RESEARCH

Hepatic nNOS impaired hepatic insulin sensitivity through the activation of p38 MAPK

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Abstract

Neuronal nitric oxide synthase (nNOS) interacts with its adaptor protein NOS1AP through its PZD domain in the neurons. Previously, we had reported that NOS1AP enhanced hepatic insulin sensitivity through its PZD-binding domain, which suggested that nNOS might mediate the effect of NOS1AP. This study aimed to examine the role and underlying mechanisms of nNOS in regulating hepatic insulin sensitivity. nNOS co-localized with NOS1AP in mouse liver. The overexpression of NOS1AP in mouse liver decreased the level of phosphorylated nNOS (p-nNOS (Ser1417)), the active form of nNOS. Conversely, the liver-specific deletion of NOS1AP increased the level of p-nNOS (Ser1417). The overexpression of nNOS in the liver of high-fat diet-induced obese mice exacerbated glucose intolerance, enhanced intrahepatic lipid accumulation, decreased glycogen storage, and blunted insulin-induced phosphorylation of IRbeta and Akt in the liver. Similarly, nNOS overexpression increased triglyceride production, decreased glucose utilization, and downregulated insulin-induced expression of p-IRbeta, p-Akt, and p-GSK3beta in the HepG2 cells. In contrast, treatment with N^ω-propyl-L-arginine (L-NPA), a selective nNOS inhibitor, improved glucose tolerance and upregulated insulin-induced phosphorylation of IRbeta and Akt in the liver of ob/ob mice. Furthermore, overexpression of nNOS increased p38MAPK phosphorylation in the HepG2 cells. In contrast, inhibition of p38MAPK with SB203580 significantly reversed the nNOS-induced inhibition of insulin-signaling activity (all $P < 0.05$). This indicated that hepatic nNOS inhibited the insulin-signaling pathway through the activation of p38MAPK. These findings suggest that nNOS is involved in the development of hepatic insulin resistance and that nNOS might be a potential therapeutic target for diabetes.

Key Words

- ▶ neuronal nitric oxide synthase (nNOS)
- ▶ insulin resistance
- ▶ p38 MAPK
- ▶ obesity

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Introduction

Non-alcoholic fatty liver disease (NAFLD), a common chronic liver disease, manifested as hepatic steatosis and insulin resistance. The pathogenesis of NAFLD has many similarities with type 2 diabetes (T2D), and might underline the basis of the development of T2D (Loria

et al. 2013). However, the mechanisms underlying NAFLD development have not been elucidated. Currently, there is a need to identify effective therapeutic targets for NAFLD.

Nitric oxide synthase (NOS) is an enzyme that catalyzes nitric oxide (NO) synthesis. Excessive production of

NO decreases glycogen synthase activity and increases glucose production in the liver (Borgs *et al.* 1996, An *et al.* 2008, Sansbury & Hill 2014). Inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) (also called NOS1) are the three isoforms of NOS. The liver-specific overexpression of iNOS induced hepatic insulin resistance through S-nitrosylation of Akt (Shinozaki *et al.* 2011, Yang *et al.* 2015), whereas, the inhibition of iNOS reversed hyperglycemia and elevated insulin sensitivity in the liver of genetic obese mice (Fujimoto *et al.* 2005). eNOS, highly expressed in endothelial cells, played a role in regulation of vasorelaxation through the activation of insulin-signaling pathway (Muniyappa *et al.* 2007). The animal models of NAFLD are associated with liver endothelial cell dysfunction (Tateya *et al.* 2011), which resulted in the downregulation of eNOS activity and exacerbation of hepatic steatosis, fibrosis, and insulin resistance under obese conditions (Pasarín *et al.* 2011, Sheldon *et al.* 2014). nNOS is highly expressed in neurons and skeletal muscle, and moderate expressed in heart and pancreas (Bachar *et al.* 2010, Mezghenna *et al.* 2011, Lu *et al.* 2013, Baldelli *et al.* 2014). While the inhibition effect of iNOS on hepatic insulin sensitivity and the activation effect of eNOS on endothelial insulin sensitivity are well-documented, the reported role of nNOS in the regulation of insulin activity is controversial at the present. Mezghenna *et al.* reported that nNOS expression decreased in skeletal muscle of obese Zucker (fa/fa) rats, overexpression of nNOS promoted insulin-stimulated Glut4 translocation and glucose uptake in the muscle cells (Mezghenna *et al.* 2014). However, lipid infusion-induced activation of hypothalamus nNOS resulted in enhanced hepatic glucose production (Marsollier *et al.* 2009). The exact role of nNOS within the hepatocytes is not clear. Recently, we demonstrated that NOS1 adaptor protein (NOS1AP) regulates hepatic insulin sensitivity through its PZD-binding domain (Mu *et al.* 2019). Several proteins, including nNOS, are reported to bind to the PZD-binding domain of NOS1AP (Jaffrey *et al.* 1998, Zhu *et al.* 2014). According to Jaffrey's reports, the binding of NOS1AP to nNOS in neurons diminished nNOS activity (Jaffrey *et al.* 1998). While previous study has revealed the dependency of NOS1AP on its PZD-binding domain on insulin-sensitizing effect in liver of genetic and diet-induced obese (DIO) mice, more work is required to explore the role of nNOS in the regulation of insulin activity in liver, and the underlying mechanisms.

Materials and methods

Recombinant adenoviruses, materials and antibodies

Recombinant adenoviruses encoding nNOS (Ad-nNOS) and control virus (EGFP) were generated using the Gateway system (Invitrogen). Briefly, nNOS with HA as a tag was constructed into adenoviral plasmids (Invitrogen). Multiplicity of infection (MOI) of the generated adenoviruses was determined by an end-point dilution assay. Palmitate (PA) and oleate (OA) were purchased from Sigma. N ω -Propyl-L-arginine (L-NPA) was from Cayman Chemical (Ann Arbor, MI, USA). SB203580 was purchased from MedChem Express (New Jersey, USA). Protein A/G agarose beads were from GE Healthcare. The antibodies used in this study were obtained from Cell Signaling Technology (rabbit anti-tubulin, anti-beta-actin, anti-IR, anti-phospho-IR (Tyr1150/1151), anti-GSK3beta, anti-phospho-GSK3beta (Ser9), anti-phospho-Akt (Ser473) antibodies, anti-p38 mitogen-activated protein kinase (MAPK), anti-phospho-p38 MAPK (Thr180/Tyr182), anti-PPARgamma, anti-FoxO1 and anti-PCK1), Novus Biologicals (rabbit anti-DGAT1), Sigma (rabbit anti-phospho-nNOS/NOS1(Ser1417)), Abcam (rabbit anti-phospho-nNOS (Ser1417), anti-nNOS and -iNOS antibodies), Santa Cruz (anti-NOS1AP) and KangChen Bio-tech Inc. (Shanghai, China) (Mouse anti-GAPDH).

Animal studies

The ob/ob and db/db mice which were purchased from Shanghai Laboratory Animal Center (Shanghai, China) were fed with a chow diet. The C57BL/6 male mice (aged 6 weeks) were obtained from Shanghai Slaccas Company (Shanghai, China) and fed either with chow diet or with a high-fat diet (HFD: 20% kcal protein, 60% kcal fat and 20% kcal carbohydrates; Research Diets, New Brunswick, USA) for 8 weeks. NOS1AP liver condition knockout and liver overexpression mice, and recombinant adenoviruses carrying NOS1AP were generated as previously reported (Mu *et al.* 2019). All the mice were housed at 23°C $\pm$ 1°C with a 12 h light:12 h darkness cycle and had free access to food and water. Procedures involving the care and use of animals were performed according to the Guidelines for the Care and Use of Laboratory Animals of Shanghai Jiao Tong University and approved by the Animal Ethics Committee of the University Affiliated Sixth People's Hospital (DWSY2011-096/DWLL2019-0274). To overexpression of nNOS in the liver, HFD- or chow diet-fed C57BL/6 mice (aged 15 weeks for HFD and 10 weeks

for chow diet-fed mice) were injected with Ad-nNOS (10⁸ plaque-forming units) through the tail vein. Virus infection in liver of mice fed with chow diet had no obvious effect on mouse liver morphology (data not shown). Glucose tolerance test (GTT), insulin tolerance test (ITT), and insulin-stimulation assays were performed for 10–14 days post-transfection. To perform GTT, mice were fasted for 12 h and intraperitoneally injected with glucose at a dose of 1.5 g/kg body weight. ITT was performed with mice of 6 h fasting and 1.5 U/kg insulin injection. For *in vivo* insulin-stimulation assay, mice were fasted for 16 h and injected with 5 U/kg insulin intraperitoneally thereafter. The mice were sacrificed through cervical dislocation after 10 min and the liver was collected, frozen in liquid nitrogen and stored at –80°C. For *in vivo* nNOS inhibition, ob/ob mice (aged 8–9 weeks) or chow diet-fed C57BL/6 male mice (aged 10 weeks) were intraperitoneally injected with L-NPA (20 mg/kg), a nNOS specific inhibitor. After 2 h, GTT or *in vivo* insulin-stimulation assay were performed.

Human samples

Human liver tissue was collected from patients of benign focal hepatic lesions undergoing liver surgery at the Department of Liver Surgery (Zhongshan Hospital, Fudan University, Shanghai, China) (Li *et al.* 2010). The protocol was approved by the Ethics Committee of Shanghai Jiao Tong University (2018-KY-026 (K)). Informed consent was given by all subjects and principles of the Declaration of Helsinki were followed.

Cell culture and treatments

The HepG2 (a kind gift from Dr F Liu, University of Texas Health Science Center at San Antonio, TX, USA from ATCC) was cultured in DMEM (G25) (Gibco) (Dong *et al.* 2015). The overexpression of nNOS in the HepG2 cells was achieved by infecting the cells with 10 multiplicity of infection (MOI) of Ad-nNOS or EGFP (control) for 16 h. The virus-containing medium was, then, removed, and cells were cultured for additional 24 h. To detect triglyceride production, the Ad-nNOS transfected HepG2 cells were treated with 0.4 mM PA in combination with OA or 1% BSA for 24 h. The insulin-stimulation experiment was performed by culturing the Ad-nNOS transfected HepG2 cells in the presence or absence of 20 µM SB203580 for 30 min, followed by treatment with 100 nM insulin for 30 min. Glucose consumption assay was conducted by transfecting the HepG2 cells with 5, 10, 15 and 20 MOI of Ad-nNOS. The glucose concentration was measured using a glucose oxidase-peroxidase assay kit (Applygen, Beijing, China).

Quantitative real-time PCR, Western blotting and co-immunoprecipitation (Co-IP)

Liver or cell total RNA was obtained using TRIzol reagents (Invitrogen). Quantitative real-time PCR was performed using Roche LightCycler 96 (Roche Ltd.) with SYBR Green Real-time PCR Master Mix-Plus (Toyobo, Osaka, Japan) at the annealing temperature of 60°C. The relative expression level of each sample was calibrated by the comparative Ct method, using *GAPDH* as an endogenous control. Primers for each target gene were examined (data not shown). For Western blotting, the tissue or cell samples were homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of protein were subjected to SDS-PAGE (8% or 10%), transferred to a nitrocellulose membrane. The targeted proteins were detected using specific antibodies. The images were captured with ImageQuant LAS 4000 mini (GE Healthcare Life Science). To perform Co-IP, mouse liver tissues overexpression of NOS1AP were lysed using RIPA buffer. NOS1AP in the tissue lysates was precipitated using the anti-NOS1AP antibody bound to protein A/G agarose beads. The beads were washed with RIPA buffer, boiled in loading buffer and subjected to denaturing SDS-PAGE.

Histology study and triglyceride measurements

Liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin wax and subjected to hematoxylin and eosin (H&E) staining. The frozen liver sections were subjected to Oil Red O staining. For periodic acid-Schiff (PAS) staining, the liver sections were incubated in 0.5% periodic acid solution after deparaffinization and hydration. A Zeiss Axio-Imager Standard Microscope (Carl Zeiss) was used to acquire digital images. Triglycerides in HepG2 cells were extracted with NP40, while those in the tissue were extracted using a heptane isopropanol-tween mixture as previously described (Mu *et al.* 2019). Triglycerides were measured using a Triglyceride assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Statistical analysis

The data are shown as means ± S.E.M. Statistical differences between two groups of variables were determined using Student's t-test, and among multiple groups of variables were determined by one-way ANOVA, followed by Bonferroni's multiple comparison post-tests. The correlation between two groups was determined by Pearson test. *P*-value of less than 0.05 was considered statistically significant.

Results

Hepatic nNOS activity was associated with insulin resistance in ob/ob mice

The expression levels of nNOS in different mouse tissues and human liver were examined. Figure 1A and B show the mRNA and/or protein expression levels of nNOS in the mouse brain, muscle, liver, pancreas, and heart with strong nNOS production in brain and muscle. Additionally, nNOS expression was detectable in human liver (Fig. 1C). In neurons, the activity of nNOS was regulated through interaction with NOS1AP by its C-terminal domain. Next,

the physical interaction between NOS1AP and nNOS was examined. As shown in Fig. 1D, NOS1AP co-localized with nNOS but not with iNOS in mouse liver. The liver-specific overexpression of NOS1AP in mouse liver lowered hepatic phosphorylation of nNOS at Ser1417, an active form of nNOS (Fig. 1E). In contrast, the liver-specific deletion of NOS1AP increased p-nNOS expression (Ser1417) (Fig. 1F). Moreover, p-nNOS was increased in livers of db/db diabetic mice compared with that of chow diet C57BL/6 mice (Fig. 1G). Treatment with L-NPA (20 mg/kg), a specific nNOS inhibitor, improved glucose tolerance in ob/ob mice (aged 8–9 weeks) (Fig. 1H). The area under the curve of GTT in the L-NPA-treated ob/ob mice was significantly lower than

Figure 1

nNOS activity was associated with hepatic insulin resistance. (A and B) nNOS mRNA (A) and protein (B) expression levels in mouse tissues. F, fat; L, liver; M, muscle; P, pancreas; H, heart; B, brain. (C) Human liver nNOS protein expression. L1 and L2, human liver tissue; Hep, HepG2 cells; MB, mouse brain. (D) Co-localization of NOS1AP and nNOS in mouse liver. Mice were injected with adenovirus encoding NOS1AP. IB, immunoblot; iNOS, inducible nitric oxide synthase; IP, immunoprecipitation; NOS1AP, nitric oxide synthesis adaptor protein. (E and F) Western blotting of phosphorylation of nNOS at Ser1417 in mouse liver with NOS1AP overexpression (E) and NOS1AP deletion (CKO) (F). (G) Phosphorylation of nNOS at Ser1417 expression in mouse liver from C57BL/6 (C57) and db/db diabetic mice (aged 12 weeks). (H and J) ob/ob mice (aged 8–9 weeks) were injected with L-NPA (nNOS specific inhibitor, 20 mg/kg) or saline (control). Glucose tolerance test (GTT) was performed 2 h later (H), and the AUC of GTT was calculated (I) ($n = 5$). (J) L-NPA treated ob/ob mice were injected with insulin (5 U/kg) for 10 min. Western blot analysis was conducted to detect p-IR, p-Akt and p-GSK3 expression (left). Quantification of protein phosphorylation levels are normalized to total levels (right) ($n = 5$). Data represent means $\pm$ s.e.m., $*P < 0.05$, $**P < 0.01$ vs control (Student's *t*-test).

that in the saline-treated ob/ob mice (Fig. 1I). No difference was observed in glucose tolerance between L-NPA-treated and saline-treated chow diet-fed C57BL/6 mice (data not shown). These findings indicated a correlation between nNOS activity and insulin resistance in the liver of obese mice. To confirm, phosphorylation of IR (Tyr1150/1151), AKT (Ser473) and GSK3 β (Ser9), three components of insulin-signaling, were detected, among which p-AKT is a key mediator of hepatocellular insulin action. As expected, treatment with L-NPA significantly enhanced the insulin-stimulated expression of p-IR and p-Akt, but not that of p-GSK3 β , in ob/ob mice (Fig. 1J).

Liver-specific overexpression of nNOS impaired glucose and insulin tolerance in HFD-induced obese mice

To confirm the findings above, the adenoviral vectors encoding nNOS were generated and delivered to C57BL/6 mice fed with HFD for 8 weeks. The liver-specific overexpression of nNOS was confirmed using Western blotting (Fig. 2A). Overexpression of nNOS in mouse liver did not affect body weight and fasting blood glucose levels (Fig. 2B and C). However, injection of Ad-nNOS resulted in impaired glucose tolerance (Fig. 2D and E) and insulin sensitivity in the HFD-induced obese mice (Fig. 2F and G). The areas under the GTT and ITT curve in mice transfected with adenovirus encoding nNOS was much higher than in control mice (Fig. 2E and G).

Overexpression of nNOS exacerbated HFD-induced hepatic steatosis, impaired hepatic glycogen storage and HepG2 cell glucose consumption

The effect of nNOS overexpression on hepatic steatosis in the HFD-induced obesity mouse model was examined. Liver sections revealed more lipid droplets shown by H&E and Oil Red O staining after overexpression of nNOS (Fig. 3A and B). Consistently, hepatic triglyceride content significantly increased in the mice with nNOS overexpression (Fig. 3C). Moreover, the overexpression of nNOS increased the expression of mRNA and protein levels of Ppargamma and Dgat1 in liver of the HFD-fed obese mice (Fig. 3D and E). Similar phenomena were observed in HepG2 cells. Treatment with PA and OA significantly increased the triglyceride production in of the HepG2 cells. The overexpression of nNOS further enhanced triglyceride production in the presence of PA and OA (Fig. 3F).

Furthermore, nNOS overexpression decreased the glycogen storage in the liver of the HFD-induced obese mice, and glucose consumption in HepG2 cells (Fig. 3G and H). In consistency, the mRNA and protein expression levels of Pck1 and/or Foxo1 were upregulated in the liver upon nNOS overexpression (Fig. 3I and J). Similar phenomena were observed in HepG2 cells (data not shown). These data suggested a role of nNOS on hepatic glucose metabolism regulation in liver of obese mice.

Figure 2

Overexpression of nNOS in liver impaired glucose and insulin tolerance of high-fat diet-induced obese mice. C57BL/6 mice were injected with adenoviruses encoding nNOS or EGFP (control) after 8 weeks of high-fat diet. (A) Western blot analysis of nNOS in liver. (B) Body weight ($n = 7-12$) and (C) fasting blood glucose ($n = 5-6$) were detected before and after injection. (D and E) Glucose tolerance tests (GTT) ($n = 6-8$) and (F and G) insulin tolerance tests (ITT) ($n = 5-8$) were conducted 9-12 days after injection, and the areas under curve (AUC) for GTT (E) and ITT (G) were calculated. Data represent means $\pm$ S.E.M., * $P < 0.05$ vs control (Student's t -test).

Figure 3

The overexpression of nNOS enhanced hepatocyte lipid accumulation, lowered glycogen storage and glucose consumption. C57BL/6 mice were injected with adenoviruses encoding nNOS (Ad-nNOS) after 8 weeks of high-fat diet (HFD). (A) H&E staining (scale bar, 100 μm) and (B) Oil Red O staining (scale bar, 25 μm) were conducted in liver of the HFD-fed mice. (C) Triglyceride (TG) content was detected in the liver of the mice ($n = 7-10$). (D) Real-time PCR and (E) Western blot analyses were performed on the liver extracts. Quantifications of mRNA ($n = 5-6$) and protein ($n = 4$) expression were normalized by Gapdh. Data represent means $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ vs indicated groups (Student's t-test). HepG2 cells were exposed to Ad-nNOS. After 16 h, the cells were treated with 0.4 mM palmitate (PA) in combination with oleate (OA) for 24 h. (F) TG content was detected from the HepG2 cells ($n = 9$). (G) PAS glycogen staining of the liver of the HFD-fed mice (scale bar, 100 μm). (H) The HepG2 cells were exposed to different concentrations of Ad-nNOS (5, 10, 15 and 20 MOI). Glucose consumptions of the cells were detected at the end of experiments. ** $P < 0.01$ for trend (Pearson test, $n = 7$). (I) Real-time PCR ($n = 6$) and (J) Western blot analyses were performed on liver extracts from HFD-fed mice, quantification of Western blotting was normalized by Gapdh ($n = 4$). Data represent means $\pm$ S.E.M. * $P < 0.05$ vs indicated groups (Student's t-test). *cd36*, fatty acid translocase; *fas*, fatty acid synthase; *acc1*, acetyl-CoA carboxylase1; *scd1*, stearoyl-Coenzyme A desaturase; *dgat*, diacylglycerol O-acyltransferase; *gpat*, glycerol-3-phosphateacyltransferase; *mtfp*, microsomal triglyceride transfer protein; *hsl*, hormone-sensitive lipase; *atgl*, adipose triglyceride lipase; *srebp1*, Sterol-regulatory element binding proteins1; *ppar*, peroxisome proliferator activated receptor; *aco1*, acyl-Coenzyme A oxidase 1; *g6pc*, glucose-6-phosphatase; *pck1*, phosphoenolpyruvatecarboxykinase1; *foxo1*, forkhead box protein O1.

Overexpression of nNOS inhibited insulin-signaling activity in HepG2 cells and mouse liver

To confirm the effect of nNOS on hepatic insulin sensitivity, the activity of insulin-signaling pathway in hepatocytes with nNOS overexpression was examined *in vitro* and *in vivo*. Treatment with PA significantly lowered the levels of insulin-stimulated phosphorylation of IR (Tyr1150/1151), Akt (Ser473) and GSK3 β in HepG2 cells (Fig. 4A). The effect of PA on insulin signaling was mimicked by nNOS overexpression alone in the cells. Transfection with

Ad-nNOS significantly reduced insulin-induced expression of p-IR β , p-Akt and p-GSK3 β expression in the cells (Fig. 4A). Similar phenomena could be viewed in the mouse liver with nNOS overexpression (Fig. 4B).

nNOS overexpression activated p38 MAPK signaling pathway in HepG2 cells

Previous studies have reported that p38MAPK activation contributed to insulin resistance in liver and that nNOS regulates p38MAPK in neuronal PC12 cells. Then, the

effect of nNOS overexpression on p38MAPK activity in the hepatocytes was examined. As shown in Fig. 5, transfection with Ad-nNOS significantly increased the phosphorylation of p38MAPK and decreased insulin-stimulated activation of insulin-signaling pathway. Inhibition of p38MAPK activity with its specific inhibitor SB203580 significantly lowered phosphorylation of p38MAPK, and mitigated the nNOS-induced inhibition of insulin signaling (Fig. 5). These results suggested that the inhibition effect of nNOS on insulin-sensitivity in HepG2 cells is dependent on the activation of p38MAPK.

Discussion

In the present study, we provide evidences demonstrating that nNOS played a role in inducing hepatic insulin resistance. The nNOS protein were detectable in human

and mouse liver. The *in vivo* evidences revealed that the elevation of hepatic nNOS in obese mice impaired glucose tolerance, reduced hepatic glycogen storage, increased hepatic triglyceride content, and inhibited insulin-induced signaling activation in mouse liver and HepG2 cells. In contrast, inhibition of nNOS activity improved glucose tolerance and hepatic insulin sensitivity in ob/ob mice. Moreover, the inhibition effects of nNOS on insulin sensitivity was through the activation of p38MAPK in the HepG2 cells.

Hepatic insulin resistance contributes to the development of T2D. The findings of the present study established a link between the activation of nNOS and elevation of hepatic glucose production through the inhibition of insulin-induced signaling activity. nNOS activity was positively correlated with hepatic insulin resistance. Liver-specific nNOS overexpression exacerbated glucose and insulin intolerance in DIO mice.

Figure 5

Overexpression of nNOS activated p38 MAPK activity. HepG2 cells were exposed to adenovirus encoding nNOS or EGFP (control) and treated with insulin (100 nM). (A) Western blot analysis of p-IR, p-AKT and p-GSK3beta expression in the presence or absence of p38 MAPK inhibitor SB203580 (20 uM) for 30 min. (B) Quantification of protein phosphorylation levels are normalized to total levels ($n = 5-7$). Data represent means $\pm$ S.E.M., * $P < 0.05$, ** $P < 0.01$ between indicated groups (One-way ANOVA with Bonferroni's multiple comparison test).

The overexpression of nNOS in liver reduced hepatic glycogen storage in mice and glucose utilization in the HepG2 cells. These changes were associated with upregulation of Pck1 and FoxO1 expression. PCK1 is a rate-limiting enzyme of gluconeogenesis in liver (Gomez-Valades *et al.* 2008). FOXO1, a transcription factor, mediates activation of PCK1 and downstream targets of insulin-signaling pathway (O-Sullivan *et al.* 2015). FoxO1-overexpressing mice exhibited impaired glucose tolerance and downregulated hepatic glycogen levels. In contrast, FoxO1 knockout mice exhibited enhanced glucose utilization and insulin sensitivity (Qu *et al.* 2006, Lu *et al.* 2012). Consistent with the findings of previous studies, this study demonstrated that nNOS overexpression upregulated the hepatic Foxo1 expression and downregulated the insulin-induced phosphorylation of proteins involved in the insulin-signaling pathway (IRbeta and Akt) both *in vivo*. Insulin exerts its effects by binding to its receptor, which promotes the autophosphorylation at the Tyr1150/1151 site of the insulin receptor beta subunits. The activation of insulin receptor, in turn, recruits and phosphorylates signaling of proteins, such as phosphoinositide-3-kinase/AKT, which are critical for hepatocyte insulin function. The downstream substrates of AKT include FOXO1 and GSK3beta. The activation of GSK3beta released the inhibition of glycogen

synthase, which leads to the production of glycogen in liver (Petersen & Shulman 2018). Glycogen can also be synthesized in the liver through a GSK3-independent pathway (Li *et al.* 2019). In contrast, inhibition of nNOS activity with L-NPA, a specific inhibitor, reversed glucose intolerance of ob/ob mice, and significantly improved insulin-stimulated hepatic signaling pathway activity in these mice. These findings suggested that nNOS exerts an inhibitory effect on the insulin-signaling pathway in the hepatocytes. The findings of the study were in consistent with these reported by Marsollier *et al.* (Marsollier *et al.* 2009) but not those reported by Shankar *et al.* (Shankar *et al.* 2000), and Nakata *et al.* (Nakata *et al.* 2008). Marsollier *et al.* reported that activation of hypothalamic nNOS activity leads to hepatic insulin resistance (Marsollier *et al.* 2009). Shankar *et al.* demonstrated that systemic nNOS knockout in mice resulted in mild insulin resistance (Shankar *et al.* 2000). Nakata *et al.* found normal glucose tolerance after systemic nNOS deletion (Nakata *et al.* 2008). The reasons for the contradictory results between different studies are unclear. It is worth noticing that in the present study the expression of nNOS in muscle tended to decrease following nNOS overexpression in the liver of obese mice (data not shown). Based on the findings from Mezghenna *et al.* that nNOS expression decreased in skeletal muscle of

obese Zucker (fa/fa) rats (Mezghenna *et al.* 2014), the downregulation of nNOS in muscle might be secondary to the whole-body insulin resistance induced by liver-specific nNOS overexpression. It is expected that nNOS might play different roles on insulin sensitivity in different tissues. More work is needed to clarify the possibility. iNOS regulates hepatic insulin resistance through NO release and subsequent S-nitrosylation of the insulin-signaling pathway-related proteins (Carvalho-Filho *et al.* 2005, Shinozaki *et al.* 2011, Sansbury & Hill 2014). In contrast to iNOS overexpression, the overexpression of nNOS did not result in the upregulation of NO production in the liver of obese mice (Data not shown). This suggested that the role of nNOS on insulin sensitivity inhibition in liver might be through NO-independent pathway. It is true that the activation of nNOS is not always associated with enhanced NO production (Sansbury *et al.* 2014).

Moreover, this study showed that nNOS is involved in the regulation of hepatic lipid accumulation, which is an early characteristic of NAFLD, in HFD-fed mice. In association with the increase in triglyceride accumulation upon nNOS overexpression in liver, the protein levels of Ppargamma and Dgat1 were significantly upregulated. DGAT1, a key enzyme in liver, catalyzes the terminal step of triacylglycerol synthesis. The expression of *DGAT1* was significantly increased in patients with NAFLD (Kohjima *et al.* 2007). The overexpression of DGAT1 in mice resulted in the elevation of intrahepatic triglyceride synthesis (Millar *et al.* 2006). PPARgamma is a transcription factor that promotes lipid synthesis and regulates multiple enzymes involved in the uptake and synthesis of fatty acids (Gross *et al.* 2017). NAFLD is associated with the upregulated expression of PPARgamma (Moran-Salvador *et al.* 2011, Pettinelli & Videla 2011). The knockout of PPARgamma decreased the expression of triglyceride synthesis-related enzymes DGAT1 and DGAT2 (Zhang *et al.* 2016), suggesting that hepatic nNOS might function as an aggravator for steatosis in obese mice. However, the mechanisms linking the expression of nNOS and PPARgamma are not clear.

Lastly, the results presented in the study demonstrated that the inhibitory effect of nNOS on insulin signaling was through the activation of p38 MAPK signaling in HepG2 cells. In the present study, the phosphorylation of p38MAPK was upregulated in the Ad-nNOS-transfected HepG2 cells, and the inhibition effect of nNOS on insulin-signaling pathway in HepG2 cells was significantly blunted when p38 MAPK activity was blocked with its specific inhibitor. This indicated that nNOS exerts an inhibitory effect on the insulin-signaling pathway in the HepG2 cells through p38MAPK. Previous studies have reported that

p38MAPK is activated in the liver of ob/ob or DIO mice (Gum *et al.* 2003, Sun *et al.* 2016). Activated p38MAPK inhibits the insulin-signaling pathway by downregulating the phosphorylation of insulin receptor substrate and AKT, which promotes insulin resistance. Moreover, activation of p38 MAPK participated in the regulation of liver glucose metabolism, promoted liver gluconeogenesis, and reduced glycogen synthesis and storage (Hemi *et al.* 2011). It is worth noticing that nNOS was reported to be the upstream mediator of phosphorylation of p38 MAPK induction and mediated excitotoxicity in the nervous system (Cao *et al.* 2005, Kajiwara *et al.* 2013, Lai *et al.* 2014). The findings of the present study that the inhibitory effect of nNOS on insulin signaling was dependent on p38MAPK were in line with the findings of previous studies on the neurons. The mechanisms underlying nNOS-mediated activation of p38MAPK in the HepG2 cells are unclear. In the nervous system, nNOS-mediated NO production enhances Ca^{2+} influx, which results in the activation of calmodulin kinase II and the phosphorylation of p38MAPK (Kajiwara *et al.* 2013, Lai *et al.* 2014). In this study, the overexpression of nNOS in the HepG2 cells or mouse liver did not significantly upregulate NO levels. This indicated that other factors were involved in nNOS overexpression-induced p38MAPK activation. Previously, we had reported that

Figure 6

Regulatory effect of NOS1AP-nNOS on hepatic insulin sensitivity under obesity. Obesity induced triglyceride accumulation, and resulted in downregulation of NOS1AP activity and upregulation of nNOS activity in the liver. nNOS activation, in turn, promoted p38 MAPK activation and inhibited insulin-signaling pathway through the downregulation of the phosphorylation levels of Akt, which lead to increased hepatic glucose production (HGP), decreased glycogen synthesis and hepatic insulin resistance.

the overexpression of NOS1AP enhanced hepatic insulin sensitivity and downregulated p-p38MAPK expression in the livers of ob/ob mice (Mu *et al.* 2019). In this study, the expression of NOS1AP in the liver was inversely correlated with that of nNOS active forms. The binding of NOS1AP to nNOS in the neurons promotes the activity of nNOS on its target protein. Hence, NOS1AP may exert its effect in the liver through the inactivation of nNOS.

Taking together, the findings of this study indicated that nNOS plays an inhibitory role in liver insulin sensitivity. The overexpression of nNOS promoted fat accumulation, decreased glycogen storage, and inhibited the insulin-stimulated signaling pathway in the liver of obese mice. Moreover, the overexpression of nNOS promoted p38 MAPK activation in HepG2 cells, which was essential for the inhibitory effects of nNOS on insulin signaling (Fig. 6). The inhibitory effects of nNOS on hepatic insulin-signaling activity highlight the importance of nNOS in the pathogenesis of NAFLD, and suggested that nNOS could be utilized as a potential therapeutic target to prevent diabetes by improving hepatic insulin sensitivity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

TZ, QL, QM and KM contributed to experiment performance, and data analysis. TZ contributed to the manuscript drafting. CW designed the research, analyzed and interpreted the data, and critically revised the manuscript for important intellectual content, and is 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 revised and approved the final version of the manuscript.

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