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Serum thioredoxin reductase levels increase in response to chemically induced acute liver injury

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ABSTRACT

Background: Mammalian thioredoxin reductases (TrxR) are selenoproteins with important roles in antioxidant defense and redox regulation, principally linked to functions of their main substrates thioredoxins (Trx). All major forms of TrxR are intracellular while levels in serum are typically very low.
Methods: Serum TrxR levels were determined with immunoblotting using antibodies against mouse TrxR1 and total enzyme activity measurements were performed, with serum and tissue samples from mouse models of liver injury, as triggered by either thioacetamide (TAA) or carbon tetrachloride (CCl_4).
Results: TrxR levels in serum increased upon treatment and correlated closely with those of alanine aminotransferase (ALT), an often used serum biomarker for liver damage. In contrast, Trx1, glutathione reductase, superoxide dismutase or selenium-containing glutathione peroxidase levels in serum displayed much lower increases than TrxR or ALT.
Conclusions: Serum TrxR levels are robustly elevated in mouse models of chemically induced liver injury.
General significance: The exaggerated TrxR release to serum upon liver injury may reflect more complex events than a mere passive release of hepatic enzymes to the extracellular milieu. It can also not be disregarded that enzymatically active TrxR in serum could have yet unidentified physiological functions.

1. Introduction

All major isoforms of mammalian thioredoxin reductases (TrxR) are homodimeric selenocysteine-containing oxidoreductases, including ubiquitously expressed cytosolic TrxR1, mitochondrial TrxR2, and one variant (TGR) mainly found in testis [1]. The importance of these enzymes is mainly attributed to the catalysis of thioredoxin (Trx) reduction, whereby the active site disulfide of cytosolic Trx1 or mitochondrial Trx2 using NADPH is kept reduced by TrxR1 or TrxR2, respectively, thereby constituting the main players of the Trx system. This system, in turn, has a large number of redox functions in cells due to many diverse redox regulatory roles of Trx [1]. In addition to reduction of Trx, TrxR can also directly reduce several low molecular weight compounds [1], including dithio-bis-nitrobenzoic acid (DTNB) that is used as a model substrate in assays of TrxR activity [2]. TrxR is furthermore readily inhibited by several electrophilic compounds that easily react with the selenocysteine residue of the enzyme, e.g. with gold compounds such as aurothioglucose or auranofin being highly efficient inhibitors at virtually stoichiometric amounts [3]. TrxR enzymatic activity can thus be measured by NADPH dependent DTNB reduction using gold inhibition for specificity control [4–6], or, alternatively, by an insulin reduction assay that is based upon Trx as a TrxR substrate coupled to reduction of insulin disulfides by Trx [7].

Although TrxR1 was found to be secreted by certain cells, its activity in healthy human plasma is very low [8]. NADPH-dependent protein-disulfide reductase activity in serum of tumor-bearing mice was assumed early to be TrxR [9,10], but this assumption has not yet been further studied. Elevated levels of human serum Trx1, however, were identified early as ADF (Adult T-cell Derived Factor) [11] and is observed in numerous diseases, including several forms of liver pathology [12,13]. In contrast to cytokine-like effects of Trx1, no biological function of any TrxR isoform in serum has yet been reported. Its possible presence in serum has been much less studied and, hitherto, serum TrxR has not been studied in any toxicological models. Since TrxR1 is abundantly expressed in liver from where it was originally purified [10,14], we investigated here whether alterations of serum TrxR levels may occur as a response to chemically induced liver injury. We found that serum TrxR levels were indeed highly increased upon treatment with hepatotoxic agents in mice and these increases correlated well with those of alanine aminotransferase (ALT), a well-known indicator

Abbreviations: ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; DTNB, dithio-bis-nitrobenzoic acid; GPx, glutathione peroxidase; GR, glutathione reductase; i.p., intraperitoneally; Nrf2, nuclear factor erythroid 2-related factor 2; PBS, phosphate buffer saline; SOD, superoxide dismutase; TAA, thioacetamide; Trx, thioredoxin; TrxR, thioredoxin reductase

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for liver injury [15,16]. A number of other hepatic enzymes were, however, not increased to the same extent, thus suggesting unique kinetics or mechanisms in TrxR release from the liver.

2. Materials and methods

2.1. Chemicals and drugs

NADPH, DTNB, reduced glutathione, oxidized glutathione (GSSG), glutathione reductase (GR, from *Escherichia coli*), and rat TrxR1 were all obtained from Sigma (St. Louis, Missouri, USA). Auranofin was purchased from Santa Cruz Biotechnology, Inc. (California, USA). Other chemicals were of the highest grade available.

2.2. Animals

Healthy male Kunming-strain mice (20–22 g) and their diet were purchased from Shanghai SLAC Laboratory Animal Co. Ltd., China. The mice were housed in plastic cages in a room with controlled temperature (22 ± 1 °C) and humidity (50 ± 10%) and 12 h light/dark cycle. The mice were given food and water ad libitum. All protocols for the animal experiments complied with guidelines of the Anhui Agricultural University for care and use of laboratory animals.

2.3. Animal treatments

To first investigate serum TrxR levels upon TAA-triggered liver injury, ten mice were divided into two groups (5/group). The mice were intraperitoneally (i.p.) injected with saline as control or 200 mg/kg thioacetamide (TAA) dissolved in saline (10 mL/kg per injection), whereupon they were sacrificed 24 h later by cervical dislocation. Individual serum samples were obtained following the procedures described below. An aliquot of 0.3 mL serum from each mouse that received identical drug treatment was also used to pool the serum, which was subsequently denoted either “control serum” or “TAA serum” and utilized for the immunoblot analysis reported in Fig. 1 and validation of the DTNB-based assay of TrxR activity.

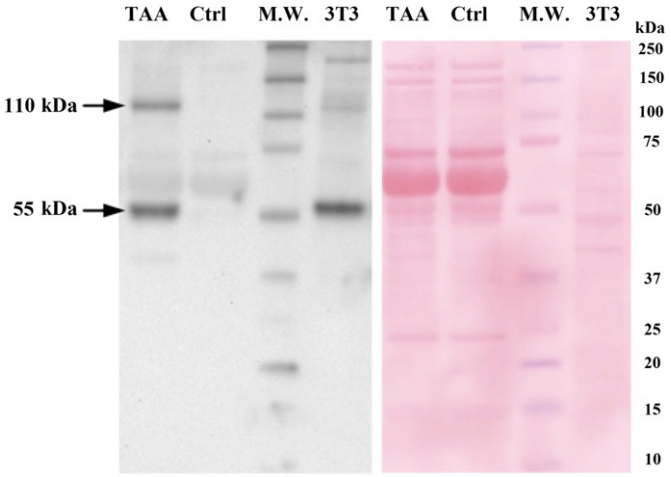

Fig. 1. TrxR protein levels in mouse serum are increased upon TAA treatment. The presence of TrxR protein in 0.5 μL serum samples of control (Ctrl) or TAA (200 mg/kg, sacrificed after 24 h) treated mice were compared with a whole cell lysate of mouse NIH 3T3 cells (3T3) as positive control using reducing SDS-PAGE and immunoblotting (left panel), with sample loading and molecular weight markers as indicated in the figure (M.W.; numbers to the right show M_r in kDa). The arrows indicate two bands that were clearly immunoreactive with a polyclonal anti-mouse TrxR1 antibody, with one band migrating slightly above 55 kDa, corresponding to the TrxR1 monomeric subunit, and one approximately at 110 kDa, most likely representing a covalently linked dimer of TrxR1. Total serum protein loading was visualized by Ponceau S staining as shown in the right panel. Both of the analyzed samples were pooled from serum of 5 mice handled under identical conditions and these pooled samples were also further analyzed in Fig. 2A–D.

To investigate 1) the impact of TAA on hepatic TrxR and ALT activities at 12 h post treatment, and 2) the impact of TAA and carbon tetrachloride (CCl_4) on serum TrxR and ALT activities as well as other enzyme activities at 24 h post treatment, thirty mice were randomly divided into five groups (6/group). Mice were i.p. injected with saline, 200 mg/kg TAA or 200 μL/kg CCl_4 dissolved in peanut oil (10 mL/kg per injection). At 12 h, mice subjected to saline and TAA treatments were sacrificed to collect liver tissues. At 24 h, the remaining mice subjected to saline, TAA and CCl_4 treatments were sacrificed to collect serum samples.

To delineate TAA-triggered time-dependent changes of serum enzyme activities, thirty mice were randomly divided into five groups (6/group). Mice in group I were injected i.p. with saline as control and sacrificed at 24 h. Mice in groups II–V were i.p. injected with 200 mg/kg TAA and sacrificed at either 6, 12, 24 or 36 h to collect serum samples.

To analyze CCl_4-caused dose-dependent changes of serum enzyme activities, twenty-four mice were randomly divided into four groups (6/group). Mice in group I were i.p. injected with peanut oil as control whereas mice in groups II–IV were i.p. injected with 40, 200 and 1,000 μL/kg CCl_4, respectively. All mice were sacrificed at 24 h to collect serum.

To investigate changes of serum enzyme activities during chemically induced liver fibrosis, twenty-four mice were randomly divided into three groups (8/group). Mice in groups II and III were i.p. injected with 200 mg/kg TAA or 1,000 μL/kg CCl_4, twice weekly for a total of 6 weeks [17,18]. Four mice in group I were i.p. injected with saline as TAA control and another four mice in group I were i.p. injected with peanut oil as CCl_4 control. The enzyme levels in the two vehicle groups were not significantly different from each other and the values were thus merged as one control. All animals were sacrificed at 24 h after their last treatment to collect serum. Although the doses used here for CCl_4 were close to its generally accepted LD50 value no lethality was observed for the duration of the treatments, which may possibly be explained by the known variability between different mice in CCl_4 susceptibility [19].

2.4. Serum and tissue preparations

At the end of each set of experiments, mice were sacrificed by cervical dislocation. Peripheral blood from both ophthalmic veins was collected into Eppendorf tubes without anticoagulant. After standing for 30 min at room temperature to allow for complete coagulation, serum was obtained by centrifugation (9000 *g* at 4 °C for 10 min). All serum samples used in the present study were clear and yellow with hemoglobin levels being 0.35 ± 0.04 g/L as estimated from the absorbance at 540 nm, and thus lacked noticeable hemolysis. Whole livers were excised, rinsed in ice-cold saline and instantly homogenized in ice-cold 150 mM, pH 7.2 phosphate buffer saline (PBS) containing 1 mM EDTA-Na_2 (1:9, w/v) using a tissue homogenizer. After centrifugation (15,000 *g* at 4 °C for 15 min), the resultant supernatants were immediately used for the assessments of hepatic TrxR and ALT activities.

2.5. Immunoblotting

Serum samples, 0.5–1 μL, were diluted in deionized water, NuPAGE LDS sample buffer (Invitrogen) and 12 mM dithiothreitol to a final volume of 50 μL. Samples were then denatured by boiling in water for 5 min. Subsequently, 20 μL of the sample was loaded onto a 4–12% NuPAGE Bis-Tris SDS-PAGE gel (Invitrogen) and subjected to electrophoresis. Separated proteins were electroblotted to a nitrocellulose membrane and equal protein loading was confirmed by Ponceau S staining. Membranes were blocked for 1 h at room temperature using 5% non-fat dry milk dissolved in PBS containing 0.1% Tween 20 (PBS-T). Membranes were then probed for TrxR1 using a polyclonal antibody raised against the mouse TrxR1 protein (kindly