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TRAM1 protect HepG2 cells from palmitate induced insulin resistance through ER stress-JNK pathway

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ABSTRACT

Excess serum free fatty acids (FFAs) are fundamental to the pathogenesis of insulin resistance. Chronic endoplasmic reticulum (ER) stress is a major contributor to obesity-induced insulin resistance in the liver. With high-fat feeding (HFD), FFAs can activate chronic endoplasmic reticulum (ER) stress in target tissues, initiating negative crosstalk between FFAs and insulin signaling. However, the molecular link between insulin resistance and ER stress remains to be identified. We here reported that translocating chain-associated membrane protein 1 (TRAM1), an ER-resident membrane protein, was involved in the onset of insulin resistance in hepatocytes. TRAM1 was significantly up-regulated in insulin-resistant liver tissues and palmitate (PA)-treated HepG2 cells. In addition, we showed that depletion of TRAM1 led to hyperactivation of CHOP and GRP78, and the activation of downstream JNK pathway. Given the fact that the activation of ER stress played a facilitating role in insulin resistance, the phosphorylation of Akt and GSK-3β was also analyzed. We found that depletion of TRAM1 markedly attenuated the phosphorylation of Akt and GSK-3β in the cells. Moreover, application with JNK inhibitor SP600125 reversed the effect of TRAM1 interference on Akt phosphorylation. The accumulation of lipid droplets and expression of two key gluconeogenic enzymes, PEPCK and G6Pase, were also determined and found to display a similar tendency with the phosphorylation of Akt. Glucose uptake assay indicated that knocking down TRAM1 augmented PA-induced down-regulation of glucose uptake, and inhibition of JNK using SP600125 could block the effect of TRAM1 on glucose uptake. These data implicated that TRAM1 might protect HepG2 cells against PA-induced insulin resistance through alleviating ER stress.

1. Introduction

The incidence of type 2 diabetes (T2D) worldwide has increased evidently and constitutes one of the major threats to public health. Insulin resistance is a hallmark of T2D [1]. Increasing evidence suggests that chronic endoplasmic reticulum (ER) stress in the liver has emerged as a major contributor to obesity-induced insulin resistance and diabetes mellitus [2]. However, the molecular mechanisms linking obesity-induced insulin resistance and ER stress remains not fully understood.

The ER is the principal organelle responsible for various cellular functions including protein folding and maturation, and the maintenance of cellular homeostasis [3]. ER plays an important role in the regulation of protein synthesis, folding and transport, calcium homoeostasis, and lipid synthesis [4]. Malfunction of ER has been linked to various human diseases. Under a variety of pathological conditions, aberrant ER functionality leads to rapid accumulation of unfolded proteins in the organelles, triggering the activation of unfolded protein response (UPR), termed as ER stress [3]. ER stress induces the activation of several downstream pathways, including GRP78 (78 kDa glucose-regulated protein precursor), PERK (protein kinase R-like ER kinase)/eIF2α (eukaryotic initiation factor 2 a kinase) and CHOP(C/EBP homologous protein) pathways. Therefore, expression of GRP78 and CHOP could be used as the markers of ER stress [5].

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In our study, we addressed the role TRAM1 (translocation associated membrane protein 1), a protein involved in translocation of nascent polypeptides and the dislocation of a type I membrane protein under viral influences, during an ER stress response [6]. It encodes a multi-pass membrane protein that is part of the mammalian endoplasmic reticulum. Previous reports have shown that TRAM1 up-regulated under conditions of ER stress [7]. TRAM1 participates in the stability of ER membrane degradation substrates, but not participates in degradation of soluble substrates. Studies have identified that TRAM1 participates in human cytomegalovirus US2- and US11-mediated dislocation of an endoplasmic reticulum membrane glycoprotein [8]. TRAM1 knockdown cells also robustly induce NF-κB activity [6]. Above all, TRAM1 is characterized for its role in relieving ER stress response. Decreasing TRAM1 expression can highly activate UPRE [6].

However, whether TRAM1 prevents excess nutrient-induced hepatocyte's insulin resistance through alleviating ER stress remains unknown. The present study was designed to elucidate the mechanism. Hyperactivation of JNK through phosphorylation is another marker of ER stress and plays a role in linking ER stress and insulin resistance [5]. In this study, we found that abundant TRAM1 resides in the human hepatocarcinoma HepG2 cells and protects HepG2 cells against PA-induced insulin resistance through alleviating ER stress-JNK signaling. TRAM1 can protect HepG2 cells from PA-induced suppression of glucose uptake, increase of gluconeogenesis and intracellular lipid accumulation.

2. Materials and methods

2.1. Reagents and antibodies

Pharmacological inhibitor SP-600125 was purchased from Sigma (Aldrich, St. Louis, MO, USA). Antibodies anti-phospho-Akt (Ser473), anti-phospho-GSK3β and anti-phospho-JNK were purchased from Cell Signaling, anti-Akt, anti-GSK3β, anti-JNK, anti-TRAM1, anti-CHOP, and anti-GRP78 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GAPDH antibody was purchased from Sigma.

2.2. Fatty acid preparation

FFAs were prepared with the methods of protein adsorption: A 100 mM stock of PA was prepared in 0.1 nM NaOH by heating to 70 °C. PA was then complexed with BSA at a 1:1 M ratio to make a 50 mM working stock via dropwise addition to 10% endotoxin/fatty acid-free BSA, while vortexing. This conjugation is needed to increase PA solubility. The PA/BSA mixture was sterile filtered before use and kept at −20 °C.

2.3. Cell culture and treatments

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT, USA).The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed every other day. For mRNA and protein analysis, HepG2 cells were cultured for 24 h in DMEM supplemented with 1% FBS in the presence or absence of PA (25 mM). Before harvest, HepG2 cells were treated with insulin (100 nM) for 20 min.

2.4. Mice

Male C57BL/6J mice were approved by the Experimental Animal Center of Nantong University. Mice were housed in groups at 22–24 °C on a 12 h light/dark cycle with free access to water and given a normal diet (ND) or high fat diet (HFD) containing 50% carbohydrate, 20% protein and 25% fat for 10 weeks. All animal procedures were followed the National Institutes of Health Animal Care and Use Guidelines.

2.5. Quantitative reverse transcription-PCR (qRT-PCR)

Quantitative real time PCR was performed with SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Otsu, Shiga, Japan). The Roche Light Cycler 480 System (Roche Diagnostics, Burgess Hill, UK) was used for analysis. Target gene mRNA level was normalized to that of GAPDH in the same sample. The primers used for qRT-PCR assay were as follows: TRAM1 (F) 5′-CTGGTCAGCTTAGTGCGTTC-3′; (R) 3′-CTCCAGCAATGTGGAAGAGG-5′; GAPDH, (F)5′-TGATGACATCAAGAAGGTGGTGAAG-3′ and (R)5′-TCCTTGGAGGCCATGTGGGCCAT-3′.

2.6. Measurement of intracellular lipid

The intracellular lipid contents of cultured HepG2 cells were evaluated by Oil Red O staining. Briefly, the cells were washed gently twice with ice-cold PBS (pH 7.4) and fixed with 10% formalin at room temperature for 1 h in room temperature. After removal of the 10% formalin, wells were washed exhaustively with PBS. Wells were allowed to dry completely before the addition of filtered Oil Red O solution for 30 min at room temperature. The staining of lipid droplets in HepG2 cells were exhaustively rinsed three times with PBS. Stained oil droplets were extracted with 100% isopropanol for 10min to quantify intracellular lipids.

2.7. Glucose uptake assay

Insulin-stimulated glucose uptake in HepG2 cells was determined by measuring glucose in various samples using Glucose Colorimetric/Fluorometric Assay Kit (BioVision). Briefly, HepG2 cells were treated as described. And, then stimulation with insulin (100 nM) for 20min. Add 30 μl test samples to a 96-well plate. Adjust the volume to 50μl/well with Glucose Assay Buffer. Mix enough reagents for the number of assays to be performed: For each well, prepare a total 50 μl Reaction Mix containing Glucose Assay Buffer 46 μl, Glucose Probe** 2 μl, Glucose Enzyme Mix 2 μl. Mix well. Add 50 μl of the Reaction Mix to each well containing the Glucose Standard and test samples. Mix well. Incubate the reaction for 30 min at 37 °C, protect from light. Measure absorbance (OD 570 nm) or Fluorescence (Ex/Em = 535/590 nm) for in a microplate reader.

2.8. Small interfering RNA (siRNA) and transfection

For targeting of human TRAM1, a custom-designed siRNA was chemically synthesized and annealed (Genechem, Shanghai, China). TRAM1siRNA and control siRNA were transfected by Lipofectamine 2000 (Invitrogen, Shanghai, China) and Plus reagents in OptiMEM (Invitrogen, Shanghai, China) as suggested by the manufacturer. Twenty hours after transfection of siRNA, 25 mM PA-BSA was added to treat the HepG2 cells for 24 h. Before harvest, HepG2 cells were treated with insulin (100 nM) for 20min.

2.9. Western blot analysis

After the indicated treatments, cells were washed three times with cold phosphate-buffered saline (PBS) and lysed in a cell lysis buffer for 30 min on ice. The lysates were then centrifuged at 12,000 rpm, 4 °C for 15 min. The protein concentration of the

supernatant obtained was measured by the Bradford assay (Bio-Rad, Hercules, CA, USA), equal amounts of supernatant from each sample were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membrane was blocked in 20 mM Tris·HCl, 137 mM NaCl, and 0.1% Tween 20 (pH 7.4) containing 5% nonfat milk for 2 h at room temperature. Then the membranes were immunoblotted with primary antibodies for 6–8 h followed by incubation with a horseradish peroxidase secondary antibody (1:2000; Southern-Biotech) for 1–2 h. And then, they were visualized using an enhanced chemiluminescence system (ECL; Pierce Company, Woburn, MA, USA). ImageJ (NIH) was used to analyze the densities of the bands.

2.10. Statistical analyses

All data are expressed as mean ± SEM. Differences between experimental groups were performed using a one-way analysis of variance (ANOVA), followed by individual post hoc comparisons. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. TRAM1 was up-regulated in insulin-resistant livers and hepatocytes

To track with whether TRAM1 played a role in the regulation of insulin resistance, we first examined the expression of TRAM1 in control and insulin-resistant liver tissues (Fig. 1A). SD rats were fed with high-fat dietary for 12 weeks.

To further determine the role of TRAM1, we then analyzed the expression of TRAM1 in insulin-resistant hepatocytes. Human HepG2 cells were treated with PA to induce insulin resistance [9]. Before harvest, the cells were treated with insulin (100 nM) for 20 min. Insulin-stimulated phosphorylation of Akt on Ser473 residues, the commonly used marker of insulin signaling, was evaluated. PA suppressed insulin-induced phosphorylation of Akt on Ser473 at 24 h (Fig. 1B). Consistently, the phosphorylation of Akt downstream target GSK-3β was also suppressed at 24 h (Fig. 1B). We found that PA-induced TRAM1 was up-regulated (Fig. 1B). But insulin or BSA alone did not induce TRAM1. We also measured the mRNA levels of TRAM1 in HepG2 cells treated with PA (Fig. 1C). And these results are consistent with the results shown in Fig. 1A and B.

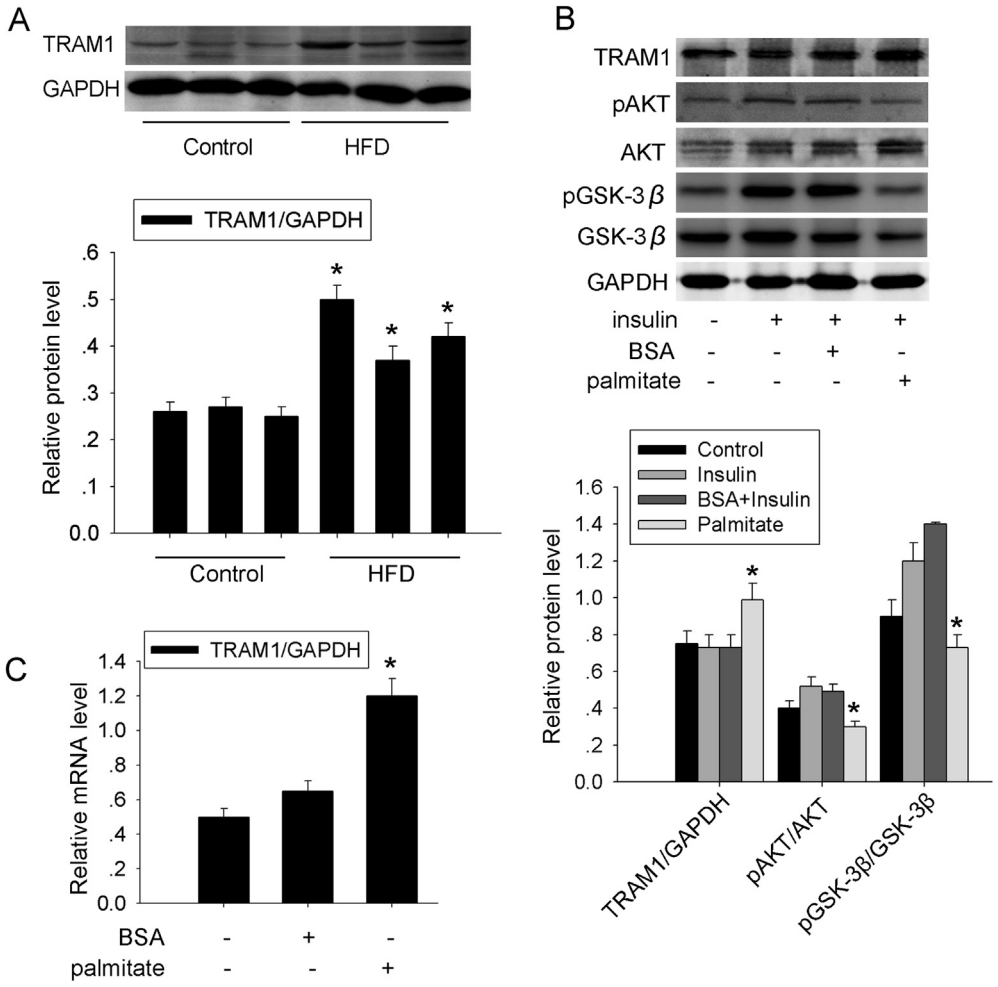

Fig. 1. TRAM1 was up-regulated in insulin-resistant livers and hepatocytes. A: The protein levels of TRAM1 in HFD mice detected by Western blotting and quantitative analysis of the intensity of protein expression relative to GAPDH in the indicated groups. B: The protein levels of TRAM1 levels and Insulin-stimulated phosphorylation of AKT and GSK-3β in PA-induced HepG2 cells and quantitative analysis of the intensity of protein expression in the indicated groups. C: TRAM1 mRNA levels in HepG2 cells were detected by qRT-PCR. Values were mean ± SEM ($n = 3$, $^*p < 0.05$, significantly different from respective controls).

3.2. TRAM1 protect HepG2 cells from PA-induced insulin resistance

To further explore whether TRAM1 plays a functional role in PA-induced insulin resistance in HepG2 cells, we used a gene silencing approach targeting TRAM1. As depicted in Fig. 2A, Four independent siRNAs were tested for their abilities to knockdown TRAM1 after stable transduction into HepG2 cells. Compared with scrambled siRNA, TRAM1 siRNA4 was most effective, with 30% knockdown. So we used TRAM1siRNA4 to define the importance of endogenous levels of TRAM1 in regulating PA-induced insulin resistance. Depletion of TRAM1 resulted in 30% decrease in PA-induced insulin resistance. This was demonstrated by decreased of insulin-induced phosphorylation of Akt on Ser473 and GSK3β (Fig. 2B). These data demonstrate that endogenous levels of TRAM1 protect HepG2 cells against PA-induced insulin resistance.

3.3. The protection of TRAM1 to HepG2 cells is through ER stress-JNK pathway

Chronic ER stress in the liver is a major contributor to obesity-induced insulin resistance. We used free fatty acid PA to induce the insulin resistance of hepatocyte and trigger ER stress. In PA-induced HepG2 cells, the markers of ER stress including GRP78, CHOP were up-regulated (Fig. 3A). We have known that TRAM1 protect HepG2 cells from PA-induced insulin resistance. And previous studies reported that TRAM1 knockdown cells highly activate UPRE. To further investigate the regulation of the UPR by TRAM1 in HepG2 cells, we introduced exogenous TRAM1siRNA into HepG2 cells and treated infected cells with scramble siRNA or with PA to induce chromic ER stress. As shown in Fig. 3B, Transduction of HepG2 cells with TRAM1siRNA resulted in a significant increase of the UPR regulators CHOP, GRP78. These data indicate that endogenous levels of TRAM1 are critical in protect HepG2 cells from PA-induced insulin resistance and that this protection is mediated through its protection against PA-induced ER stress.

To further explore potential mechanisms underlying the protection of TRAM1 against ER stress-induced insulin resistance in HepG2 cells, we determined whether the protection of TRAM1 might through activation of JNK. Phosphorylation of JNK was increased significantly in cells treated with PA compared with the control group as shown in Fig. 3A. Then we knocked down TRAM1 with TRAM1siRNA, and found that phosphorylation of JNK increased in cells transfected with TRAM1siRNA compared with cells transfected with scramble siRNA (Fig. 3B). We treated HepG2 cells with SP600125, a potent, selective and reversible inhibitor of JNK [10]. From Fig. 3C, we found that pAKT (ser473) was increased significantly in cells transduction with TRAM1-siRNA treated with SP600125 (20 μM, 30 min) compare to cells transduction with TRAM1siRNA but not treated with SP600125. Thus, we speculated that the protection of TRAM1 might alleviate PA-induced insulin resistance through modulating ER stress-JNK signaling.

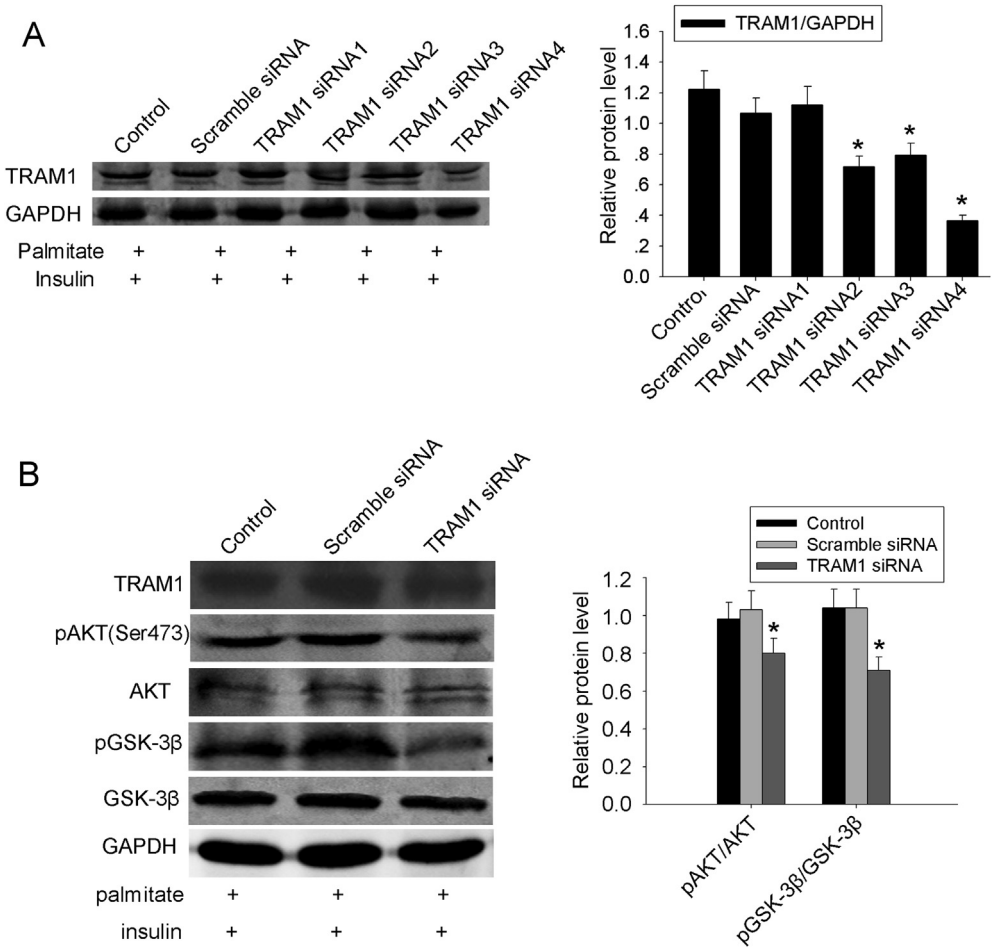

Fig. 2. Knockdown of TRAM1 accelerated PA-induced insulin resistance in HepG2 cells. A: The protein levels of TRAM1 in insulin-resistant HepG2 cells transfected with scramble siRNA or TRAM1siRNA and quantification of TRAM1 levels in the indicated groups. B: TRAM1 down-regulation recused the effects of PA on the activation of AKT and GSK-3β. The TRAM1 levels in the indicated groups were quantificated relative to GAPDH. Values were mean ± SEM (n = 3, *p < 0.05, significantly different from respective controls).

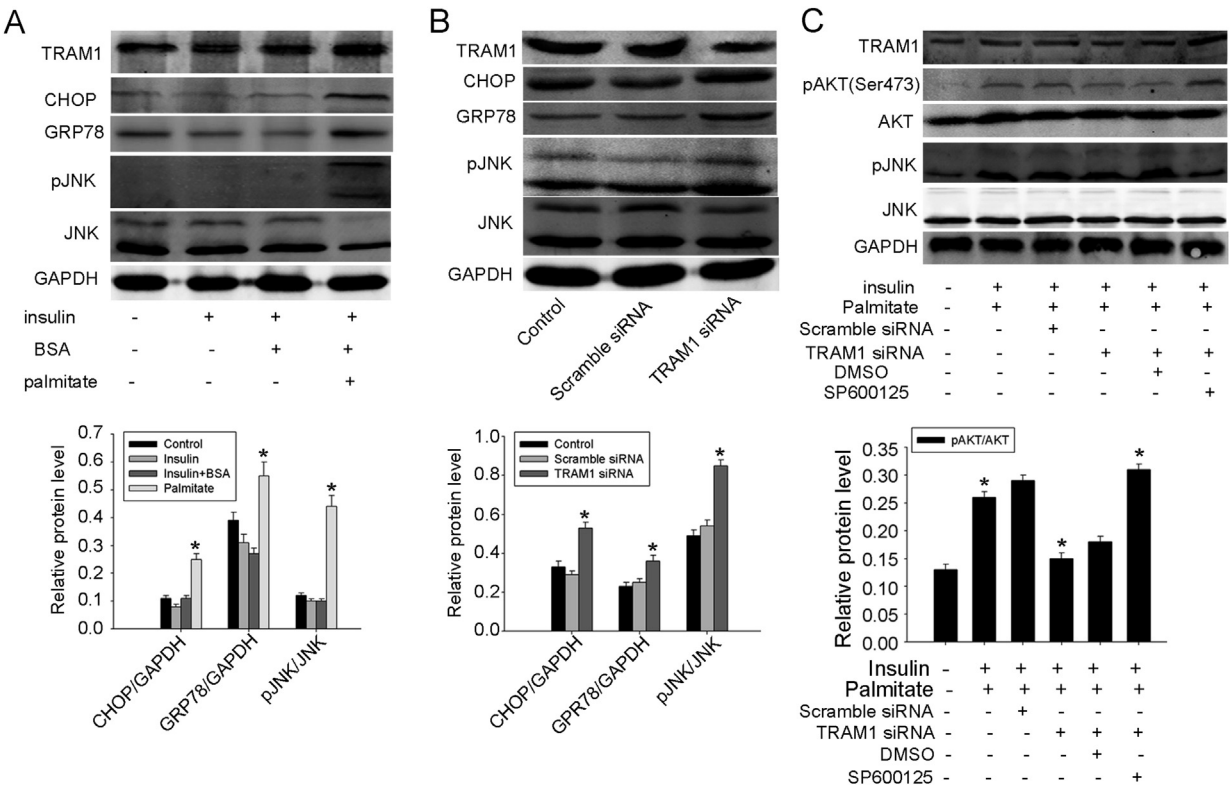

Fig. 3. ER-stress-JNK signaling was required for the protection of TRAM1 against PA-induced insulin resistance in HepG2 cells. A: The protein levels of CHOP and GRP78 in PA-induced HepG2 cells and quantification of protein levels in the indicated groups. B: HepG2 cells were transfected with scramble siRNA or TRAM1siRNA and then incubated in the presence of PA for 24 h. Levels of CHOP and GRP78 were examined by Western analysis and quantified by densitometry. C: Twenty hours after transfected HepG2 cells with TRAM1siRNA, PA was added to treat the cells for 24 h. Pretreated HepG2 cells with SP600125 (20uM) for 30min reversed PA-induced decreased p-AKT. Values were mean ± SEM ($n = 3$, $^*p < 0.05$, significantly different from respective controls).

3.4. TRAM1 protect HepG2 cells from PA-induced intracellular lipid accumulation and gluconeogenesis, and suppression of glucose uptake

As lipid metabolism occurs mainly at the endoplasmic reticulum, where many of the enzymes related to lipid metabolism reside [11]. The ER stress response is a mechanistic link between excess nutrients and lipid accumulation [12]. We determine whether TRAM1 have a role in regulation lipid metabolism. So we also examined intracellular lipid accumulation. Oil red O staining revealed a clear increase in total lipid levels in the cells exposed in PA (Fig. 4C). When TRAM1siRNA was transfected into hepatocyte, the intracellular lipid accumulation even got worse (Fig. 4E). But, the intracellular lipid accumulation of cells transduction with scramble siRNA wasn't change a lot (Fig. 4D). Pretreated HepG2 cells with JNK inhibitor SP600125 reversed PA-induced intracellular lipid accumulation although transfection of TRAM1siRNA (Fig. 4F). So, TRAM1 protect HepG2 cells from PA-induced enhance of intracellular lipid accumulation through ER stress-JNK pathway.

To further explore whether attenuated insulin signaling induced by PA can be protected by TRAM1. We evaluated insulin-induced glucose uptake by measuring insulin-stimulated glucose uptake into the HepG2 cells. PA-induced insulin resistance was confirmed by the lower glucose uptake in the PA-treated HepG2 cells compared to the normal control cells. Insulin treatment induce glucose uptake in the control cells, but was suppressed by about 40% after PA treatment (Fig. 4G). Knockdown TRAM1 of HepG2 cells can even alleviate the suppression of PA on cells' glucose uptake. The effect of TRAM1 on glucose uptake could be blocked by SP600125 (Fig. 4G).

In addition, we analyzed two genes about gluconeogenesis, G6Pase and PEPCK. The data were got through qRT-PCR and then corrected with the corresponding GAPDH mRNA measurement as a surrogate for total mRNA (Fig. 4H). These data are consistent with the effects of PA on insulin signaling shown in Fig. 3.

4. Discussion

Excessive intake of nutrients is a major cause of the metabolic syndrome, including obesity, diabetes, and cardiovascular disease [13]. HFD and obesity are associated with increased flux of FFAs into the circulation, thereby leading to enhanced uptake of the fatty acids into multiple tissues, including the liver [14]. Excessive intake of FFAs causes lipid accumulation in fat tissue, skeletal muscle, and the liver, which leads to the development of obesity and insulin resistance [15]. PA is one of the most abundant saturated fatty acids in plasma and is substantially elevated following an HFD [16]. FFAs activate various signaling pathways that inhibit the intracellular actions of insulin; chief among these is the ER stress response [17]. Thus, identification of the mechanistic link between insulin resistance and ER stress might help to define novel nutritional and pharmacological approaches for the treatment of obesity and diabetes. In the present study, we showed that TRAM1 was up-regulated in insulin-resistant liver tissues and cells, and up-regulated TRAM1 played a protective role to prevent insulin resistance and excessive FFA accumulation through the action of ER stress-induced JNK signaling. Therefore, our data implied that enforced expression of TRAM1 might serve as a promising therapeutic strategy to prevent the onset of insulin resistance and diabetes.

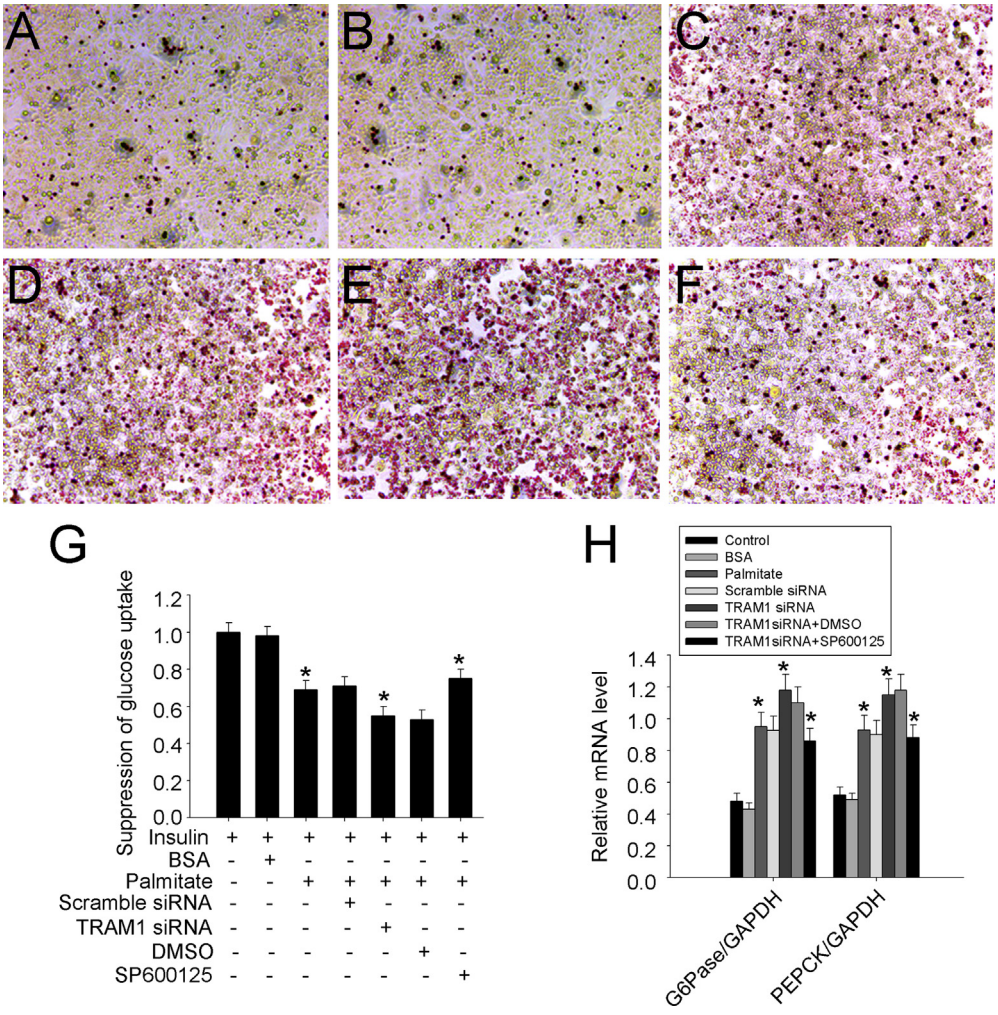

Fig. 4. Knockdown of TRAM1 alleviated PA-induced intracellular lipid accumulation and alleviated PA-induced suppression of glucose uptake and gluconeogenesis in HepG2 cells through activating JNK. Knockdown of TRAM1 Lipid accumulation was determined by oil red staining. Images are representative of results of six independent experiments. (A–C): PA(C) or BSA only (B) or none of these (A) was added to treat the HepG2 cells for 24 h. (D–F)Twenty hours after transfected HepG2 cells with scramble siRNA (D) or TRAM1siRNA (E), 25 mM PA was added to treat the HepG2 cells for 24 h. Pretreated HepG2 cells with SP600125 (F) reversed PA-induced intracellular lipid accumulation. G: The glucose uptake levels of HepG2 cells. H: The mRNA levels of G6Pase and PEPCK in HepG2 cells. Values were mean ± SEM ($n = 3$, $^{*}p < 0.05$, significantly different from respective controls).

Our findings provide mechanistic insights regarding TRAM1 protect HepG2 cells from PA-induced insulin resistance through the ER stress signaling axis to reduce the phosphorylation of Akt on serine473 and more distal GSK-3β, those are essential for the proper biological effects of insulin [18]. ER stress has been linked to insulin resistance. During the pathogenesis of T2DM, ER stress is activated by various factors, for example, excessive intake of glucose, free fatty acids and amino acids [19]. The major role of the ER stress response is to restore ER homeostasis, including refolding of misfolded proteins. However, when the ER stress response is prolonged, insulin resistance can result [17]. However, the precise functional and mechanistic relationships between these processes remain unclear. We use PA, to treat HepG2 cells to mimic the pathogenesis of T2DM. The activation of ER stress induced by PA can impede the insulin signaling pathway, which is indicated by insulin-stimulated phosphorylation of Akt and GSK3β, consequent expression of downstream target-genes and glucose intake. What's more, the activation of ER stress also promotes PA-induced lipid accumulation.

It has been reported that TRAM1-depleted cells displayed highly activated UPRE, through which XBP-1 was spliced and ATF6 was cleaved [6]. Therefore, we proposed the possibility that TRAM1 may regulate insulin resistance through alleviating ER stress. Using Western blot analysis, we fought that TRAM1 was up-regulated in PA-exposed HepG2 cells. Gene silencing of TRAM1 impaired insulin-induced phosphorylation of Akt (Ser473) and GSK3β, as well as glucose intake and lipid accumulation. These results suggested that TRAM1 might serve as a critical regulator of insulin signaling. It remains unclear, however, whether insulin resistance is modulated through the action of TRAM1 on UPRE. We observed that PA exposure induced ER stress response in HepG2 cells, as indicated by up-regulated expression of GRP78, CHOP. In addition, the effect of PA on ER stress was exacerbated by TRAM1 knockdown.

Previous reports shown that hyperactivation of JNK is a key intermediate linking ER stress and insulin resistance [5]. To prove the functional link between TRAM1 and JNK-induced insulin resistance, we used SP600125 to abrogate the activity of JNK. We found that the effect of TRAM1 on insulin resistance was markedly attenuated after JNK inhibition. Therefore, we speculated that TRAM1 might achieve its role in modulating insulin signaling mainly through the suppression of JNK activity.

In summary, we showed that TRAM1 played a protective role in the prevention of FFA-induced hepatic insulin resistance and lipid

accumulation. The effect of TRAM1 on insulin signaling was probably mediated through its negative regulation on ER stress-dependent JNK signaling. These findings highlight the importance of the TRAM1-ER stress-JNK axis in the regulation of insulin resistance and lipid accumulation under nutritional overload conditions, and provide novel insights into the mechanism by which UPRE activation alleviates hepatic lipid accumulation and insulin resistance. These data further support evidence that up-regulation of hepatic TRAM1 contributes to the improvement of insulin and leptin resistance. TRAM1 may have potential merit in the treatment of chronic metabolic diseases, including obesity, T2DM, and cardiovascular disease.

Conflict of interest

The authors declare no conflict of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.027.

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