many secreted proteins that have a disulfide bond. This system includes the TRX family of proteins. It is thus an intriguing possibility that a similar thiol-mediated redox-sensitive release mechanism is present in mammalian cell systems. Such a mechanism may be used in the release of leaderless proteins in various cell types in the immune and endocrine systems.

In terms of the kinetics of release after the addition of H2O2, TRX-WT was rapidly released from the transfectant within 1 h and its level in the culture supernatant was almost unchanged for 6 h (Fig. 1A). Although the concentration of intracellular TRX was ~10-fold higher than that of extracellular TRX as a result of Western blot analysis using anti-FLAG mAb (data not shown), the concentration of the TRX released did not increase any further after 1 h. We speculate that the release is regulated by a sensing mechanism, which may monitor the concentration of extracellular TRX. This hypothesis may be supported by the result that the H2O2-induced TRX release from the Jurkat transfectant was reduced in the presence of rTRX-WT whose concentration in the culture was comparable to that of endogenous TRX released from ATL2 cells (Fig. 2 and Table I.). It is likely that the TRX transmits an inhibitory signal to shut off further release via an autocrine and/or paracrine feedback loop because the H2O2-induced TRX release was completely suppressed by preincubation with rTRX at 37°C, but not fully at 0°C (Fig. 3A). Therefore, it seems that Jurkat cells or HTLV-I-transformed cells are equipped with TRX-sensing molecules on the cell surface, which may specifically recognize TRX protein, transmit signals into the cell, and regulate the release of TRX from the cells under physiological conditions.

It is quite natural that the extracellular rTRX can function to quench hydroxyl radicals in the culture using a TRX-dependent system. In fact, the level of ROS production after the preincubation with rTRX was significantly reduced (Fig. 3B). However, we expect that the amount of extracellular rTRX was not enough to quench both 50 μM H2O2 and subsequent ROS production since the concentration of rTRX-WT added to the culture, 10 μg/ml (~0.8 μM), was much lower than that of H2O2 (32). It is also reported that an isoform of TRX-dependent peroxidase, peroxiredoxin IV, which can scavenge hydroxyl radicals in culture, exists on the cell surface. This system may be partly involved in the suppression of TRX release. Thus, it appears that the signal transduction pathways may regulate H2O2-induced TRX release. In support of this hypothesis, it is reported that LPS-induced pro-IL-1 production and IL-1 secretion corresponded to the production of ROS and activation of c-Jun N-terminal kinase, p38, and extracellular signal-regulated kinase (33). Further study is required to elucidate the signal transduction pathways involved in the regulation of the H2O2-induced release of TRX.

As another mechanism accompanying a negative feedback loop, we speculate that the intracellular TRX that accumulated with the entry of extracellular TRX regulates the release. In fact, we demonstrated that extracellular rTRX-WT could enter ATL2 cells or Jurkat cells within 24 h by the confocal microscopic observation and Western blot analysis (Fig. 4). However, we failed to visualize the entry of extracellular rTRX-WT at a concentration of 100 μg/ml after the preincubation at 37°C for 1 h (data not shown). In this experiment, the concentration of rTRX-WT used in Fig. 3 was not appropriate for confocal microscopy because of the high fluorescence intensity of the culture medium containing Alexa-labeled rTRX-WT. Although it is possible that an undetectable amount of rTRX-WT enters the cells for 1 h, it is unlikely that this entry mechanism of TRX mainly functions to inhibit the TRX release by the preincubation with rTRX.

In summary, we suggest that the release of TRX from T lymphocytes is regulated by a negative feedback loop sensing the concentration of TRX in and/or out of cells for the purpose of maintaining the physiological condition of the cells. We are now proceeding to identify the target molecules on the plasma membrane for extracellular TRX, which might be involved in the signal transduction and the entry into the cells.

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**References**


