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Research paper

Mechanistic insights into the inhibitory effects of palmitoylation on cytosolic thioredoxin reductase and thioredoxin

Huijun Qin [a], Wei Liang [a], Zhiyu Xu [a], Fei Ye [b], Xiaoming Li [c], Liangwei Zhong [a, *]

[a] College of Life Sciences, University of Chinese Academy of Sciences, Yuquan Road 19(A), 100049 Beijing, China
[b] Institute of Materia Medica, Chinese Academy of Medical Sciences & Perking Union Medical College, 100050 Beijing, China
[c] The State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, 100101 Beijing, China

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ABSTRACT

Overnutrition can lead to oxidative stress, but its underlying mechanism remains unclear. In this study, we report that human liver-derived HepG2 cells utilize cytosolic thioredoxin reductase (TrxR1) and thioredoxin (hTrx1) to defend against the high glucose/palmitate-mediated increase in reactive oxygen species. However, enhanced TrxR1/hTrx1 palmitoylation occurs in parallel with a decrease in their activities under the conditions studied here. An autoacylation process appears to be the major mechanism for generating palmitoylated TrxR1/Trx1 in HepG2 cells. A novel feature of this post-translational modification is the covalent inhibition of TrxR1/hTrx1 by palmitoyl-CoA, an activated form of palmitate. The palmitoyl-CoA/TrxR1 reaction is NADPH-dependent and produces palmitoylated TrxR1 at an active site selenocysteine residue. Conversely, S-palmitoylation occurs at the structural Cys62/Cys69/Cys72 residues but not the active site Cys32/Cys35 residues of hTrx1. Palmitoyl-CoA concentration and the period of incubation with TrxR1/hTrx1 are important factors that influence the inhibitory efficacy of palmitoyl-CoA on TrxR1/hTrx1. Thus, an increase in TrxR1/hTrx1 palmitoylation could be a potential consequence of high glucose/palmitate. The time-dependent inactivation of the NADPH-TrxR1-Trx1 system by palmitoyl-CoA occurs in a biphasic manner - a fast phase followed by a slow phase. Kinetic analysis suggests that the fast phase is consistent with a fast and reversible association between TrxR1/hTrx1 and palmitoyl-CoA. The slow phase is correlated with a slow and irreversible inactivation, in which selenolate/thiolate groups nucleophilically attack the α-carbon of bound palmitoyl-CoA, leading to the formation of thioester/selenoester bonds. hTrx1 can enhance rate of fast phase but limits the rate of slow phase when it is present in a preincubation mixture containing NADPH, TrxR1 and palmitoyl-CoA. Therefore, hTrx1 may provide palmitoylation sites or partially protect the TrxR1 active site selenol/thiol group(s) from palmitoylation. Our data suggest that Se/S-palmitoylation acts as an important modulator of TrxR1/hTrx1 activities, representing a novel potential mechanism that underlies overnutrition-induced events.

1. Introduction

Hyperglycemia and elevated free fatty acids (FFA) have many negative consequences [1,2], including the accumulation of reactive oxygen species (ROS) [3], oxidative damage [4] and cytotoxic effects [5]. However, the underlying mechanism remains unclear. Mammalian cytosolic thioredoxin reductase (TrxR1), thioredoxin (Trx1) and NADP(H) compose the Trx1 system that is critical for scavenging free radicals and maintaining cellular redox balance [6,7]. We have recently found that the levels of liver TrxR1 and Trx1 are decreased in mice fed a high-fat diet, which was accompanied by an increased amount of TrxR1/Trx1 palmitoylation [8]. However, it is still unclear whether palmitoylation directly regulates TrxR1/Trx1 activity.

TrxR1 is a homodimeric selenoprotein [9,10]. Its active site is composed of a conserved "-Cys-$(X)_4$-Cys-" motif near the N-

Abbreviations: TrxR1, cytosolic thioredoxin reductase; Trx1, cytosolic thioredoxin; ROS, reactive oxygen species; 5-IAF, 5-iodoacetamidofluorescein; NEM, N-ethylmaleimide; 2-BrP, 2-bromo-palmitate; HGHP, high glucose and high palmitate.

* Corresponding author. Tel./fax: +86 10 88256266.
E-mail address: liazho@ucas.ac.cn (L. Zhong).

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terminus of one subunit and a conserved C-terminal "-Gly-Cys-Sec-Gly-OH" motif from the opposite subunit. Here, X represents any amino acid residue, and Sec represents a selenocysteine residue. The N-terminal Cys residues and the C-terminal Cys/Sec pair may form a reversible disulfide and a selenenylsulfide bond, respectively [11]. In the absence of NADPH, TrxR1 is tightly folded, and contains the active site disulfide and selenenylsulfide bonds [10]. Upon accepting electrons from NADPH, these covalent bonds are reduced into dithiol and selenolthiol groups. The selenolthiol groups are exposed and serve as a nucleophile to attack Trx1 or other substrates [11,12] and as sensitive targets for thiol-specific modifications [9,13].

Human Trx1 (hTrx1) is a ubiquitous 12 kDa protein that is a natural substrate of TrxR1. The active site of hTrx1 contains two Cys residues (Cys32 and Cys35) that are present as dithiol groups in the reduced form of hTrx1, or form a disulfide in the non-reduced form of hTrx1. Through a thiol-disulfide exchange, hTrx1 transfers electrons from TrxR1 to many substrates, which, in part, protects the cell against oxidative injury [6]. In addition, hTrx1 has structural Cys residues (Cys62, Cys69 and Cys73) that are implicated in post-translational modifications. Cys62/Cys73 can be nitrosylated [14] and Cys73 can be glutathionylated [15]. These post-translational modifications significantly altered the activity of hTrx1 [16]. Currently, hTrx1 glutathionylation and nitrosylation have been well-described, but little is known about its palmitoylation.

Protein S-palmitoylation is the addition of long-chain fatty acids (mainly palmitic acid) through thioester linkages. This process may occur either enzymatically through a family of DHHC protein acyltransferases [17] or non-enzymatically (autoacylation) [18]. A large body of evidence has revealed that palmitoylation may regulate protein stability and trafficking [19]. In this study, we report the novel finding that post-translational Se/S-palmitoylation of TrxR1/Trx1 inhibits its activity. (In this study, the term Se/S-palmitoylation is used exclusively to describe Se/S-acylation.)

2. Materials and methods

2.1. Materials

Mammalian TrxR1 was purified from calf liver as described previously [20]. Calf liver TrxR1 shares a common structure with human TrxR1 [9]. Recombinant hTrx1 was purified according to a previously described method [21]. Monoclonal antibodies against TrxR1, Trx1 or GADPH were purchased from Santa Cruz Biotechnology (Germany). Palmitate, palmitoyl-CoA, 2-bromo-palmitate (2-BrP), insulin, 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), guanidine hydrochloride (Gua·HCl), phenylmethylsulfonyl fluoride (PMSF), NADPH and thiol-reactive 5-iodoacetamidofluorescein (5-IAF) were purchased from Sigma (USA). The ROS assay kit was purchased from Applygen Technologies Inc. (China).

2.2. Cell culture

The human liver-derived HepG2 cell line was used as a model for hepatocytes. The cells were cultured in low glucose Dulbecco's modified Eagle's medium (DMEM-LG, Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS and were maintained at 37 °C in an incubator containing 5% CO_2. To examine the effects of different treatments, the cells were grown until they reached approximately 70% confluence, followed by treatment for 24 h. Then, the cells were harvested and lysed, followed by centrifugation at 10,000 g for 30 min to collect the supernatants that were used as cell extracts. The protein concentration of each sample was determined using the Bradford assay with BSA as the standard.

2.3. Detection of cellular ROS

The cells were cultured in a 6-well plate until they reached 70% confluence and were then incubated with high glucose (25 mM)/palmitate (400 μM, referred to as HGHP) or normal levels of glucose (5.5 mM, referred to as LG), respectively, for 24 h. Next, the cells were washed twice with PBS. Serum-free medium (1 ml) containing DCFH-DA was added into each well at a final concentration of 10 μM, and the cells were incubated at 37 °C for 30 min. Then, the cells were trypsinized and harvested. The cell pellet was washed three times with cold PBS and resuspended in 500 μl PBS, followed by flow cytometry analysis (BD FACSCalibur, USA). Fluorescence data from 50,000 cells were collected and analyzed using CellQuest Pro Software.

2.4. Cellular knock-down of hTrx1

The cells were seeded onto 6-well plates and grown until they reached 70% confluence. The hTrx1-shRNA plasmid was constructed as described previously [22] and transfected using Lipofectamine™ 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. The control cells were transfected with the control pGeneclip plasmid. After 48 h, the cells were harvested to analyze the efficiency of hTrx1 knock-down by Western blotting and activity assays.

2.5. Determination of cellular TrxR/Trx activity

The activity of cellular TrxR/Trx was determined using the modified super-insulin assay [23]. The reactions were carried out in a 96-well plate, and the activity was detected using a microplate reader (Multiskan MK3, Thermo, USA). The assay mixture contained 0.25 M phosphate buffer (pH 7.5), 10 mM EDTA, 2 mM NADPH, 1.07 mM bovine insulin, and 40 μl TrxR1 (for measuring Trx) or hTrx1 (for measuring TrxR). The working solution of hTrx1 or TrxR1 was standardized by diluting each stock solution with 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA (PE buffer) to yield a final TrxR1 activity of Δ150 mA412 nm/min or a final Trx1 activity of Δ10 mA340 nm/min in 100 μl reaction volume. The reaction was started by mixing 20 μl of cell extract with 40 μl assay mixture, and incubated at room temperature for 30 min. The reaction was terminated by adding 150 μl of 0.1 mM DTNB, 8 M Guanidine-HCl in 0.2 M phosphate buffer, pH 7.5. The TrxR/Trx activity was determined by monitoring the increase in absorbance at 412 nm due to TrxR/Trx-dependent reduction of DTNB to TNB^- and was expressed as ΔA412 nm/mg protein min. A value for the control reaction, in which the cell extract was not added until the reaction terminated, was determined to correct for TrxR/Trx-independent reduction of DTNB.

2.6. Western blotting

The expression of cellular TrxR1/Trx1 was determined by Western blotting. The cell lysates (80 μg per lane) were subjected to 12% SDS-PAGE using a Tris-glycine gel. The proteins were then transferred onto a polyvinylidene difluoride membrane (Novex, USA). The target proteins were detected using primary antibodies raised against mouse TrxR1 (1:1000 dilution), Trx1 (1:1000 dilution) or GADPH (1:10,000 dilution), respectively. The bound antibodies were detected with a horseradish peroxidase-labeled secondary antibody (1:1000 dilutions). Protein bands were visualized using an enhanced chemiluminescene detection system (ECL) according to the manufacturer's instructions.