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Original Contribution

Depletion of cytosolic or mitochondrial thioredoxin increases CYP2E1-induced oxidative stress via an ASK-1–JNK1 pathway in HepG2 cells

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ABSTRACT

Thioredoxin is an important reducing molecule in biological systems. Increasing CYP2E1 activity induces oxidative stress and cell toxicity. However, whether thioredoxin protects cells against CYP2E1-induced oxidative stress and toxicity is unknown. SiRNA were used to knockdown either cytosolic (TRX-1) or mitochondrial thioredoxin (TRX-2) in HepG2 cells expressing CYP2E1 (E47 cells) or without expressing CYP2E1 (C34 cells). Cell viability decreased 40–60% in E47 but not C34 cells with 80–90% knockdown of either TRX-1 or TRX-2. Depletion of either thioredoxin also potentiated the toxicity produced either by a glutathione synthesis inhibitor or by TNFα in E47 cells. Generation of reactive oxygen species and 4-HNE protein adducts increased in E47 but not C34 cells with either thioredoxin knockdown. GSH was decreased and adding GSH completely blocked E47 cell death induced by either thioredoxin knockdown. Lowering TRX-1 or TRX-2 in E47 cells caused an early activation of ASK-1, followed by phosphorylation of JNK1 after 48 h of siRNA treatment. A JNK inhibitor caused a partial recovery of E47 cell viability after thioredoxin knockdown. In conclusion, knockdown of TRX-1 or TRX-2 sensitizes cells to CYP2E1-induced oxidant stress partially via ASK-1 and JNK1 signaling pathways. Both TRX-1 and TRX-2 are important for defense against CYP2E1-induced oxidative stress.

Introduction

The thioredoxin system plays a key role in modulating redox signaling pathways which regulate physiological as well as patho-physiological processes [1,2]. The thioredoxin system includes thioredoxin, thioredoxin reductase, and thioredoxin peroxidases. Thioredoxin has a conserved catalytic site (−Cys-Gly-Pro-Cys-Lys-) that undergoes reversible oxidation to the cystine disulfide. Oxidized thioredoxin is a major substrate for thioredoxin reductase, and reduced thioredoxin serves as an electron carrier to reduce peroxiredoxins. The oxidized thioredoxin is reduced back to the reduced form by thioredoxin reductase [3,4]. There are two main thioredoxins: thioredoxin-1 (TRX-1), a cytosolic form; and thioredoxin-2 (TRX-2), a mitochondrial form [3]. Activity has been found: outside the cell, where thioredoxin plays a role in regulating cell growth and chemotaxis [5]; in the cytoplasm, where it functions as an antioxidant and a reductant cofactor [6]; in the nucleus, regulating transcription factor activity [7]; and in the mitochondria, where it also functions as an antioxidant [8].

Thioredoxin is important because of its reducing power and antioxidant activity and also because modification of thiols in thioredoxin interrupts signaling mechanisms involved in cell growth, proliferation, and apoptosis. The role of thioredoxin in the regulation of the activation of apoptosis signal-regulating kinase-1 (ASK-1) and downstream apoptosis pathways has been reported in multiple studies [9–11]. Thioredoxin can associate with the N-terminal portion of ASK-1 in vitro and in vivo. Expression of thioredoxin inhibited ASK-1 kinase activity and the subsequent ASK-1-dependent apoptosis [10]. In resting cells, endogenous ASK-1 constitutively forms a complex which includes thioredoxin. On ROS stimulation, the ASK-1 unbinds from thioredoxin and forms a fully activated higher-molecular-mass complex [12]. TNFα increases oxidative stress in mice with elevated CYP2E1, with subsequent activation of ASK-1 via a mechanism involving thioredoxin-ASK-1 dissociation, followed by activation of downstream MKK and MAPK [11]. A study with troglitazone also showed that increased intramitochondrial oxidant stress activates the TRX-2/ASK-1 pathway, leading to mitochondrial membrane permeabilization [13].

Both TRX-1 and TRX-2 are involved in the protection from oxidative stress. TRX-2 plays an important role in protecting the

Abbreviations: CYP2E1, cytochrome P4502E1; ASK-1, apoptosis signal-regulating kinase-1; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; ER, endoplasmic reticulum; TNFα, tumor necrosis factor α; 4-HNE, 4-hydroxynonenal; GSSE, glutathione ethyl ester; BSO, L-buthionine-[*R*,*S*]-sulfoximine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ROS, reactive oxygen species; TRX-1, cytosolic thioredoxin; TRX-2, mitochondrial thioredoxin; PDI, prolyl disulfide isomerase; DHE, dihydroethidine.

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mitochondria against oxidative stress and in protecting cells from ROS-induced apoptosis. It is required for normal development of mice embryos and actively respiring cells. The absence of TRX-2 causes massive apoptosis and early embryonic lethality in homozygous mice [14]. Also, TRX-2 haploinsufficiency in mice results in impaired mitochondrial function and increased oxidative stress after diquat treatment [8]. Overexpression of human TRX-2 confers resistance to the oxidant *tert*-butylhydroperoxide-induced apoptosis in human osteosarcoma cells [15]. As for TRX-1, overexpression of human TRX-1 reduces oxidative stress in the placenta of transgenic mice and promotes fetal growth [16]. Supplementation of human recombinant TRX-1 to mice fed a Lieber DeCarli ethanol diet decreased several markers of oxidative stress, inflammatory cytokine expression, and apoptosis in liver [17].

CYP2E1 is of interest in liver injury because of its ability to metabolize and activate many toxicological substrates, including ethanol, to more reactive toxic products. Levels of CYP2E1 are elevated under a variety of physiological and pathophysiological conditions, and after acute and chronic alcohol treatment. CYP2E1 is an effective generator of reactive oxygen species [18]. Since thioredoxin is a reducing molecule which can decrease oxidative stress, the goal of this study was to evaluate whether thioredoxin can inhibit the oxidative stress induced by CYP2E1, and whether there is any difference in the function of TRX-1 versus TRX-2 in blunting CYP2E1 oxidant stress. SiRNA for either TRX-1 or TRX-2 was added to HepG2 cells with CYP2E1 expression (E47 cells) or without CYP2E1 expression (C34 cells) to test: (1) whether thioredoxin decreases oxidative stress and injury induced by CYP2E1; (2) considering the compartmentation of thioredoxin, whether TRX-1 or TRX-2 has a stronger protective effect in preventing against this injury and oxidative stress? (3) what is the mechanism of the protection by thioredoxin from cell death in CYP2E1 expressing cells?

Materials and methods

Reagents and chemicals

ON-TARGETplus Non-targeting pool siRNA, TRX (TRX-1) ON-TARGETplus SMARTpool siRNA, TRX-2 ON-TARGETplus SMARTpool siRNA, and Dharmafect I transfection reagent were from Dharmacon Research (Lafayette, CO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), glutathione ethyl ester (GSSE), cycloheximide, and L-buthionine-[R,S]-sulfoximine (BSO) were purchased from Sigma (St. Louis, MO, USA). Murine TNFα was from Fitzgerald (Concord, MA, USA). Antibody against TRX-1, pASK-1, and PDI was from Cell Signaling Technology (Beverly, MA, USA). Antibody against TRX-2 was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Antibody against LC3 was purchased from Thermo Scientific (Rockford, IL, USA). Antibody against CYP2E1 was a generous gift from Dr. Jerry Lasker. Fluorescence-conjugated secondary antibodies used in the Odyssey infrared imaging system were from Li-Cor Biosciences (Lincoln, NE, USA). All other antibodies were from Santa Cruz Biotechnology Inc. Total ROS detection kit was purchased from ENZO Life Sciences (Plymouth Meeting, PA, USA). Histostain Plus Broad Spectrum (ACE) kit was from Invitrogen (Camarillo, CA, USA). The JNK inhibitor, L-JNKI1, was purchased from EMD Chemicals (San Diego, CA, USA). Dihydroethidine (DHE) was purchased from Invitrogen. MitoSOX Red reagent was purchased from Molecular Probes (Eugene, OR, USA). ApoAlert annexin V was from Clontech (Mountian View, CA, USA).

Cell culture and treatments

C34 and E47 HepG2 cells were cultured either on 96-well plates or 6-well plates or on glass cover slides according to the requirements of the experiments. E47 cells are HepG2 cells which were transfected with PCi plasmid containing human CYP2E1 cDNA. These cells constitutively express CYP2E1. C34 cells are HepG2 cells transfected with the empty plasmid. These cells express low or zero CYP2E1. E47 and C34 cells were maintained in DMEM containing 10% FBS plus 1% penicillin-streptomycin-glutamine mixture plus 0.1 mg/ml G418 [19]. Cells were plated at a density of 1×10^4 cells/ml in DMEM containing 10% FBS and 1% antibiotics (penicillin plus streptomycin). SiRNA of TRX-1, TRX-2, TRX-1 and TRX-2 together or control were used at 10 nM concentration in DMEM containing 2% FBS and were added 24 h after cells were plated. The treatment was continued for 5, 24, 48, and 72 h. The respective siRNA was present during the entire culture period. The transfection reagent was Dharmafect I used at a concentration of 1 μl/ml. For cells treated with either BSO or TNFα, 200 μM BSO or 2 ng/ml TNFα with 10 μg/ml cycloheximide was added after 24 h of siRNA treatment. The cells continued to be incubated with the appropriate siRNA and either BSO or TNFα for another 48 h (total siRNA treatment for 72 h). To test the protection effect of glutathione ethyl ester, 5 mM glutathione ethyl ester was added to the E47 cell culture medium after 24 h siRNA treatment and the cells were incubated with the respective siRNA and glutathione ethyl ester for another 48 h. To test the effect of a JNK inhibitor on cell viability, E47 cells were treated with 5 μM L-JNKI1 for 3 h, and then cells were treated either with control siRNA or with the respective thioredoxin siRNAs for another 72 h. Cells were collected and various analyses were carried out as described below.

Cell viability assay

Cells grown on 96-well plates and treated with siRNA for 72 h were incubated with MTT for 3 h. Cell culture medium was aspirated, and isopropanol was added and plates were shaken for 30 min. Absorbance at 590 and 630 nm was detected on a plate reader. Cell viability was calculated as the absorbance difference between 590 and 630 nm. Cell viability in the control siRNA group of both C34 and E47 cells was taken as 100%, and cell viability in the thioredoxin knockdown groups was expressed as the percentage of viability relative to that of the control siRNA group in corresponding C34 and E47 cells.

Propidium iodide and annexin V staining

Cells on 6-well plates were treated with siRNA for 72 h. For PI staining, 5 μg/ml PI was added to the culture medium and the cells were incubated for 10 min at 37 °C. PI staining was analyzed using fluorescence microscopy. Images shown were the merged ones taken with both light and fluorescence microscopy. The ApoAlert annexin V detection kit was used for annexin V staining. After siRNA treatment for 72 h, cells were digested with trypsin, and pelleted by centrifugation. Cells were washed twice with binding buffer supplied in the detection kit, resuspended with 200 μl binding buffer, treated with 5 μl annexin V stock solution (20 μg/ml), and then incubated for 15 min in the dark. Annexin V staining was analyzed by flow cytometry in the FITC channel.

ROS detection by microscopy

ROS was detected with the Total ROS Detection Kit from Enzo. Cytosolic ROS was detected by fluorescence of DHE, and mitochondrial ROS was detected by fluorescence of mitoSOX Red. Briefly, cells were allowed to attach to the glass cover slides for 24 h. Cells were treated with either TRX-1 siRNA or TRX-2 siRNA or both for 72 h. Cells were loaded with ROS detection solution or 40 μM DHE or 5 μM mitoSOX Red reagent at 37 °C and incubated in the dark. Cover slides were washed with wash buffer twice, overlaid on slides, and then observed immediately under a