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New insights into the posttranslational regulation of human cytosolic thioredoxin by S-palmitoylation

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ABSTRACT

High level of palmitate is associated with metabolic disorders. We recently showed that enhanced level of S-palmitoylated cytosolic thioredoxin (Trx1) in mouse liver was new characteristic feature of insulin resistance. However, our understanding of the effect of S-palmitoylation on Trx1 is limited, and the tissue specificity of Trx1 S-palmitoylation is unclear. Here we show that S-palmitoylation also occurs at Cys73 of Trx1 in living endothelial cells, and the level of S-palmitoylated Trx1 undergoes regulation by insulin signaling. Trx1 prefers thiol-thioester exchange with palmitoyl-CoA to acetyl-CoA. S-palmitoylation alters conformation or secondary structure of Trx1, as well as decreases the ability of Trx1 to transfer electrons from thioredoxin reductase to S-nitrosylated protein–tyrosine phosphatase 1B and S-nitroso-glutathione. Our results demonstrate that S-palmitoylation is an important post-translational modification of human Trx1.

1. Introduction

High levels of palmitate are usually associated with metabolic disorders, such as insulin assistance [1]. Palmitoyl-CoA, an activated form of palmitate, can modify proteins by S-palmitoylation [2]. Protein S-palmitoylation usually influences protein stability and traffic [3]. We recently showed that enhanced level of S-palmitoylated cytosolic thioredoxin (Trx1) in mouse liver was new characteristic feature of insulin resistance [4]. Such a modification had negative effect on Trx1 reductase activity [5]. However, compared with well-studied S-glutathionylation and S-nitrosylation of Trx1 [6–9], much less is known about the properties of S-palmitoylated Trx1.

Trx1 is involved in a broad range of physiological functions [10]. Human Trx1 has molecular weight about 12 kDa with five Cys residues. Two of them are located in the active-site (Cys32 and Cys35) that catalyze reduction of target disulfide. Three structural Cys residues (Cys62, Cys69 and Cys73) are located close to helix α3 [11]. These structural Cys residues make Trx1 a prime target for S-glutathionylation [8], S-nitrosylation [6,7] and S-palmitoylation [5]. The consequences of S-glutathionylation and S-nitrosylation on Trx1 were known to represent contributing factors for regulating Trx1 functions reversibly. Whereas our understanding of the effect of S-palmitoylation on Trx1 is limited, and the tissue specificity of Trx1 S-palmitoylation is unclear.

Accumulating evidence suggests a potential relationship between Trx1 and palmitate in endothelium because Trx1 is an important regulator of vascular redox homeostasis [12] and palmitate could promote over-generation of intracellular reactive oxygen species (ROS) [13]. We noticed that investigations on agents affecting the role of Trx1 in vascular endothelial cells have not reported the linkage between palmitate and endothelial Trx1. Moreover, cellular Trx1 exists mainly in the reduced form. The five Cys residues can be modified under oxidative stress or nitrosative stress in different ways [12]. It remains unknown whether S-palmitoylation of Trx1 in living cells occurs at the structural Cys residue(s), similar to the results with purified Trx1 [5]. The current study has addressed the above issues.

2. Materials and methods

2.1. Materials

Rabbit anti-human Trx1 monoclonal antibody was purchased from Sino Biological Inc. Mouse anti-human TrxR1 monoclonal

Abbreviations: ACSL, long-chain acyl-CoA synthetase; 2-BP, 2-bromopalmitate; FAS, fatty acid synthase; Gluc-N, glucosamine; GSNO, S-nitroso-glutathione; HAM, hydroxylamine; pNPP, para-Nitrophenylphosphate; PTP1B, protein–tyrosine phosphatase 1B; Trx1, thioredoxin.

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antibody was purchased from Santa Cruz Biotechnology, Inc. Recombinant Trx1 was prepared according to a previously reported method [14]. His-tag construct of protein-tyrosine phosphatase 1B (PTP1B) was kindly provided by Professor Fei Ye (Institute of Materia Medica, Chinese Academy of Medical Sciences & Perking Union Medical College). Both purified proteins showed a single band on SDS-PAGE. S-nitrosylated PTP1B (PTP1B-SNO) was prepared according to the reported method [15].

2.2. Cell culture

EA.hy926, used as a model for endothelial cells, was cultured in Dulbecco's Modified Eagle Medium (DMEM; Corning, USA) containing 1 g/L glucose in a humidified atmosphere of 5% CO_2/air at 37 °C. The medium was supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and 100 units/ml penicillin.

2.3. Analysis of cellular S-palmitoylated Trx1

Acyl-RAC assay [16] was used.

2.4. LC-MS/MS analysis

The method employed for sample preparation was the same as described previously [5]. Each (5 μg) of samples was loaded onto self-packed C18 RP capillary columns (100 mm × 0.075-mm inner diameter) with buffer A (0.1% formic acid, 99.9% H_2O). The peptides were eluted with 5–30% buffer B (0.1% formic acid, 99.9% ACN; flow rate, 300 nL/min) for 120 min. Each sample was run three times. Eluted peptides were detected in a survey scan from 350 to 1250 amu followed by 30 data-dependent MS/MS scans (isolation width, 0.7 amu; 35% normalized collision energy; dynamic exclusion for 15 s) in a completely automated fashion on an AB SCIEX Triple TOF 5600 mass spectrometer. All MS/MS data were analyzed using PEAKS with a fragment ion mass tolerance of 0.050 Da and a parent ion tolerance of 0.050 Da. Carbamidomethyl of Cys and NEM of Cys modification were set as a variable modification. Four miscleavage sites were allowed. The peptide false discovery rate (FDR) was set to 1% on the peptide level. Protein identification was accepted at FDR less than 1.0% on protein level and contained at least two identified peptides.

2.5. Fluorescence measurements

All experiments were carried out at room temperature with a fluorescence spectrophotometer (Hitachi F-4600). Native or S-palmitoylation Trx1 (5 μM) was incubated with 150 μM 8-anilino-1-naphthalenesulfonic acid (ANS) at 37 °C for 1 h. Samples were taken in a quartz glass cuvette, and excited at 365 nm while scanning the emission spectra from 400 to 600 nm.

2.6. Preparation of S-palmitoylated Trx1

Palmitoyl-CoA was incubated with purified Trx1 in a molar ratio of 4:1 overnight at 37 °C, followed by dialysis to remove excess of palmitoyl-CoA. A fluorescence-based assay [17] was used to monitor autopalmitoylation of purified Trx1.

2.7. Circular dichroism

Protein sample was diluted to 10 μM in 50 mM Tris, pH 7.5, 1 mM EDTA (TE buffer). Measurements were carried out on a Jasco J720 spectropolarimeter at room temperature. All spectra were recorded between 200 and 300 nm using a 2 mm path-length cuvette.

2.8. Measurement of Trx1 activity

In the experiment with GSNO as substrate, the method previously reported [18] was used. In the experiment with PTP1B—SNO as substrate, PTP1B-SNO was incubated with native or S-palmitoylated Trx1 (5 μM), respectively, in the presence of 450 μM NADPH and 50 nM TrxR1. At the indicated time intervals, aliquots were withdrawn and mixed with substrate para-Nitrophenylphosphate (pNPP, a final concentration of 6 mM) for measuring an increase in absorbance at 405 nm. The activity was defined as the change in absorbance at 405 nm per min·mg protein.

3. Results

3.1. S-palmitoylated Trx1 in endothelial cells

We used LC-MS/MS mass spectrometry system to identify the Cys residues where S-palmitoylation of Trx1 may occur in the living cells. In this experiment, the free thiols in cell extracts were blocked by NEM. The resulting proteins were dialyzed to remove excess of NEM, followed by treatment with HAM that breaks thioester bonds. The nascent thiols were then modified by IAM. Therefore, IAM-modified Cys residues were originally modified by S-palmitoylation. The identified peptide contains Cys73, as shown in (Fig. 1A). The detected mass of $b2+$ ion was 292.08 Da, which are 58.04 Da over theoretical mass (234.04 Da), matching to the Cys modified with IAM (57.07 Da). Moreover, the mass difference (291.1 Da) between precursor ion (1205.56 Da) and $y7++$ ion (914.48 Da) is 57.06 Da over the theoretical mass, confirming that Cys73 is modified by IAM, that is, S-palmitoylation of Trx1 in the cells occurred at Cys73.

To know the proportion of S-palmitoylated Trx1 in total Trx1, we followed the method [19] in making estimates based on immunoblots with purified Trx1 standard. The result showed that 400 μg total protein contained about 5.3 ng S-palmitoylated Trx1(Fig. 1B). The average level of Trx1 in different cell lines was about 17 ng/10 μg total protein [19]. Thus, about 0.8% of Trx1 was present in S-palmitoylated form under basal conditions.

At present, there are proposed mechanisms elucidating the occurrence of protein S-palmitoylation [20]. Of them, autopalmitoylation occurs *via* a nucleophilic attack of the thiolate anion on the α-carbon of palmitoyl-CoA, and protein acyltransferases (PATs) catalyze the addition of palmitate. The latter is inhibited by 2-bromopalmitate (2-BP) [21]. The level of Trx1 S-palmitoylation in the living cells was not decreased in the presence of 2-BP (Fig. 1C), but 2-BP treatment could lower the degree of cytosolic thioredoxin reductase (TrxR1) S-palmitoylation (Fig. 1D). This result indicates that S-palmitoylation of Trx1 is independent of PATs.

3.2. Response of Trx1 S-palmitoylation to insulin stimulation

The endothelium has now emerged as a potentially important target tissue for insulin [22]. Interestingly, insulin regulated the level of palmitoylated Trx1 in a biphasic manner. A significant elevation of S-palmitoylated Trx1 appeared in the cells exposured to insulin for half an hour, whereas the enhancement went back down to original afterwards (Fig. 2A), and yet insulin-induced changes in the level of palmitoylated TrxR1 was not observed under these conditions (Fig. 2B). This result indicates a potential role of S-palmitoylated Trx1 in mediating insulin function.

To test further whether insulin signaling is involved in S-palmitoylation of Trx1, glucosamine (Gluc-N) was used to impair the response of the cells to insulin [23]. The level of palmitoylated Trx1 was significantly lower in the cell treated with Gluc-N than that in the absence of Gluc-N (Fig. 2C).

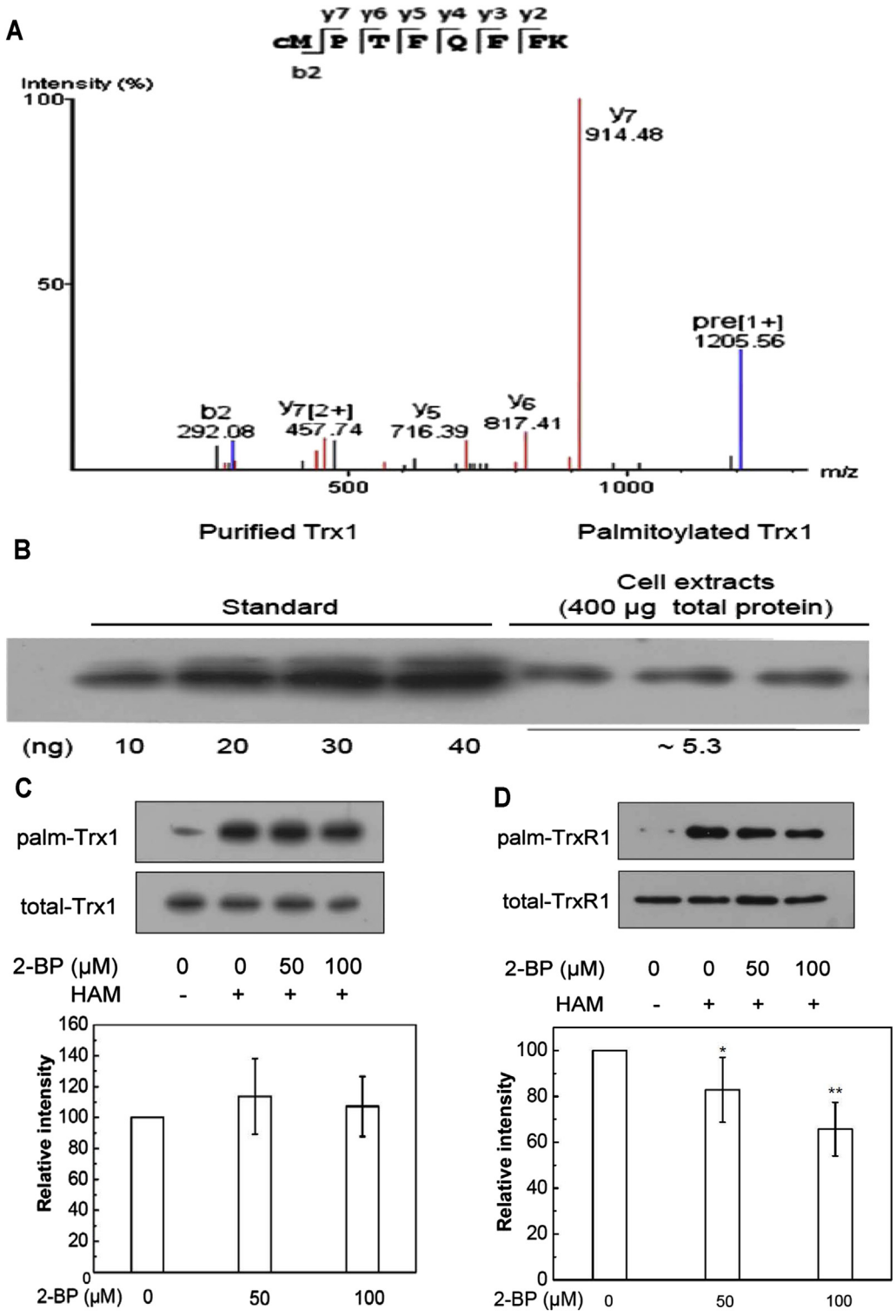

Fig. 1. S-Palmitoylated Trx1 in endothelial cells. (A) S-palmitoylated proteins were isolated from cell lysates by Acyl-RAC assay, from which S-palmitoylated Trx1 was isolated by immunoprecipitation, and analyzed by LC-MS/MS. (B) Level of S-palmitoylated Trx1 in the cells was estimated by immunoblots and standard curves with purified Trx1. The calculated amounts of palmitoylated Trx1 was estimated by densitometry analyses. (C,D) The cells were treated with 2-BP for 12 h. S-Palmitoylation of Trx1/TrxR1 was analyzed by Acyl-RAC assay. Palm-Trx1/TrxR1, S-palmitoylated Trx1/TrxR1; *, $P \leq 0.05$; **, $P \leq 0.01$.

3.3. Long-chain Acyl-CoA synthetase mediated the insulin effect

Since insulin may promote the expression of long-chain acyl-CoA synthetase (ACSL) [24], the latter is critical for the activation of palmitate to palmitoyl-CoA. When the cells were treated with 5 μM Triacsin C (an inhibitor of ACSL) for 24 h, a significant decrease in the levels of S-palmitoylated Trx1 was observed, which could be hardly protected if insulin was present in the cell cultures (Fig. 3A). This finding supports a physiological role of ACSL in mediating the insulin effect on Trx1 S-palmitoylation (Fig. 3D).

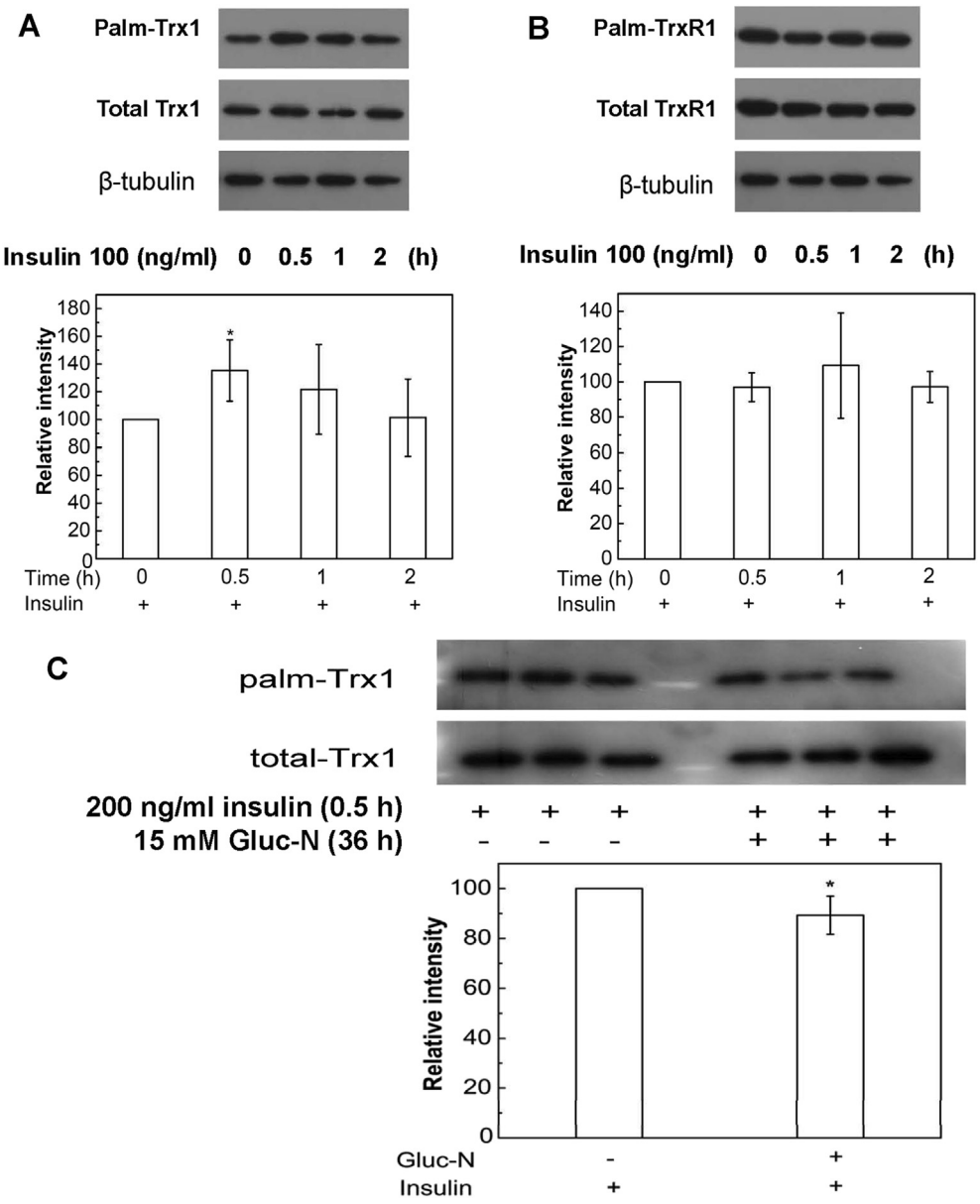

Fig. 2. Effect of insulin on the level of S-palmitoylated Trx1 in the cells. (A,B) The cells were treated with insulin before subjected to Acyl-RAC assay. Captured proteins were analyzed by Western blotting. Upper panel, a representative result of Western blot using anti-Trx1 monoclonal antibody/anti-TrxR1 monoclonal antibody, respectively. Lower panel, quantification of the protein bands by scanning densitometry and expressed as a percentage of the insulin-untreated sample. (C) Upper panel, Western blot. Palm-Trx1 was detected by Acyl-RAC. Lower panel, quantification of the protein bands. Data are means ± SEM ($n = 3$). Palm-Trx1/TrxR1, S-palmitoylated Trx1/TrxR1; *$P < 0.05$.

Recently, fatty acid synthase (FAS) was reported to participate in protein palmitoylation [25,26], and insulin was reported to acutely decrease FAS activity [27]. To address the relationship between FAS activity and level of S-palmitoylated Trx1, tannin acid (TA) and α-mangostin which are inhibitors of FAS [28,29] were used. Although α-mangostin exhibited an inhibitory effect on Trx1 expression, the proportion of S-palmitoylated Trx1 in total Trx1 was not decreased, but instead increased (Fig. 3B). TA did not show any effect either on Trx1 S-palmitoylation or on Trx1 expression (Fig. 3C). These data preclude the role of FAS in mediating effect of insulin on Trx1 S-palmitoylation, as illustrated by Fig. 3D.

3.4. Characters of S-palmitoylated Trx1

Since acetyl-CoA is also important in metabolic reaction, and may participate thiol-thioester exchange. To compare the rate of thiol in Trx1/palmitoyl-CoA exchange with that of thiol in Trx1/acetyl-CoA exchange, NADH fluorescence-based assay was used [17]. In this assay system, HSCoA generated from the reaction of Trx1 with palmitoyl-CoA or acetyl-CoA serves as cofactor of α-ketoglutarate dehydrogenase (α-KDH): α-ketoglutarate + NAD + HSCoA → succinyl-CoA + CO_2 + NADH. Fluorescence of NADH was then measured. When a Trx1 to palmitoyl-CoA molar ratio was 1:1, an increase in NADH was detected in a time-dependent manner, and yet NADH generation was not detected when palmitoyl-CoA was replaced with acetyl-CoA (Fig. 4A). Apparently, no thiol-thioester exchanges occur between Trx1 and acetyl-CoA. This indicates that S-palmitoylation of Trx1 would not be influenced by acetyl-CoA.

To examine the effect of S-palmitoylation on Trx1 conformation, ANS was incubated with native, S-palmitoyl-CoA/acetyl-CoA treated Trx1, respectively. ANS is commonly used extrinsic

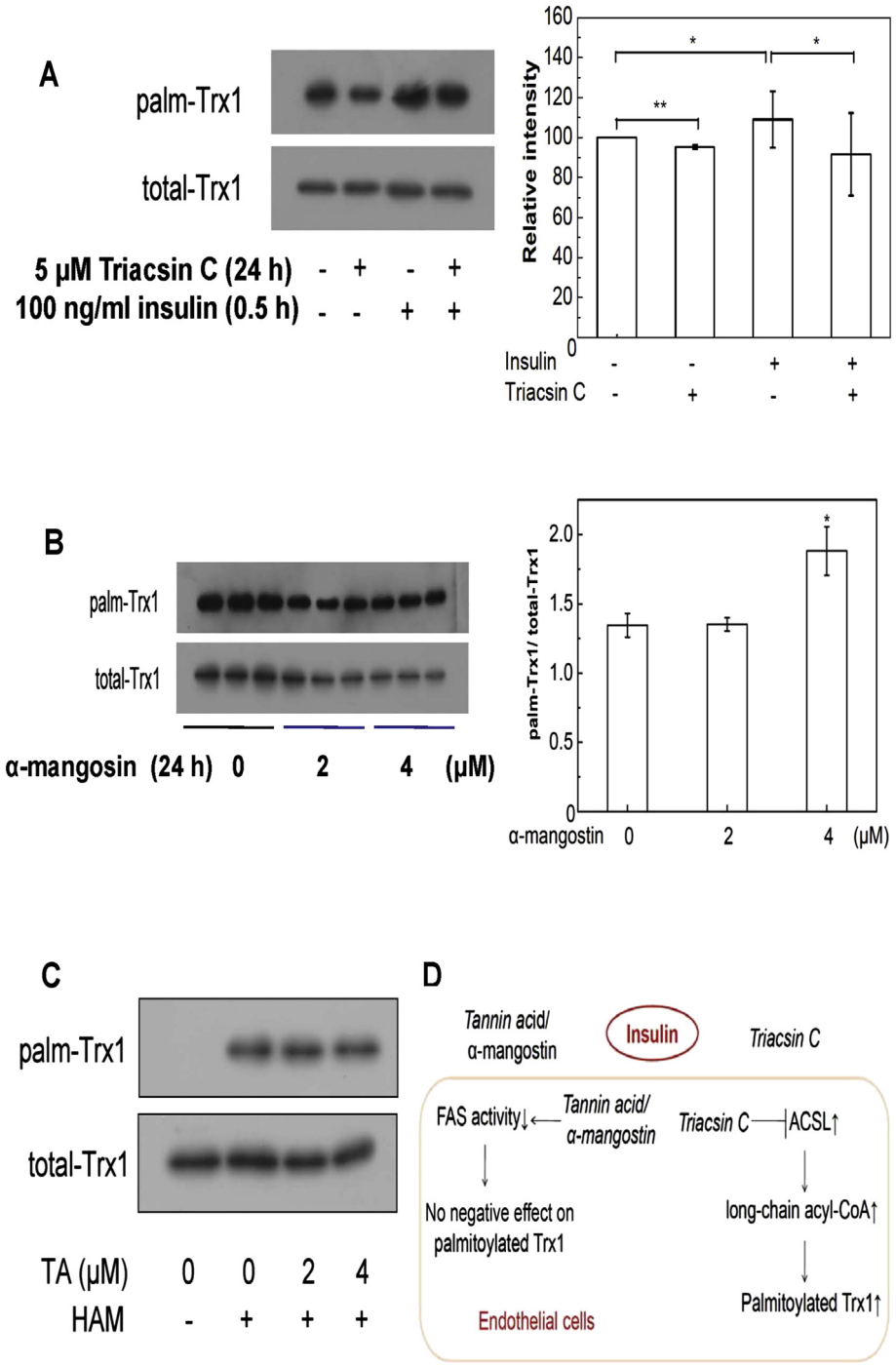

Fig. 3. ACSL mediated the effect of insulin on S-palmitoylation of Trx1. S-palmitoylated Trx1 was analyzed by Acyl-RAC assay, followed by Western blotting. (A) The inhibitory effect of Triacsin C was hardly attenuated by insulin. (B,C) α-mangostin or TA appeared no inhibitory effects. (D) Illustration for ACSL to mediate the insulin effect on the levels of palmitoylated Trx1 in the cells. Palm-Trx1, S-palmitoylated Trx1; *, $P < 0.05$; **, $P < 0.01$.

fluorescent probe that selectively binds to the partially folded and molten globule states of a protein. The binding of ANS is accompanied by a blue shift of the emission maximum (from ~530 nm to ~475 nm) and an increase in the quantum yield of ANS [30]. Under these conditions, incubation of ANS with palmitoyl-CoA treated Trx1, but not with native or acetyl-CoA treated Trx1, led to an increase in the fluorescence intensity of ANS by ~6.6-fold at ~480 nm, accompanied by blue-shift in λ_{max} of ANS from ~510 nm to ~480 nm (Curves 2,3 in Fig. 4B). Such an enhancement induced by palmitoyl-CoA was no longer present if S-palmitoylated Trx1 was treated with HAM (Curve 4 in Fig. 4B) that can break thioester bond [16]. These data confirm that palmitoylation-induced an significant alteration in Trx1 conformation.

Next circular dichroism (CD) spectroscopy was used to measure the possible differences in the secondary structure of native Trx1 *versus* S-palmitoylated Trx1. Native Trx1 displayed a characteristic spectrum with minimum at 221 nm (Fig. 4C). Although the spectrum of S-palmitoylated Trx1 appeared to be similar to that of

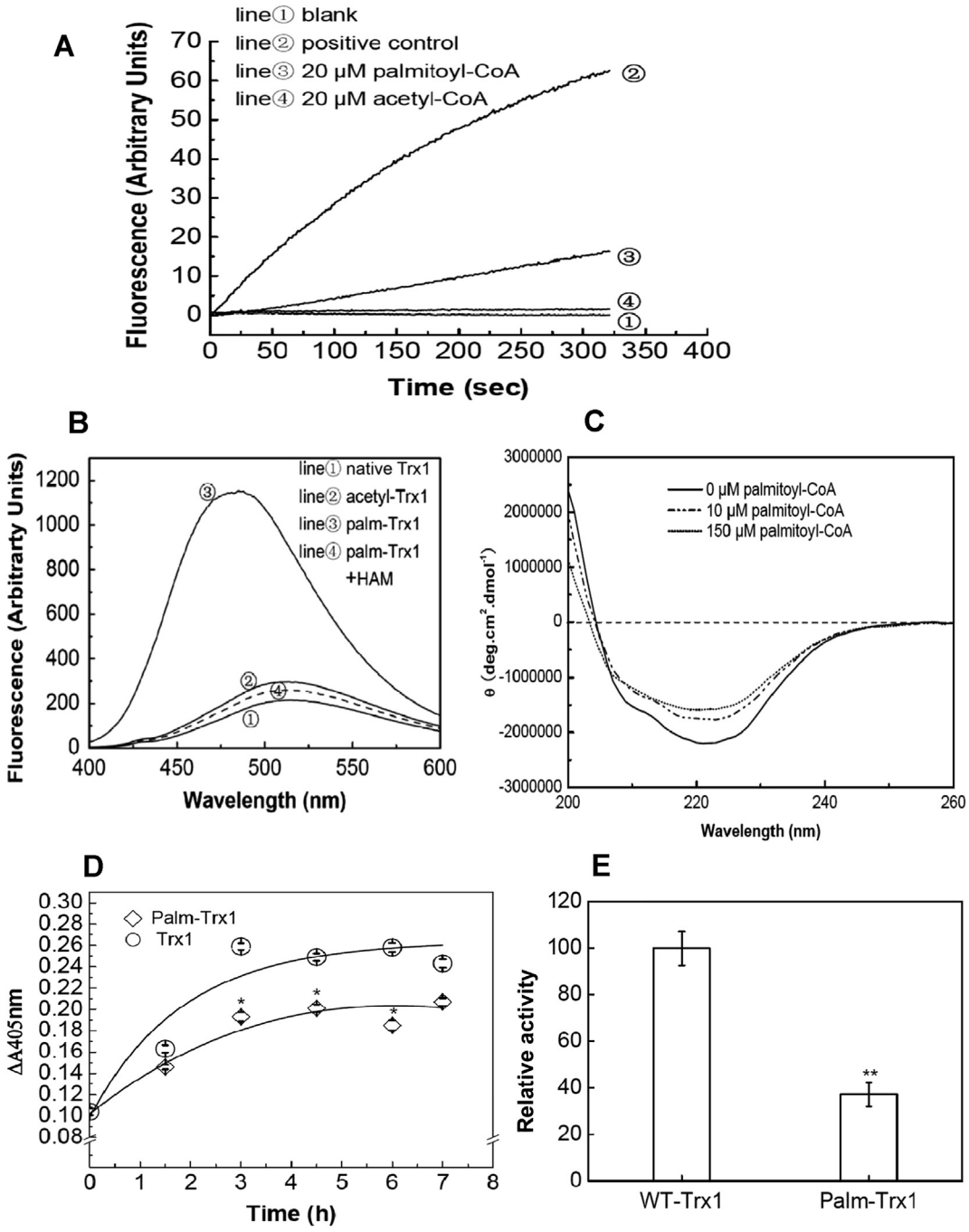

Fig. 4. Characters of S-palmitoylated Trx1. (A) Detection of thiol-thioester exchange between Trx1 and palmitoyl-CoA or acetyl-CoA using fluorescence-based coupled assay. Please see "Materials and methods" for details. Line ①, buffer. Line ②, 20 μM HSCoA as positive control. (B) The ability of ANS binding to Trx1 was significantly increased after Trx1 modified by palmitoyl-CoA rather than acetyl-CoA. Line ①, native Trx1. (C) CD spectra of native Trx1 and S-palmitoylated Trx1. (D) Effect of Trx1 S-palmitoylation on PTP1B-SNO reduction activity. (E) Effect of Trx1 S-palmitoylation on GSNO reduction activity. The data in (D,C) represent the means ± SEM from three independent experiments. Palm-Trx1, S-palmitoylated Trx1; *, $P < 0.05$; **, $P < 0.01$.

native Trx1, the mean residue ellipticity of this signal was lower for S-palmitoylated Trx1 in an S-palmitoyl-CoA concentration-dependent manner (Fig. 4C). Similar changes in CD spectrum of Trx1 were confirmed to correspond to loss of the C-terminal helix strand [31]. From these observations, we proposed a decrease in α-helix strands for S-palmitoylated Trx1.

Since Cys73 of Trx1 is also involved in transnitrosylation [32], we tested whether S-palmitoylation influences the denitrosylation activity of Trx1. Using PTP1B-SNO as a model of S-nitrosylated protein, we showed that the ability for Trx1 system containing native Trx1 to recover the phosphatase activity of PTP1B-SNO was much higher than that for Trx1 system containing S-palmitoylated Trx1 instead of Trx1 (Fig. 4D). Similarly, S-palmitoylation led to a significant decrease in the ability of Trx1 to transfer electrons from NADPH to GSNO *via* TrxR1 (Fig. 4E).

4. Discussion

For Trx1 in living endothelial cells, we show here S-palmitoylation of Trx1 at Cys73, strengthening *in vitro* observation that S-

palmitoylation occurred at the structural Cys residues of Trx1 [5]. In experiments to clarify whether or not S-palmitoylation affects all Trx1, we found that about 0.8% of Trx1 was modified by S-palmitoylation under basal conditions. The dynamic response of S-palmitoylated Trx1 to insulin is consistent with a regulatory effect of insulin on Trx1 by S-palmitoylation. There are two more lines of evidence for insulin signaling involved in regulation of Trx1 S-palmitoylation. First, blocking the response of the cells to insulin by Gluc-N, insulin-induced effect on Trx1 S-palmitoylation was significantly attenuated. Second, insulin was reported to increase ACSL activity that converts free fatty acids into long-chain acyl-CoA [33]. In our study, we observed the reduced level of S-palmitoylated Trx1 in the presence of Triacsin C, an inhibitor of ACSL, which could at least partially offset the positive effect of insulin on Trx1 S-palmitoylation (Fig. 3A). Although previous studies demonstrated a role of FAS in eNOS/mucin 2 palmitoylation [25,26], the activity of FAS under our study conditions did not show its contribution to Trx1 S-palmitoylation (Fig. 3B,C). It is likely that ACSL mediates the effect of insulin on Trx1 S-palmitoylation. Thus, insulin in the presence of intact signaling would have a regulatory role in the levels of Trx1 S-palmitoylation in endothelial cells under normal conditions. Generally, levels of Trx1 S-palmitoylation were higher under high glucose and high palmitate conditions than those under basal conditions [5]. It seems likely that under insulin-resistant conditions, insulin loses its ability to regulate Trx1 S-palmitoylation. For instance, the mice with high-fat diet-induced insulin resistance showed a significant increase in the proportion of S-palmitoylated Trx1 in total Trx1 [4].

In the present study, we also analyzed the effect of S-palmitoylation on biochemical and structural properties of Trx1. Trx1 is critical for Trx1 system to catalyze the reduction of ribonucleotide reductase that is involved in cell survival [10], and protect PTP1B from oxidative inactivation [34,35]. The transient inactivation of PTP1B contributes to the efficiency and temporal control of insulin signaling [36]. Thus, the results from this study add important information to our previous study [5] and reveal potential biochemical and structural mechanisms involved in the dysfunction of Trx1 under insulin-resistant conditions [4]. The major findings are: i) modification of Trx1 resulted in an alteration in conformation of this protein; ii) S-palmitoylation of Trx1 reduced its α-helix content; iii) the ability of Trx1 system to reactivate PTP1B-SNO was specifically impaired by Trx1 S-palmitoylation; iv) Trx1 S-palmitoylation lowered the activity of Trx1 system in cleavage of GSNO.

Trx1 primarily reacts with palmitoyl-CoA through structural Cys residues [5] including Cys73. The latter is responsible for Trx1 transnitrosylation [32]. S-palmitoylated Trx1 is ineffective in transferring electrons from TrxR to insulin [5], PTP1B-SNO and GSNO. Therefore one reason for the compromised ability of S-palmitoylated Trx1 to reduce substates could be due to increased space steric hindrance of Trx1. This is in line with our previous observation that in the experiment with the structural Cys residues modified by iodoacetamide that is small and water soluble, the resulting Trx1 remains the ability to transfer electrons from TrxR to substrate [5].

Moreover, our data show that the structural Cys residues of Trx1 are susceptible to palmitoyl-CoA, but not to acetyl-CoA (Fig. 4A). This selectivity indicates that hydrophobic region(s) around these Cys residue may be involved in binding to palmitate moiety. Since S-palmitoylated Trx1 could be separated as a soluble protein from liver tissue [4] and S-palmitoylated Trx1 remains soluble, the palmitate moiety might insert into a hydrophobic crevice of Trx1. As the structural Cys residues are located close to helix α3 [11], their modification possibly influences stability of helix α3, whereas unraveling of helix α3 has been demonstrated to give rise to an elongated hydrophobic pocket [37]. This may help explain the observation shown in Fig. 4B.

In summary, S-palmitoylation could modulate Trx1 *via* influencing conformation state and functional state, so that Trx1 may respond to nutrient state in such a unique way. The emerging picture is that S-palmitoylation, like S-glutathionylation and S-nitrosylation, is an important post-translational modification of human Trx1.

Conflict of interest

None.

Acknowledgments

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Transparency document

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