EBioMedicine 47 (2019) 352–364

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

EBioMedicine
Published by THE LANCET

# Hepatic nitric oxide synthase 1 adaptor protein regulates glucose homeostasis and hepatic insulin sensitivity in obese mice depending on its PDZ binding domain

Kaida Mu [a,b,c,1,2], Yun Sun [a,b,c,2], Yu Zhao [a,b,c], Tianxue Zhao [a,b,c], Qian Li [a,b,c], Mingliang Zhang [a,b,c], Huating Li [a,b,c], Rong Zhang [a,b,c], Cheng Hu [a,b,c], Chen Wang [a,b,c,*,3], Weiping Jia [a,b,c,3]

[a] Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, People's Republic of China
[b] Shanghai Diabetes Institute, Shanghai Jiao Tong University, People's Republic of China
[c] Department of Endocrinology and Metabolism, Shanghai Jiao Tong University Affiliated Sixth People's Hospital, 600 Yishan Road, Shanghai 200233, People's Republic of China

ARTICLE INFO

*Article history:*
Received 11 May 2019
Received in revised form 11 August 2019
Accepted 16 August 2019
Available online 28 August 2019

*Keywords:*
Nitric oxide synthase 1 adaptor protein (NOS1AP)
Insulin sensitivity
p38 MAPK
ER stress

ABSTRACT

*Background:* NOS1AP is an adaptor protein and its SNP rs12742393 was associated with type 2 diabetes (T2D). However, it remains uncertain whether NOS1AP plays a role in regulation of insulin sensitivity. Hepatic insulin resistance contributed to the development of T2D. Here, our investigation was focused on whether NOS1AP is involved in the regulation of hepatic insulin sensitivity and its underlying mechanisms.
*Methods:* Liver specific NOS1AP condition knockout (CKO) and NOS1AP overexpression mice were generated and given a high fat diet. SNPs of *NOS1AP* gene were genotyped in 86 human subjects.
*Findings:* NOS1AP protein is expressed in human and mouse liver. CKO mice exhibited impaired pyruvate, glucose and insulin tolerance, and increased lipid deposits in the liver. Conversely, NOS1AP overexpression in livers of obese mice improved pyruvate and/or glucose, and insulin tolerance, and attenuated liver lipid accumulation. Moreover, hepatocytes from CKO mice exhibited an elevated glucose production and mRNA expressions of *Pc* and *Pck1*. Overexpression of NOS1AP potentiated insulin-stimulated activation of IR/Akt in livers from obese mice. The insulin sensitizing effect of NOS1AP could be mimicked by overexpression of C-terminal domain of NOS1AP in ob/ob mice. Furthermore, NOS1AP overexpression in liver significantly inhibited p38 MAPK phosphorylation, and maintained ER homeostasis through p-eIF2a-ATF4-CHOP pathway. Subjects with rsl2742393 of *NOS1AP* have higher risk to develop hepatic steatosis.
*Interpretation:* Our data demonstrate a novel role of NOS1AP in regulating hepatic insulin sensitivity and p38 MAPK inactivation in obese mice, which makes NOS1AP a potential therapeutic target for the prevention and treatment of T2D.
*Fund:* This work was supported by the National Natural Science Foundation of China (81670707, 31340072) (to C. Wang), and National Basic Research Program of China (Nation 973 Program) (2011CB504001) (to W. Jia).
© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

## 1. Introduction

The liver is an important target organ of insulin and fat accumulation in liver is strongly associated with insulin resistance and the development of type 2 diabetes (T2D) [1]. The mechanisms underlying the pathobiology of hepatic lipid abnormal deposit include PKCε activation [2], inflammatory factors [3,4] and endoplasmic reticulum (ER) stress [5,6]. ER stress activates an adaptive cellular response termed the unfolded protein response (UPR). In mammals, three canonical signaling branches of the UPR act in coordination to relieve ER stress including activating transcription factor 6 (ATF6), ER-resident transmembrane protein inositol-requiring enzyme 1α (IRE1α), and PKR-like endoplasmic reticulum kinase (PERK) [6,7]. Abnormality of the UPR to reestablish ER homeostasis causes numerous metabolic diseases as nonalcoholic fatty liver disease and T2D.

T2D and fatty liver are closely related, both result in increased morbidity and mortality rates. Subjects with fatty liver are more likely to develop T2D than those without [8]. The notion that resolution of fatty liver and improvement of liver lipid metabolism can modify the risk of

* Corresponding author at: Yishan Road, Shanghai 200233, China.
*E-mail address:* wangchen@sjtu.edu.cn (C. Wang).
[1] Present address: Department of Endocrinology and Metabolism, Shanghai University of Medicine & Health Sciences Affiliated Zhoupu Hospital, 1500 Zhouyuan Road, Shanghai 201318, People's Republic of China.
[2] K. M. and Y. S. contributed equally to this work.
[3] C. W. and W. J. contributed equally to this work.

https://doi.org/10.1016/j.ebiom.2019.08.033
2352-3964/© 2019 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

**Research in context**

*Evidence before this study*

Nitric oxide synthase 1 adaptor protein (NOS1AP) is known to possess several domains. We reported previously that *NOS1AP* SNP rs12742393 was associated with type 2 diabetes (T2D), and found in the preliminary study that NOS1AP was highly expressed in liver.

*Added value of this study*

In the present study, we found that liver specific deletion of *nos1ap* gene led to impairment of glucose, insulin, and pyruvate tolerance, and accumulation of lipids in liver from obese mice. In contrast, overexpression of NOS1AP in liver attenuated glucose and pyruvate intolerance, improved insulin sensitivity, and reduced triglyceride content in liver from obese mice. Moreover, overexpression of NOS1AP potentiated insulin-stimulated activation of IR/Akt, and inhibited the phosphorylation of p38 MAPK in the livers from obese mice. The insulin sensitizing effect of NOS1AP could be mimicked by overexpression of C-terminal domain of NOS1AP in ob/ob mice. Furthermore, deletion of NOS1AP enhanced eIF2α phosphorylation and nuclear accumulation of ATF4 and ATF6 proteins, and CHOP protein expressions. Overexpression of this protein in HepG2 cells lowered the expression of p-eIF2a, ATF4 and CHOP induced by FFA. Most importantly, we showed that NOS1AP SNP was associated with fatty liver in Chinese subjects.

*Implications of the available evidence*

NOS1AP protein plays a vital role in regulation of hepatic insulin sensitivity, gluconeogenesis, and p38 MAPK inactivation in hepatocytes of obese mice. These data, together, implicate that NOS1AP can be a therapeutic target for the prevention and treatment of diabetes through improving hepatic insulin sensitivity.

T2D through a liver-specific effect highlights the great needs for identification of therapeutic targets in liver [9]. The combination of genetic and environmental factors plays an important role in the development of both diseases. With the application of genome-wide association studies, lots of risk gene variants have been identified, which may influence hepatic and peripheral insulin sensitivity, adipogenesis, and β-cell functional mass. We reported previously that single nucleotide polymorphism (SNP) rs12742393 of nitric oxide synthase 1 adaptor protein (*NOS1AP*) was associated with the pathogenesis of T2D in Chinese, and C allele carriers had higher risk for T2D [10]. *NOS1AP* encodes the neuronal nitric oxide synthase (nNOS) adaptor protein. It is highly expressed in brain, smooth muscle and breast, and contains several protein domains [11–13]. The amino terminal phosphotyrosine-binding domain (PTB) of NOS1AP works as a tumor suppressor to regulate Hippo signaling pathway [14], and the carboxyl terminal PDZ binding motif associated with the PDZ domain of nNOS inhibits nitric oxide (NO) synthesis [11,15].

While previous studies have revealed an association of NOS1AP variants with T2D, more work is required to characterize the specific role of NOS1AP in the pathogenesis of diabetes. We found in our preliminary study that NOS1AP was highly expressed in human and mouse liver. To clarify its function in liver, we have generated a knockout mouse model that lacks this protein specifically in the liver (CKO), and hepatic NOS1AP overexpression mouse model. Using these mouse models, we identified the role of NOS1AP in regulation of glucose homeostasis and hepatic insulin sensitivity, and explored its underlying mechanisms.

## 2. Materials and methods

### 2.1. Recombinant adenoviruses, materials and antibodies

Recombinant adenoviruses for the overexpression of full-length NOS1AP, N-terminal 185aa of NOS1AP (NOS1AP-185 N) or C-terminal 125aa of NOS1AP (NOS1AP-125C), and control virus (carrying EGFP) were generated using the Gateway system (Invitrogen, Carlsbad, CA, USA) or AdMax system. Briefly, full-length or N-terminal 185aa, C-terminal 125aa of NOS1AP with HA as a tag was constructed into adenoviral plasmids (pAd CMV/V5-DEST (Invitrogen, Carlsbad, CA, USA) for full-length, mCMV-MCS-RFP-HA (Genomeditech, Shanghai, China) for N-terminal 185aa and C-terminal 125aa of NOS1AP). Multiplicity of infection of the generated adenoviruses was determined by an end-point dilution assay [16]. Forskolin (FK) (Cat# 344270), palmitate (PA) (Cat# P9009) and tunicamycin (TM) (Cat# 654380) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rabbit anti-eIF2α (Cat# 9722, RRID:AB_2230924), anti-phospho-eIF2α (Ser51) (Cat# 9721, RRID: AB_330951), anti-IRE1α (Cat# 3294, RRID:AB_823545), anti-ATF4 (Cat# 11815, RRID:AB_2616025), anti-β-Actin (Cat# 4970, RRID:AB_2223172), anti-IR (Cat# 3025, RRID:AB_2280448), anti-phospho-IR (Tyr1150/1151) (Ca# 3024, RRID:AB_331253), anti-phospho-Akt (Ser473) (Cat# 9271, RRID:AB_329825), anti-GSK3β (Cat# 12456, RRID:AB_2636978), anti-phospho-GSK3β (Ser9) (Cat# 5558, RRID: AB_10013750), anti-phospho-p38 mitogen-activated protein kinase (MAPK) (Thr180/Tyr182) (Cat# 4511, RRID:AB_2139682), anti-p38 MAPK (Cat# 8690, RRID:AB_10999090), mouse anti-C/EBP homologous protein (CHOP) (Cat# 2895, RRID:AB_2089254), anti-Akt (Cat# 2920, RRID:AB_1147620) and anti-Lamin A/C (Cat# 4777, RRID:AB_10545756) were obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-phospho-IRE1α (Ser724) (Cat# NB100–2323, RRID: AB_10145203) was obtained from Novus Biologicals (Littleton, CO, USA). Rabbit anti-ATF6 (Cat# sc-22,799, RRID: AB_2242950), anti-Actin (Cat# sc-1616, RRID: AB_630836) and anti-NOS1AP (Cat # sc-9138, RRID: AB_2251417) were obtained from Santa Cruz (CA, USA). Mouse anti-GAPDH (Cat# KC-5G4, RRID: AB_2493106) were purchased from KangChen Bio-tech Inc. (Shanghai, China).

### 2.2. Human samples

Human pancreas and adipose tissues were collected from patients undergoing resection of benign pancreatic pathology at the Department of General Surgery of Shanghai Jiao Tong University Affiliated Sixth People's Hospital. Human muscle tissues were obtained from the quadriceps femoris muscle of healthy subjects via muscle biopsy. Human liver samples were collected from patients of benign focal hepatic lesions undergoing liver surgery at the Department of Liver Surgery in Fudan University Affiliated Zhongshan Hospital as described previously [17]. Informed consent was given by all subjects. The protocol was approved by the Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital (2018-KY-026 (K)) and the principles of the Declaration of Helsinki was followed.

### 2.3. Animal studies

The homozygous floxed *nos1ap* (exon 3–5) mice on the C57BL/6 background were generated in Shanghai Model Organisms Center, Inc. (SMOC) (Shanghai, China). The mice expressing the Cre recombinase driven by the albumin promoter (*alb-cre*) (Cat# jax003574, RRID: IMSR_JAX:003574) were also obtained from SMOC. These mice were used for the generation of liver specific NOS1AP condition knockout (CKO) mice (Supplementary Fig. S1). The genotype was determined by PCR using primers that recognize the *alb-cre* (Forward, 5′-TGGCAA

ACATACGCAAGGG-3′; Reverse, 5′-CGGCAAACGGACAGAAGCA-3′) and *nos1ap-flox* (Forward, 5′-TCTCCGTGGTATTTAATTCAAATG-3′; Reverse, 5′-CACATGATCAAAACCTCTTCACTA-3′) (Supplementary Fig. S1). ob/ob mice, obtained from Shanghai Laboratory Animal Center (Shanghai, China), were given a chow diet. Male CKO and C57BL/6 male mice at 6 weeks old were fed with a high-fat diet (HFD, 60% kcal fat; Research Diets, New Brunswick, NJ, USA, Cat# D12492) for 8 weeks. The mice were raised in SPF condition at 23 °C ± 1 °C on a 12 h light-dark cycle with free access to food and water. All procedures involving the care and use of animals were carried out in accordance with Shanghai Jiao Tong University Guidelines for the care and use of laboratory animals, and the protocol was approved by the Animal Ethics Committee of the Shanghai Jiao Tong University Affiliated Sixth People's Hospital (DWSY2011-096/DWLL2019-0274). Liver overexpression of NOS1AP, NOS1AP-185N or NOS1AP-125C was achieved by tail-vein- injection of the viruses ($10^8$ plaque-forming units) in ob/ob mice at 9 weeks old and/or HFD-fed male C57BL/6 mice at 14 weeks old. Mice injected with Ad-EGFP were used as controls. Overexpression of NOS1AP had no effect on the liver function (Supplementary Fig. S2). At 14 weeks old (for CKO mice) or 10–14 days after injection (for NOS1AP overexpression mice), mice were conducted with glucose tolerance test (GTT), insulin tolerance test (ITT), pyruvate tolerance tests (PTT), insulin stimulation assay, and in vivo ER stress induction.

GTT or ITT were performed with 6 h fasting, and 1.5 g/kg glucose injection (GTT) or 1.5–2.0 U/kg regular insulin injection intraperitoneally (1.5 U/kg for C57BL/6 and 2.0 U/kg for ob/ob mice) (ITT). PTT were performed with 16 h fasting and 2 g/kg pyruvate injection in C57BL/6 mice and CKO mice. Glucose levels were detected through a glucose monitor. Insulin levels were measured using ELISA kits (Mercodia, Uppsala, Sweden, Cat# 10–1247-01, RRID: AB_2783837).

For in vivo insulin stimulation assay, mice were fasted for 16 h and injected intraperitoneally with regular insulin (5 U/kg). After 10 min, the mice were sacrificed via cervical dislocation and then liver tissues were frozen in liquid nitrogen and kept at −80 °C. For in vivo ER stress induction, TM was injected intraperitoneally at a dose of 1 mg/kg, and the mice were sacrificed after 8 h.

### 2.4. Cell culture, glucose production assay, and adenovirus-mediated gene overexpression

Primary hepatocytes were isolated from male NOS1AP CKO and control mice at 8–12 weeks old according to the procedures reported by Jiang et al. [18] and cultured in DMEM (G5.6) (HyClone, Logan, UT, USA, Cat# SH30021). Glucose production assay was conducted according to He et al. [19]. After 16 h starvation, the cells were washed with PBS, and 20uM FK was added in glucose production medium for 4 h. HepG2 cells (a kind gift from Dr. F. Liu, Department of Cellular and Structural Biology, University of Texas Health Science Center at San Antonio, TX, USA) were maintained in DMEM (G5.6). The cells were infected with 15 multiplicity of infection of adenoviruses carrying *NOS1AP* or EGFP. After 16-18 h, the virus-containing medium was changed, and cells were cultured for additional 24 h before analysis. Adenoviral infected HepG2 cells have no significant effects on cellular viability observed under a fluorescence microscopy after staining with Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA, Cat# B2261) and propidium iodide (Sigma-Aldrich, St. Louis, MO, USA, Cat# P4170).

### 2.5. Quantitative real-time PCR and western blot

Total RNA from liver sample or HepG2 cells was extracted using TRIzol reagent (Ambion, Austin, TX, Cat# 213406) and transcribed into cDNA. Quantitation of mRNA was done using Roche Light Cycler 96 (Roche Ltd., Berlin, Germany) with SYBR Green Real-time PCR Master Mix-Plus (Toyobo, Osaka, Japan, Cat# QPK-212) with an annealing temperature of 60 °C. *Gapdh* was used as an endogenous control for calculation of the relative expression level of each target gene. Primers for each gene are shown in Supplementary Table 1.

For western blot, tissue or cell samples were homogenized in ice-cold RIPA buffer containing protease inhibitors as previously described [20]. Nuclear extracts were extracted using NE-PER nuclear and cytoplasmic extraction kit (Thermo Scientific, Waltham, MA, USA, Cat# 78835). Proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and detected with specific antibodies (1:1000 dilution or according the protocol provided by manufactory). The intensities of bands of each interest protein were quantified by ImageJ and expressed in arbitrary units of optical density.

### 2.6. Hepatic triglyceride content, human genotyping and histology study

Liver tissue was homogenized in heptane-isopropanol-tween mixture (3:2:0.01 by volume). Lipid extracts were prepared as previously described by Hu et al. [21]. 86 human liver samples were genotyped by restriction enzyme PCR analysis with *NOS1AP* allele of rs12742393. All tissue samples had been evaluated by a pathologist who was blinded to the study design. Liver steatosis was graded as 0 (1–5%), 1 (6–33%), 2 (34–66%) and 3 (67–100%) [22]. For hematoxylin & eosin (H&E), liver tissue was fixed in 4% paraformaldehyde and embedded in paraffin wax.

### 2.7. Statistical analysis

Data shown are the means ± SE or ± SD as indicated. Statistical significance of differences was determined using Student's *t*-test, ANOVA followed by Bonferroni's multiple comparison posttests for continuous variables, and Chi-square test for categorical variables. *P* value of <.05 was considered statistically significant.

## 3. Results

### 3.1. NOS1AP protein was expressed in human and mouse liver

To know NOS1AP tissue expression profile, its mRNA levels and protein expressions were examined in different tissues from human and mice. As shown in Fig. 1, NOS1AP mRNA and protein were expressed in human and C57BL/6 mouse fat, liver, muscle and pancreas tissue with strong NOS1AP production in liver. Its protein expression decreased after 16 weeks of HFD (Fig. 1).

### 3.2. Hepatic conditional deletion of NOS1AP impaired glucose and pyruvate tolerance, and insulin sensitivity

To investigate the effect of hepatic NOS1AP on mouse metabolic state, we generated NOS1AP CKO mice. PCR genotyping and western blot analysis confirmed the insertion of *alb-cre* and *nos1ap-flox* (Supplementary Fig. S1), and the disruption of the *nos1ap* gene in liver in these mice (Fig. 2a). NOS1AP CKO and their age-matched littermate control mice (Cre−/−, NOS1AP flox/flox) were fed with an HFD for 8 weeks. Body weight and food intake of CKO mice were not altered when compared with the control mice (Fig. 2b and c). No changes could be viewed in fasting glucose and insulin levels between the two groups (Fig. 2d and e). However, compared with their littermate control mice, NOS1AP CKO mice exhibited impaired glucose and insulin tolerance (Fig. 2f and g). Furthermore, the blood glucose levels of CKO mice increased after pyruvate challenge compared with those of the control littermates (Fig. 2h).

### 3.3. Hepatic overexpression of NOS1AP improved glucose, pyruvate and insulin tolerance in obese mice

To confirm the above findings, adenoviruses carrying NOS1AP or control virus were generated and delivered to ob/ob or C57BL/6 mice

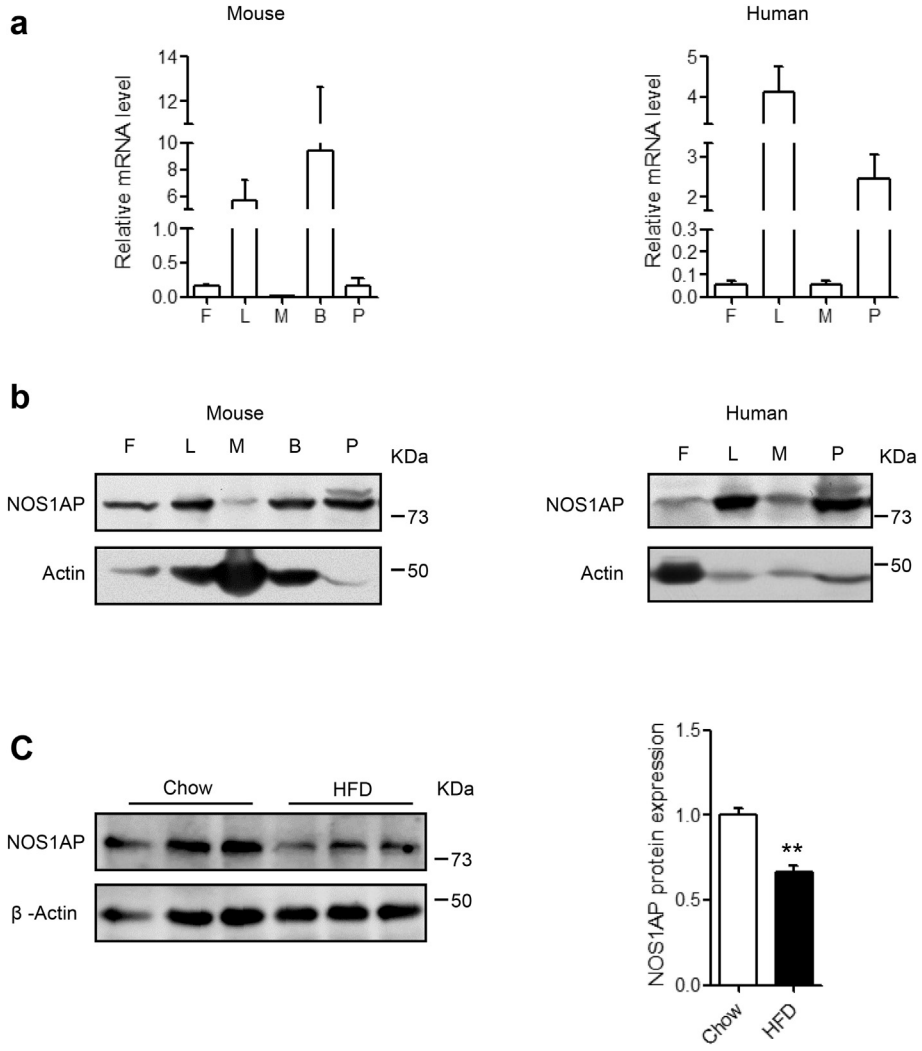



**Fig. 1.** NOS1AP is highly expressed in liver. (a and b) NOS1AP mRNA and protein levels in human and mouse tissues. $n = 3$–4. F, fat; L, liver; M, muscle; B, brain; P, pancreas. (c) NOS1AP protein expression in livers from chow and high fat diet (HFD) fed mice. $^{**}P < .01$ between chow diet mice, $n = 6$ (Student's *t*-test).

fed with an HFD for 8 weeks. Overexpression of NOS1AP in liver was confirmed by western blot analysis (Fig. 3a). Injection of adenoviruses encoding NOS1AP in ob/ob mice had no effect on mouse body weight, but reduced fasting glucose levels and improved glucose tolerance and insulin sensitivity. although no effect was observed on fasting insulin levels after NOS1AP overexpression in liver (Fig. 3b–f). Similar phenomena were viewed in HFD-induced obese mice (Fig. 3g–k). Moreover, blood glucose levels also significantly decreased after pyruvate challenge in these mice (Fig. 3l).

### *3.4. Hepatic conditional deletion of NOS1AP aggravated steatosis, while overexpression of NOS1AP reduced steatosis in liver of obese mice*

We, next, determined the changes of hepatic steatosis after deletion or overexpression of NOS1AP in the obese mice. Liver sections revealed more lipid droplets after hepatic condition deletion of NOS1AP, while overexpression of NOS1AP led to an improvement of hepatic steatosis in ob/ob or HFD-fed obese mice (Fig. 4a–c). In agreement, hepatic triglyceride content significantly increased in NOS1AP CKO mice (Fig. 4d), and decreased in NOS1AP overexpressed obese mice (Fig. 4e and f). No obvious changes in lipid metabolism related genes and ketone body were observed in NOS1AP CKO or overexpression mice (Supplementary Fig. S3).

### *3.5. Hepatic deletion of NOS1AP increased hepatic glucose production, while overexpression of NOS1AP improved insulin sensitivity in liver*

To confirm the role of NOS1AP in modulating glucose production in liver, we isolated primary hepatocytes from NOS1AP CKO and control mice. After stimulated with FK, the glucose production of hepatocytes with deletion of NOS1AP greatly increased compared with that of control cells (Fig. 5a). In line with, mRNA levels of pyruvate carboxylase (*Pc*) and phosphoenolpyruvate carboxykinase 1 (*Pck1*), two genes encoding key enzymes for gluconeogenesis, increased significantly in liver of NOS1AP CKO mice (Fig. 5b), and decreased in liver of NOS1AP overexpression obese mice (Fig. 5c and d), suggesting a role of NOS1AP on regulation of glucose production in hepatocytes.

To investigate the effect of hepatic NOS1AP on insulin sensitivity in liver, insulin stimulation test was performed. After insulin stimulation, phosphorylation of IR (Tyr1150/1151), Akt (Ser473) and GSK3β (Ser9), the 3 major components of insulin signaling, were increased in the liver from ob/ob mice with NOS1AP overexpression compared with those of the control mice (Fig. 5e and f). Interestingly, deletion of NOS1AP also greatly enhanced the phosphorylation of p38 MAPK (Thr180/182) in liver from HFD-induced obese mice. Conversely, overexpression of NOS1AP abolished p38 MAPK (Thr180/182) phosphorylation in liver from ob/ob mice (Fig. 5g and h). Taking together, our data suggested a role of NOS1AP in activation of insulin sensitivity

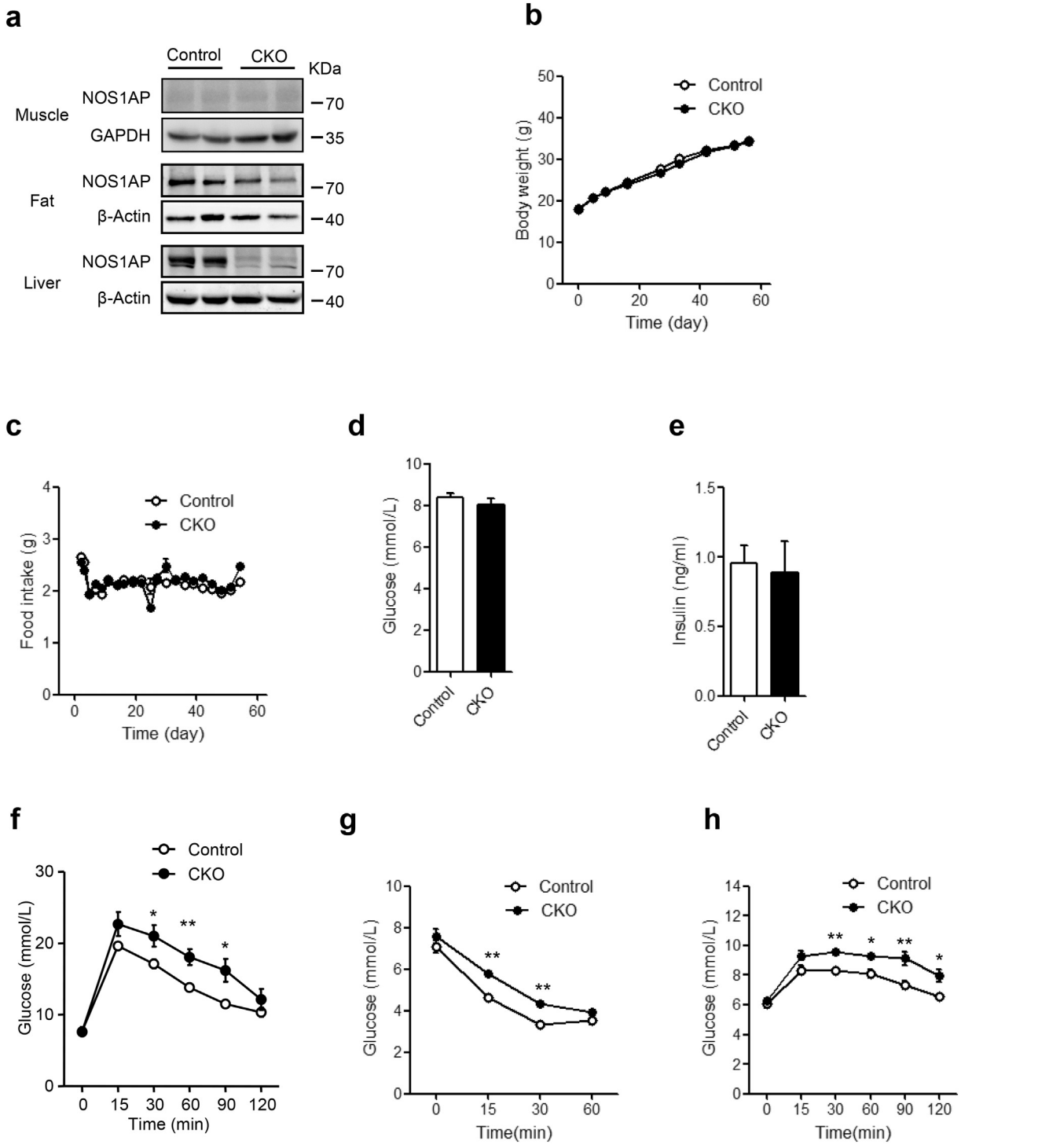



**Fig. 2.** Liver condition deletion of NOS1AP in mice impaired glucose/pyruvate tolerance and insulin sensitivity. (a) NOS1AP protein expression in skeletal muscle, fat and liver tissue homogenates from mice of NOS1AP liver condition knockout (CKO) and their age-matched littermate control mice (*Cre−/−*, *nos1apflox/flox*). Control and CKO mice were fed with a high-fat diet for 8 weeks, body weight (b), food intake (c), fasting glucose (d), fasting insulin (e), glucose tolerance (f), insulin tolerance (g), and pyruvate tolerance (h) were tested. $^{*}P < .05$, $^{**}P < .01$ between control (Student's t-test), $n = 5–10$.

and inhibition of p38 MAPK (Thr180/182) activation in liver of obese mice.

### 3.6. Hepatic conditional deletion of NOS1AP exacerbated ER stress

ER stress in hepatocytes has been reported to be associated with fatty liver and contributed to insulin resistance in liver [23]. To decipher whether the insulin sensitizing effect was associated with ER homeostasis, we treated NOS1AP CKO and its control mice with TM. As expected, after treatment with TM, phosphorylation of eIF2α and IRE1α, and the protein expression of ATF4, ATF6 and CHOP were significantly elevated (Fig. 6a–c). There was also an elevation of mRNA level of *ATF4*, *ATF6*, *CHOP* and 78 kD glucose regulated protein (*GRP78*) (Fig. 6d). Knocking out NOS1AP enhanced eIF2α phosphorylation (Fig. 6a), along with increased nuclear accumulation of ATF4 and ATF6 proteins (Fig. 6b), and CHOP protein expression (Fig. 6c). Consistently,

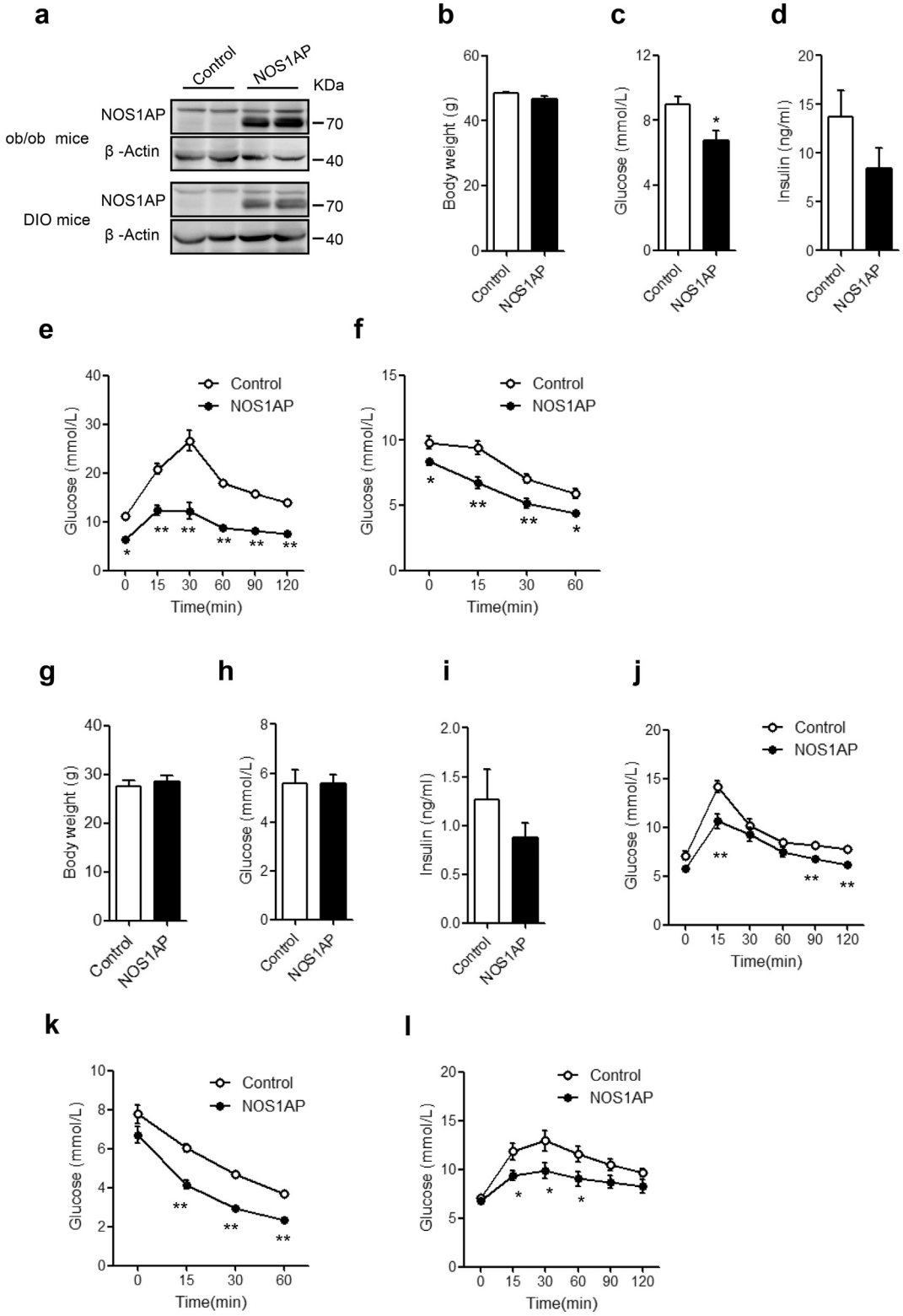



**Fig. 3.** Overexpression of NOS1AP in liver improved glucose/pyruvate, and insulin tolerance. ob/ob (a–f) and high fat diet -induced obese (C57BL/6) mice (DIO) (a, g–l) were injected with adenoviruses encoding *NOS1AP*. (a) NOS1AP protein expression in liver of ob/ob mice and DIO mice. (b and g) Body weight. (c and h) Fasting glucose levels. (d and i) Fasting insulin levels. (e and j) Glucose tolerance test. (f and k) Insulin tolerance test. (l) Pyruvate tolerance test. $^{*}P < .05$, $^{**}P < .01$ between control (Student's t-test), $n = 5–10$.

the mRNA abundance of *ATF4*, *ATF6* and *CHOP* were significantly increased in liver after NOS1AP deletion (Fig. 6d). In contrast, overexpression of NOS1AP by transfection of HepG2 cells with adenoviruses encoding NOS1AP dramatically lowered the phosphorylation of eIF2α, and mRNA accumulation and protein expression of ATF4 and CHOP from palmitate-induced ER stress in cells (Fig. 7a–c).

### *3.7. Dependence of NOS1AP on its C-terminal domain in regulation of insulin sensitivity in obese mice*

As an adaptor protein, NOS1AP was known to consist of a PTB domain at its N-terminal and a PDZ binding domain at the C-terminal. To know which domain in NOS1AP played the role in the regulation of

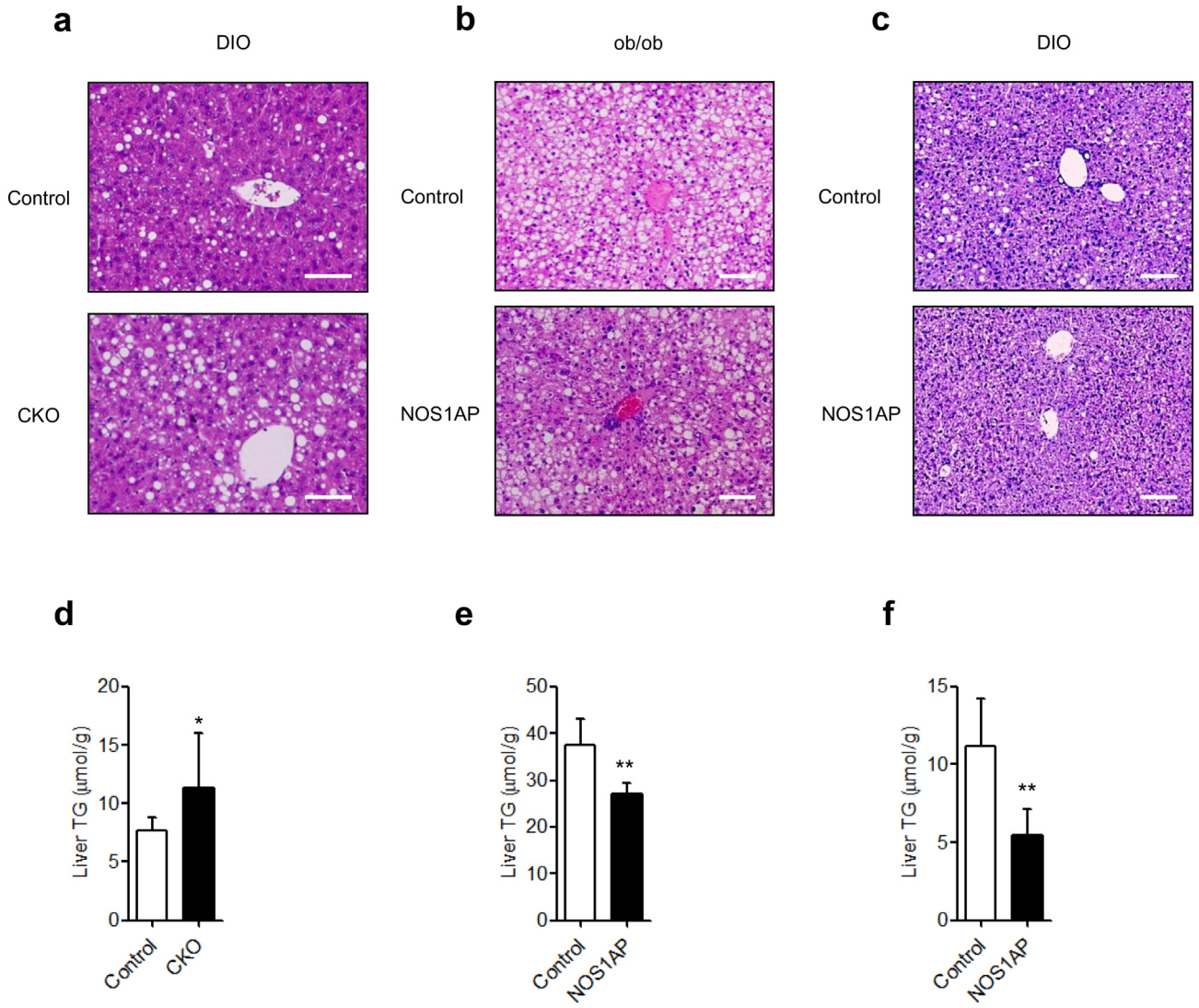



**Fig. 4.** Hepatic condition deletion of NOS1AP aggravated steatosis, while overexpression of NOS1AP reduced steatosis in liver of obese mice. NOS1AP liver condition knockout (CKO) and C57BL/6 mice were fed with a high-fat diet for 8 weeks. ob/ob mice and high fat diet -induced obese (C57BL/6) mice (DIO) were injected with adenoviruses encoding *NOS1AP*. (a-c) H&E staining (scale bar, 50 μm), and (d-f) triglyceride (TG) content extracted from liver of the obese mice. $^{*}P < .05$, $^{**}P < .01$ between control (Student's t-test), $n = 5–10$.

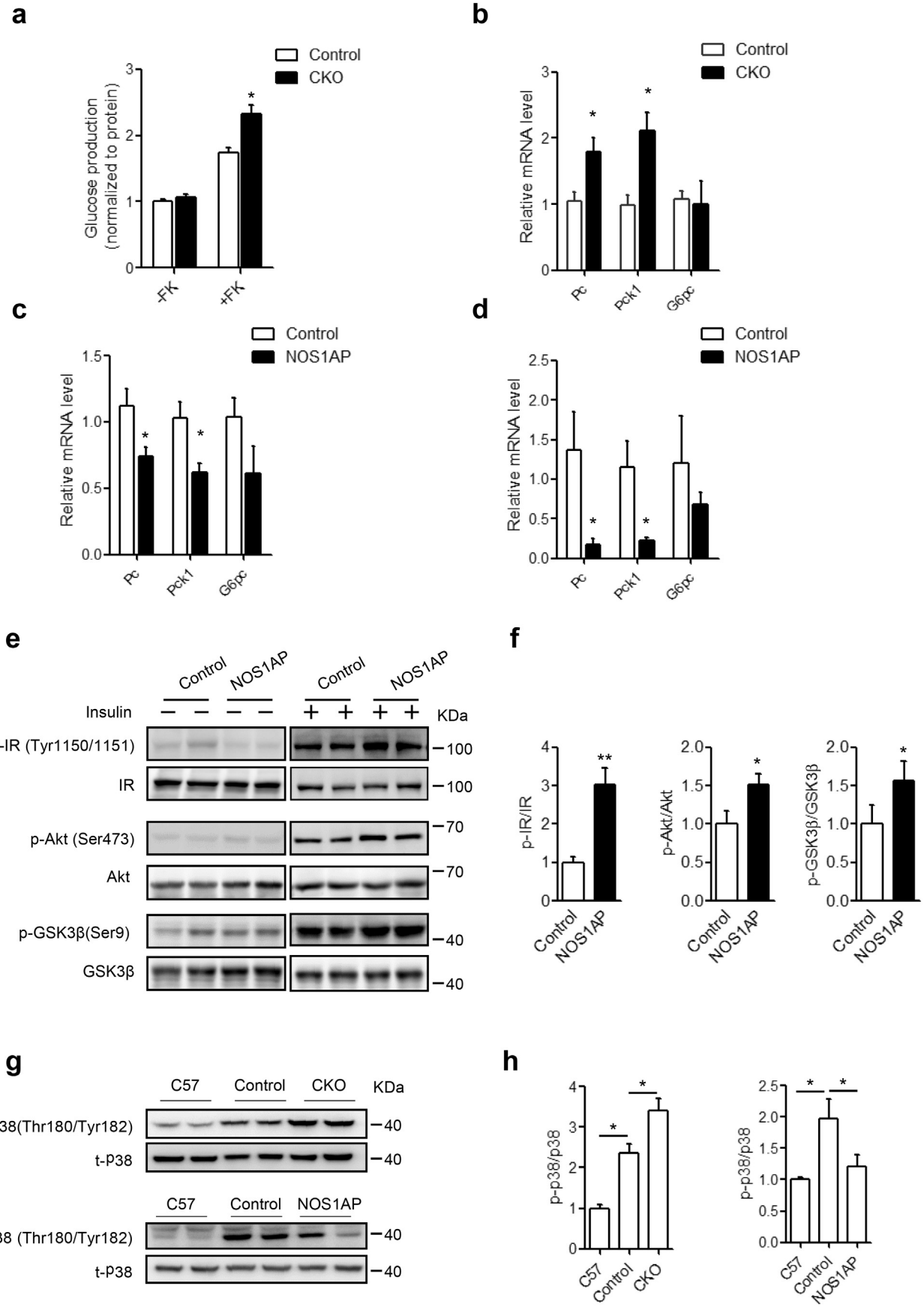
above functions, we generated adenoviral vectors expressing NOS1AP-185 N and NOS1AP-125C, respectively. Overexpression of NOS1AP-125C in liver by tail vain injects in ob/ob mice, but not NOS1AP-185 N, improved glucose and insulin tolerance in these obese mice (Fig. 8a–d). Similar to its full-length overexpression, insulin-stimulated phosphorylation of IR (Tyr1150/1151) and Akt (Ser473) was significantly increased in the liver of NOS1AP-125C overexpression mice compared with those of control mice (Fig. 8e), suggesting that the insulin sensitizing effect of NOS1AP was dependent on its C-terminal.

### *3.8. NOS1AP genetic variants (rs12742393) was associated with hepatic steatosis*

We, then, determined the association between NOS1AP SNP rs12742393 and liver steatosis in human. A total of 86 liver samples were randomly selected and genotyped. Among them, 62 were AA genotypic carriers, 21 were AC and 3 were CC genotypic carriers. The frequency of C allele observed is compatible with previous studies [10]. 48.8% of the liver samples were with grade 0, 45.3% with grade 1, and 5.8% with grade 2 steatosis. The proportion of hepatosteatosis with grade 1–2 was significantly higher in AC + CC carries (75%) than that in AA carriers (41.9%) ($P < .05$). No differences could be viewed in plasma alanine aminotransferase, aspartate aminotransferase, triglycerides, cholesterol, and LDL-cholesterol levels between AA and AC + CC carries (Table 1).

## 4. Discussion

In the present study, we found that NOS1AP protein was highly expressed in human and mouse liver, and its protein expression decreased after HFD. We provided in vivo evidences showing that liver specific deletion of *Nos1ap* gene led to impairment of glucose, insulin, and pyruvate tolerance, and accumulation of lipids in the livers from HFD-induced obese mice. In contrast, selective overexpression of NOS1AP in liver attenuated glucose and pyruvate intolerance, improved insulin sensitivity, and reduced triglyceride content in the livers of ob/ob and HFD-induced obese mice. Our study has revealed a novel role of NOS1AP against the liver gluconeogenesis and steatosis through improving insulin signaling mainly on its PDZ binding domain. Most importantly, NOS1AP SNP was associated with fatty liver in Chinese subjects. The subjects with rs12742393 of NOS1AP have higher risk for hepatic steatosis, in which the C allele carriers have higher proportion of steatosis. The finding that NOS1AP links insulin sensitivity and glucose homeostasis in liver indicated that this protein is a potential


a
Control
CKO
Glucose production (normalized to protein)
-FK
+FK
b
Control
CKO
Relative mRNA level
Pc
Pck1
G6pc
c
Control
NOS1AP
Relative mRNA level
Pc
Pck1
G6pc
d
Control
NOS1AP
Relative mRNA level
Pc
Pck1
G6pc
e
Control
NOS1AP
Control
NOS1AP
Insulin
KDa
p-IR (Tyr1150/1151)
IR
p-Akt (Ser473)
Akt
p-GSK3β(Ser9)
GSK3β
f
p-IR/IR
p-Akt/Akt
p-GSK3β/GSK3β
Control
NOS1AP
g
C57
Control
CKO
KDa
p-p38(Thr180/Tyr182)
t-p38
C57
Control
NOS1AP
p-p38 (Thr180/Tyr182)
t-p38
h
p-p38/p38
C57
Control
CKO
p-p38/p38
C57
Control
NOS1AP

therapeutic target for the treatment and/or prevention of hepatic insulin resistance and T2D.

It is well-established that hepatic insulin resistance is a key event in diabetes. Our results in the present study establish NOS1AP as a key player in the inhibition of hepatic insulin resistance, a situation which may ultimately result in T2D. This notion can be supported by the following evidences. Firstly, our previous genetic study indicated that the SNP within the *NOS1AP* gene were associated with T2D [10]. The binding capacity of NOS1AP with rs12742393-C allele, reported by Wratten et al., was much lower than rs12742393-A allele [24]. Most strikingly, the physiological relevance of NOS1AP as a key regulator in liver insulin sensitivity is supported by both loss-of-function and gain-of-function strategies in the present study in animal models. Liver specific NOS1AP deletion in mice impaired glucose, pyruvate and insulin tolerance, while selective overexpression of NOS1AP in liver resulted in dramatic improvement in insulin, glucose, or pyruvate tolerance in ob/ob and HFD-fed obese mice. Consistently, deletion of NOS1AP led to an increase of glucose production along with up-regulation of the mRNA expression of *Pck1* and *Pc*. Overexpression of NOS1AP in ob/ob mice lowered the mRNA expression of *Pck1*, and improved insulin signaling in liver evidenced by enhancing the phosphorylation of IR, Akt and GSK3β. *Pck*1 encodes cytosolic phosphoenolpyruvate carboxykinase, responsible for the rate-limiting step in the hepatic gluconeogenesis pathway [25,26]. In sum, our data indicated that NOS1AP might play a pivotal role in regulation of insulin sensitivity in liver and targeting NOS1AP may be an effective strategy for reversing obese-induced insulin resistance.

The liver plays a major role in lipid metabolism, importing FFAs, and manufacturing, storing and exporting lipids. Our data in the present study showed that the insulin sensitive regulation effect of NOS1AP in liver was associated with its reduction on lipid accumulation in liver. Liver specific deletion or overexpression of NOS1AP in obese mice increased or reduced lipid accumulation evidenced by liver triglyceride measurement and histology staining. Interestingly, *NOS1AP* gene SNP, the variant was associated with T2D, is also associated with hepatic steatosis in human in the present study. The C allele carriers in rs12742393 of *NOS1AP* have a higher proportion of hepatosteatosis than the AA allele carriers. Lipid accumulation and gluconeogenesis in the liver are two central features of hepatic insulin resistance in obesity. However, the mechanisms link the function of NOS1AP to its SNP (rs12742393) are not clear. Although Wratten et al. reported that the plasmid containing NOS1AP with C allele produced a lower expression level and binding capacity to nuclear protein than the one containing NOS1AP with A allele [24], we could not find any changes in NOS1AP mRNA and protein expression in liver between A and C allele carriers (Supplementary Fig. S4). Hepatocytes are rich in ER that is responsible for triglyceride and cholesterol synthesis, as well as protein synthesis, folding, trafficking and maturation. Studies have been shown that steatosis associated consequences of ER stress play a key role for the development of hepatic insulin resistance in obesity [27–30]. p38 MAPK is one of stress-activated MAPKs. Its activation has been reported to contribute to insulin resistance in liver [31]. In the present study, we also provide evidences showing a link between NOS1AP expression and ER homeostasis/p38 MAPK inactivation. Overexpression of NOS1AP lowered the expression of p-eIF2α, ATF4 and CHOP induced by FFA in HepG2 cells, and abolished p38 MAPK activation in liver from ob/ob mice, indicating that the role of NOS1AP on lipid accumulation and insulin sensitivity regulation was associated with its maintenance of ER homeostasis and p38 MAPK inactivation. The exact mechanism for the link between NOS1AP and ER stress/p38 MAPK in the liver is unclear. Further studies are needed to explore in this area.

Three function domains were reported in NOS1AP protein: the N-terminal (PTB), the middle and the C-terminal (PDZ binding) domains [32–34]. We reported that both full-length of NOS1AP and its C-terminal, but not N-terminal, domain improved glucose and insulin tolerance, and insulin signaling activity when specific overexpressed in the livers of obese mice, suggesting that the PDZ binding domain of NOS1AP is responsible for the regulation of hepatic insulin sensitivity. In neuron, NOS1AP was a ligand of nNOS through its PDZ binding domain [11,34]. There are three isoforms of NOS: nNOS, inducible NOS (iNOS), and endothelial NOS (eNOS). Disruption of iNOS was reported to prevent obesity-linked insulin resistance in muscle by Perreault et al. [35]. Liver-specific iNOS overexpression induced hepatic insulin resistance and hyperglycemia in mice [36], in which S-nitrosylation induced by iNOS played an important role in the impairment of ER function in hepatocytes [37]. As to nNOS, it was reported to be a pivotal signaling molecule triggering ER stress and oxidative damage in brain [38]. Disruption of nNOS protected against oxidative damage [39]. However, the role of nNOS on insulin sensitivity remained controversial. Marsollier et al. reported previously that central lipid overload increased hypothalamic nNOS activity and induced hepatic insulin resistance [40]. The mice with nNOS deletion were reported to have peripheral insulin resistance by Shankar's study [41] and normal glucose tolerance by Natata et al. [42]. The dependency of NOS1AP on its C-terminal domain for insulin sensitizing effect in our study suggested that nNOS might be involved in the effect of NOS1AP. In liver, nNOS expression was upregulated in bile duct ligation-induced cirrhosis [43]. Deletion or inhibition of nNOS with its inhibitor has been reported to protect against Acetaminophen induced-cytotoxicity in hepatocytes [44,45]. Further studies are needed to explore the role of nNOS under the effect of NOS1AP in liver.

In summary, we report for the first time that the NOS1AP is highly expressed in human and mouse liver, and this protein plays a vital role in regulating hepatic insulin sensitivity, gluconeogenesis and lipogenesis inhibition, and p38 MAPK inactivation in liver of HFD-induced and/or genetic obese mice. The insulin sensitizing effect of NOS1AP depends on its C-terminal domain. The regulatory role of NOS1AP on hepatic insulin sensitivity highlights the importance of this adaptor protein in the development of T2D and points to a therapeutic target of this protein for the prevention and treatment of diabetes through improving hepatic insulin sensitivity.

## Author contributions

K.M. and Y.S. contributed to performance the animal experiments, data analysis and manuscript drafting. Y. Z., T. Z., Q. L., M. Z., H. L., R. Z., and C.H. contributed to the performance of the experiments. C.W. designed the research, analyzed and interpreted the data, and critically revised the manuscript for important intellectual content. C. W. and W. J. are the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors approved the final version of the manuscript.

**Fig. 5.** Effect of NOS1AP on hepatic insulin sensitivity and p38 MAPK in obese mice. (a) Primary hepatocytes isolated from NOS1AP condition knockout (CKO) or their littermate control mice. After 16 h starvation, the cells were washed with PBS, and 20uM forskolin (FK) was added in glucose production medium for 4 h ($n = 8$). (b-d) Real-time PCR analyses were performed on liver extracts from NOS1AP CKO mice (b), ob/ob mice (c) and high fat diet -induced obese C57BL/6 mice (d), and mRNA expression was normalized by *Gapdh* ($n =$ 4–7). *Pc*, pyruvate carboxylase; *Pck1*, Phosphoenolpyruvate carboxykinase 1; *G6pc*, glucose-6-phosphatase. (e and f) Fasted ob/ob mice with liver specific NOS1AP overexpression were injected with insulin (5 U/kg). After 10 min, liver was taken and detected for p-IR (Tyr1150/1151), p-Akt (Ser473) and p-GSK3β (Ser9) expression. Quantification of protein phosphorylation levels are normalized to total levels (n = 4). $^{*}P < .05$, $^{**}P < .01$ between control (Student's t-test). (g) p38 MAPK expression in liver extracts from NOS1AP liver condition knockout (CKO) mice (upper) and ob/ob mice with NOS1AP overexpression (lower). C57BL/6 (C57) mice were fed with a chow diet. CKO and its control mice were fed with a high fat die. (h) Quantification of protein phosphorylation levels (n = 5–6), $^{*}P < .05$, $^{**}P < .01$ between indicated groups (One-way ANOVA with Bonferroni's multiple comparison test). p38, p38 mitogen-activated protein kinase.

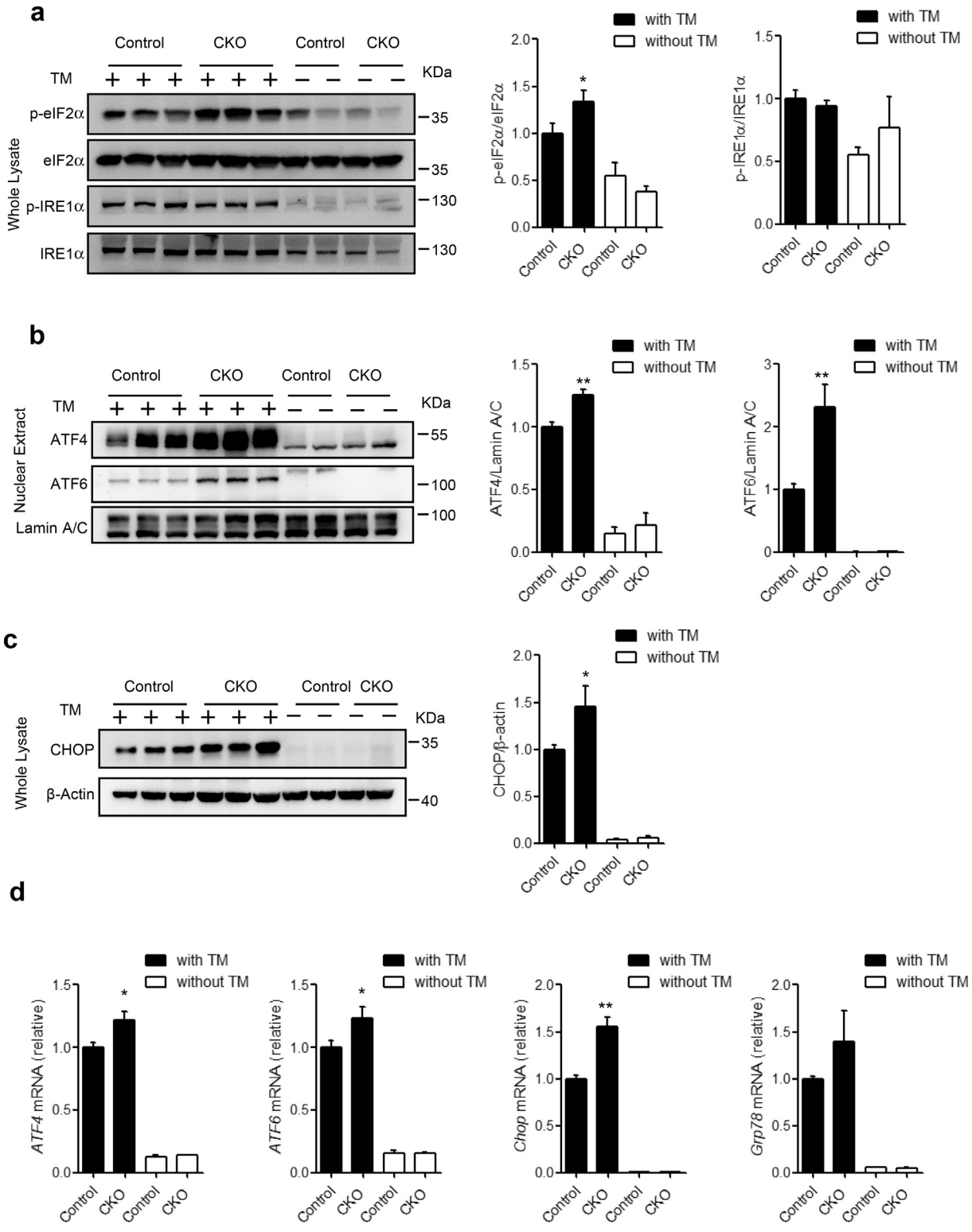



**Fig. 6.** Hepatic condition deletion of NOS1AP exacerbated tunicamycin -induced ER stress. NOS1AP liver condition knockout (CKO) or control mice were injected intraperitoneally with tunicamycin (TM) at a dose of 1 mg/kg body weight or PBS, and sacrificed after 8 h of treatment. (a–c) Protein expression of (p)-eIF2a, (p)-IRE1a (a), ATF4, ATF6 (b) and CHOP (c) were analyzed by western blot (left panels). Quantification of the protein level was normalized to total protein or β-actin (right panels) ($n = 4$–6). (d) mRNA expression of *ATF4*, *ATF6*, *Chop and Grp78* were analyzed by Real-time PCR. Quantification of the mRNA level was normalized by *Gapdh* ($n = 4$–6). ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; CHOP, C/EBP-homologous protein; GRP78, 78 kD glucose regulated protein. $^{*}P < .05$, $^{**}P < .01$ between controls (Student's t-test).

**Acknowledgements/Funding sources**

The authors thank Dr. R Wang from The University of New Orleans for English editorial support. This work was supported by the National Natural Science Foundation of China (81670707, 31340072) (to C. Wang), and National Basic Research Program of China (Nation 973 Program) (2011CB504001) (to W. Jia). The funders have no roles in study design, data collection, data analysis, interpretation, and writing of the report.

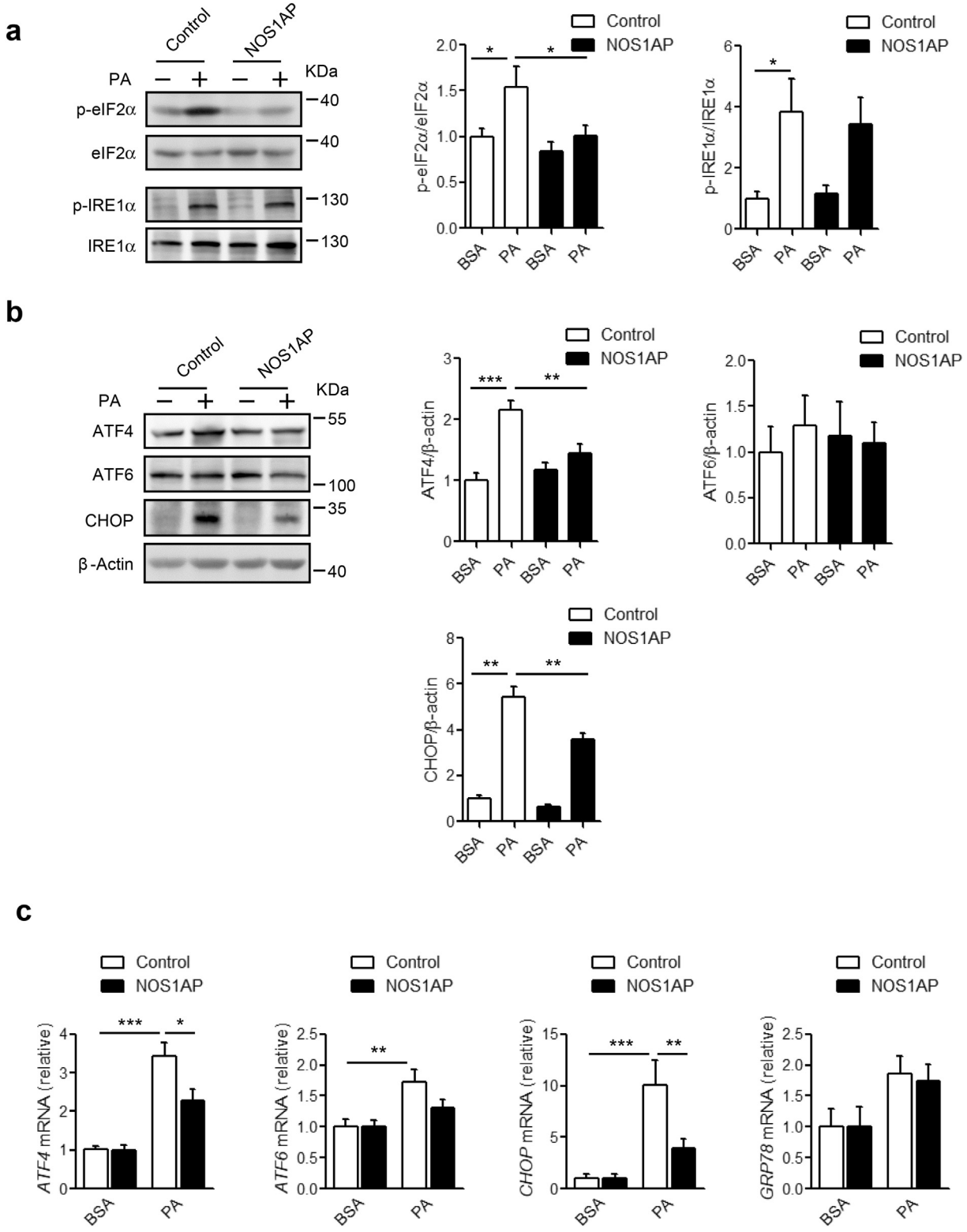



**Fig. 7.** Overexpression of NOS1AP protected hepatocytes from palmitate-induced ER stress. HepG2 cells were infected with adenovirus encoding NOS1AP. 36-42 h post-infection, cells were treated with 1% BSA or in combination with palmitate (PA) (0.4 mM). (a and b) Protein expression of (p)-eIF2a, (p)-IRE1a (a), ATF4, ATF6 and CHOP (b) were analyzed by western blot (left panels). Quantification of the protein level was normalized to total protein or β-actin (right panels) ($n = 4–5$). (c) mRNA expression of *ATF4*, *ATF6*, *CHOP and GRP78* were analyzed by Real-time PCR. Quantification of the mRNA level was normalized by *GAPDH* ($n = 5–7$). $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ between indicated groups (ANOVA followed by Bonferroni's multiple comparison).

## Declaration of Competing Interest

The authors declare no conflict of interests.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.08.033.

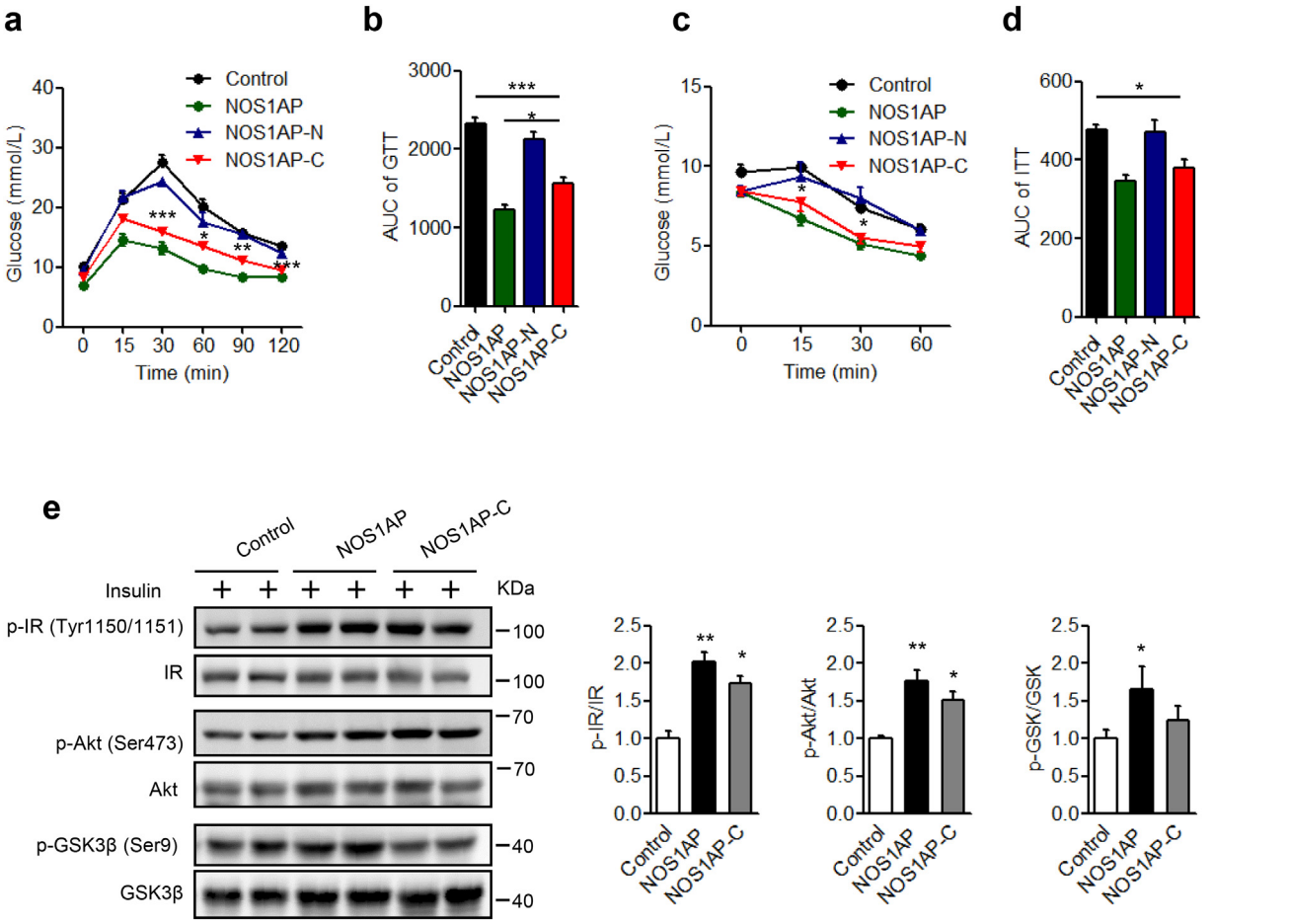



**Fig. 8.** Dependence of NOS1AP on its C-terminal domain in regulation of hepatic insulin sensitivity. (a–d) ob/ob mice were injected with adenovirus encoding full-length (NOS1AP), N-terminal (NOS1AP-N) or C-terminal of NOS1AP (NOS1AP-C) through tail vein injection. Glucose tolerance (a and b) and insulin tolerance test (c and d) were performed (n = 4–6). (e) Effect of NOS1AP expression on insulin signaling in liver. Fasted ob/ob mice with liver specific NOS1AP or NOS1AP-C overexpression were injected with insulin (5 U/kg). After 10 min, liver was taken and detected for p-IR (Tyr1150/1151), p-Akt (Ser473) and p-GSK3β (Ser9) expression. Quantification of protein phosphorylation levels are normalized to total levels ($n = 4$). $^{*}P < .05$, $^{**}P < .01$, $P < .001$ between indicated groups or control (ANOVA followed by Bonferroni's multiple comparison).

**Table 1**
Characteristics of subjects stratified according to *NOS1AP rs12742393*.

| *NOS1AP* rs12742393 | AA ($n = 60$) | AC + CC ($n = 26$) | *P* values |
|---|---|---|---|
| Genotype frequency (%) | 69.8 | 30.2 | – |
| Glucose (mmol/L) | 5.28 ± 2.67 | 4.91 ± 1.79 | 0.522 |
| ALT (U/L) | 9.36 ± 8.68 | 9.76 ± 10.03 | 0.876 |
| AST (U/L) | 26.62 ± 17.88 | 26.76 ± 12.33 | 0.971 |
| TG (mmol/L) | 0.89 ± 0.82 | 0.82 ± 0.67 | 0.703 |
| TC (mmol/L) | 2.40 ± 1.25 | 2.25 ± 1.16 | 0.634 |
| LDL-c (mmol/L) | 1.41 ± 0.81 | 1.38 ± 0.72 | 0.903 |

Data represent means ± SD. Statistical significance of differences was analyzed with student *t*-test.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; TG, triglyceride; TC, total cholesterol; LDL-c, low-density lipoprotein cholesterol; NOS1AP, nitric oxide synthase 1 adaptor protein.

## References


[1] Birkenfeld AL, Shulman GI. Nonalcoholic fatty liver disease, hepatic insulin resistance, and type 2 diabetes. Hepatology 2014;59(2):713–23.
[2] Perry RJ, Samuel VT, Petersen KF, Shulman GI. The role of hepatic lipids in hepatic insulin resistance and type 2 diabetes. Nature 2014;510(7503):84–91.
[3] Asrih M, Jornayvaz FR. Inflammation as a potential link between nonalcoholic fatty liver disease and insulin resistance. J Endocrinol 2013;218(3):R25–36.
[4] Reddy JK, Rao MS. Lipid metabolism and liver inflammation. II. Fatty liver disease and fatty acid oxidation. Am J Physiol Gastrointest Liver Physiol 2006;290(5):G852–8.
[5] Fu S, Yang L, Li P, Hofmann O, Dicker L, Hide W, et al. Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity. Nature 2011;473(7348):528–31.
[6] Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. J Hepatol 2011;54(4):795–809.
[7] Rutkowski DT, Wu J, Back SH, Callaghan MU, Ferris SP, Iqbal J, et al. UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. Dev Cell 2008;15(6):829–40.
[8] Tai FW, Syn WK, Alazawi W. Practical approach to non-alcoholic fatty liver disease in patients with diabetes. Diabet Med 2015;32(9):1121–33.
[9] Byrne CD, Targher G. NAFLD: a multisystem disease. J Hepatol 2015;62(1):S47–64 Suppl.
[10] Hu C, Wang C, Zhang R, Ng MC, Bao Y, Wang C, et al. Association of genetic variants of NOS1AP with type 2 diabetes in a Chinese population. Diabetologia 2010;53(2):290–8.
[11] Jaffrey SR, Snowman AM, Eliasson MJ, Cohen NA, Snyder SH. CAPON: a protein associated with neuronal nitric oxide synthase that regulates its interactions with PSD95. Neuron 1998;20(1):115–24.
[12] Xu B, Wratten N, Charych EI, Buyske S, Firestein BL, Brzustowicz LM. Increased expression in dorsolateral prefrontal cortex of CAPON in schizophrenia and bipolar disorder. PLoS Med 2005;2(10):e263.
[13] Richier L, Williton K, Clattenburg L, Colwill K, O'Brien M, Tsang C, et al. NOS1AP associates with scribble and regulates dendritic spine development. J Neurosci 2010;30(13):4796–805.
[14] Clattenburg L, Wigerius M, Qi J, Rainey JK, Rourke JL, Muruganandan S, et al. NOS1AP functionally associates with YAP to regulate hippo signaling. Mol Cell Biol 2015;35(13):2265–77.
[15] Segalat L, Grisoni K, Archer J, Vargas C, Bertrand A, Anderson JE. CAPON expression in skeletal muscle is regulated by position, repair, NOS activity, and dystrophy. Exp Cell Res 2005;302(2):170–9.
[16] Darling AJ, Boose JA, Spaltro J. Virus assay methods: accuracy and validation. Biologicals 1998;26(2):105–10.
[17] Li H, Fang Q, Gao F, Fan J, Zhou J, Wang X, et al. Fibroblast growth factor 21 levels are increased in nonalcoholic fatty liver disease patients and are correlated with hepatic triglyceride. J Hepatol 2010;53(5):934–40.
[18] Jiang S, Yan C, Fang QC, Shao ML, Zhang YL, Liu Y, et al. Fibroblast growth factor 21 is regulated by the IRE1alpha-XBP1 branch of the unfolded protein response and

counteracts endoplasmic reticulum stress-induced hepatic steatosis. J Biol Chem 2014;289(43):29751–65.
[19] He L, Chang E, Peng J, An H, McMillin SM, Radovick S, et al. Activation of the cAMP-PKA pathway antagonizes metformin suppression of hepatic glucose production. J Biol Chem 2016;291(20):10562–70.
[20] Wang C, Li XW, Mu KD, Li L, Wang SH, Zhu YX, et al. Deficiency of APPL1 in mice impairs glucose-stimulated insulin secretion through inhibition of pancreatic beta cell mitochondrial function. Diabetologia 2013;56(9):1999–2009.
[21] Hu X, Feng Y, Shen Y, Zhao XF, Yu JH, Yang YS, et al. Antidiabetic effect of a novel non-thiazolidinedione PPAR gamma/alpha agonist on ob/ob mice. Acta Pharmacol Sin 2006;27(10):1346–52.
[22] Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41(6):1313–21.
[23] Dara L, Ji C, Kaplowitz N. The contribution of endoplasmic reticulum stress to liver diseases. Hepatology 2011;53(5):1752–63.
[24] Wratten NS, Memoli H, Huang Y, Dulencin AM, Matteson PG, Cornacchia MA, et al. Identification of a schizophrenia-associated functional noncoding variant in NOS1AP. Am J Psychiatry 2009;166(4):434–41.
[25] Yang J, Kalhan SC, Hanson RW. What is the metabolic role of phosphoenolpyruvate carboxykinase? J Biol Chem 2009;284(40):27025–9.
[26] Mendez-Lucas A, Duarte JA, Sunny NE, Satapati S, He T, Fu X, et al. PEPCK-M expression in mouse liver potentiates, not replaces, PEPCK-C mediated gluconeogenesis. J Hepatol 2013;59(1):105–13.
[27] Fu S, Watkins SM, Hotamisligil GS. The role of endoplasmic reticulum in hepatic lipid homeostasis and stress signaling. Cell Metab 2012;15(5):623–34.
[28] Oyadomari S, Harding HP, Zhang Y, Oyadomari M, Ron D. Dephosphorylation of translationinitiation factor 2alpha enhances glucose tolerance and attenuates hepatosteatosis in mice. Cell Metab 2008;7:520–32.
[29] Birkenfeld AL, Lee HY, Majumdar S, Jurczak MJ, Camporez JP, Jornayvaz FR, et al. Influence of the hepatic eukaryotic initiation factor 2alpha (eIF2alpha) endoplasmic reticulum (ER) stress response pathway on insulin-mediated ER stress and hepatic and peripheral glucose metabolism. J Biol Chem 2011;286:36163–70.
[30] Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science 2004;306(5695):457–61.
[31] Hemi R, Yochananov Y, Barhod E, Kasher-Meron M, Karasik A, Tirosh A, et al. p38 mitogen-activated protein kinase-dependent transactivation of ErbB receptor family: a novel common mechanism for stress-induced IRS-1 serine phosphorylation and insulin resistance. Diabetes 2011;60(4):1134–45.
[32] Jaffrey SR, Benfenati F, Snowman AM, Czernik AJ, Snyder SH. Neuronal nitric-oxide synthase localization mediated by a ternary complex with synapsin and CAPON. PNAS 2002;99(5):3199–204.
[33] Fang M, Jaffrey SR, Sawa A, Ye K, Luo X, Snyder SH. Dexras1: a G protein specifically coupled to neuronal nitric oxide synthase via CAPON. Neuron 2000;28(1):183–93.
[34] Carrel D, Hernandez K, Kwon M, Mau C, Trivedi MP, Brzustowicz LM, et al. Nitric oxide synthase 1 adaptor protein, a protein implicated in schizophrenia, controls radial migration of cortical neurons. Biol Psychiatry 2015;77(11):969–78.
[35] Perreault M, Marette A. Targeted disruption of inducible nitric oxide synthase protects against obesity-linked insulin resistance in muscle. Nat Med 2001;7(10):1138–43.
[36] Shinozaki S, Choi CS, Shimizu N, Yamada M, Kim M, Zhang T, et al. Liver-specific inducible nitric-oxide synthase expression is sufficient to cause hepatic insulin resistance and mild hyperglycemia in mice. J Biol Chem 2011;286(40):34959–75.
[37] Yang L, Calay ES, Fan J, Arduini A, Kunz RC, Gygi SP, et al. METABOLISM. S-Nitrosylation links obesity-associated inflammation to endoplasmic reticulum dysfunction. Science 2015;349(6247):500–6.
[38] Zhu X, Dong J, Han B, Huang R, Zhang A, Xia Z, et al. Neuronal nitric oxide synthase contributes to PTZ kindling epilepsy-induced hippocampal endoplasmic reticulum stress and oxidative damage. Front Cell Neurosci 2017;11:377.
[39] Gerónimo-Olvera C, Tristán-López L, Martínez-Lazcano JC, García-Lara L, Sánchez-Mendoza A, Morales-Martínez A, et al. Striatal protection in nNOS knock-out mice after quinolinic acid-induced oxidative damage. Neurochem Res 2019;44(2):421–7.
[40] Marsollier N, Kassis N, Mezghenna K, Soty M, Fioramonti X, Lacombe A, et al. Deregulation of hepatic insulin sensitivity induced by central lipid infusion in rats is mediated by nitric oxide. PLoS One 2009;4(8):e6649.
[41] Shankar RR, Wu Y, Shen HQ, Zhu JS, Baron AD. Mice with gene disruption of both endothelial and neuronal nitric oxide synthase exhibit insulin resistance. Diabetes 2000;49(5):684–7.
[42] Nakata S, Tsutsui M, Shimokawa H, Suda O, Morishita T, Shibata K, et al. Spontaneous myocardial infarction in mice lacking all nitric oxide synthase isoforms. Circulation 2008;117(17):2211–23.
[43] Wei CL, Khoo HE, Lee KH, Hon WM. Differential expression and localization of nitric oxide synthases in cirrhotic livers of bile duct-ligated rats. Nitric Oxide 2002;7(2):91–102.
[44] Banerjee S, Melnyk SB, Krager KJ, Aykin-Burns N, Letzig LG, James LP, et al. The neuronal nitric oxide synthase inhibitor NANT blocks acetaminophen toxicity and protein nitration in freshly isolated hepatocytes. Free Radic Biol Med 2015;89:750–7.
[45] Agarwal R, Hennings L, Rafferty TM, Letzig LG, McCullough S, James LP, et al. Acetaminophen-induced hepatotoxicity and protein nitration in neuronal nitric-oxide synthase knockout mice. J Pharmacol Exp Ther 2012;340(1):134–42.