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High fat diet-induced oxidative stress blocks hepatocyte nuclear factor 4α and leads to hepatic steatosis in mice

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Nonalcoholic fatty liver disease (NAFLD) is the most common form of chronic liver disease with manifestation of over-accumulation of fat in liver. Increasing evidences indicate that NAFLD may be in part caused by malfunction of very low density lipoprotein (VLDL) secretion. Hepatocyte nuclear factor 4α (HNF4α), a nuclear receptor protein, plays an important role in sustain hepatic lipid homeostasis via transcriptional regulation of genes involved in secretion of VLDL, such as apolipoprotein B (ApoB). However, the exact functional change of HNF4α in NAFLD remains to be elucidated. In the present study, we found that high fat diet (HFD) induced cytoplasmic retention of HNF4α in hepatocytes, which led to down-regulation of hepatic ApoB expression and its protein level in serum, as well as reduced secretion of VLDL. We further revealed that oxidative stress, elevated in fatty liver, was the key factor inducing the cytoplasmic retention of HNF4α in hepatocytes by activating protein kinase C (PKC)-mediated phosphorylation in HNF4α. Thus, our findings reveal a novel mechanism underlying HFD-induced fatty liver that oxidative stress impairs function of HNF4α on ApoB expression and VLDL secretion via PKC activation, eventually promoting fat accumulation in the liver. Therefore, oxidative stress/PKC/HNF4α pathway may be a novel target to treat diet-induced fatty liver.

KEYWORDS
apolipoprotein B, HNF4α, NAFLD, oxidative stress, protein kinase C, VLDL secretion

Abbreviations: ApoB, apolipoprotein B; CAT, catalase; FFA, free fatty acid; H_2O_2, hydrogen peroxide; HFD, high fat diet; HNF4α, hepatocyte nuclear factor 4α; LDL, low density lipoprotein; LPS, lipopolysaccharide; MTTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; NRF2, nuclear factor-like 2; PA, palmitate; PKC, protein kinase C; PPARα, peroxisome proliferator-activated receptor-α; SOD, superoxide dismutase; TAG, triglycerides; VLDL, very low density lipoprotein.

Dongsheng Yu and Gang Chen contributed equally to this work.

1 | INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is caused by over-deposit of hepatic fat in the absence of excess alcohol intake, and often associated with obesity, insulin resistance, and metabolic syndrome (Gaggini et al., 2013; Loomba & Sanyal, 2013). Nonalcoholic steatohepatitis (NASH), characteristic of liver damage after steatosis, is the more virulent form of NAFLD, and can lead to cirrhosis (Michelotti, Machado, & Diehl, 2013). Hepatic lipid levels are the results of balance between multiple physiological processes, including lipid uptake, lipogenesis, fatty acid-oxidation, and very low density lipoprotein (VLDL) secretion. Free fatty acid (FFA) flux from lipolysis in adipose tissue, dietary intake, and de novo lipogenesis are usually either oxidized in the mitochondria (β-oxidation) or esterified to triglycerides (TAG), which in turn are packaged as VLDL for export (Browning & Horton, 2004; Cohen, Horton, & Hobbs, 2011). VLDL secretion is the main pathway to transport hepatic TAG to periphery tissues. When the biosynthesis of TAG exceeds the rate of FFA oxidation of TAG secretion via VLDL, excessive TAG accumulates into lipid droplets in the liver resulting in steatosis. Steatosis may also originate when the packaging process of TAG into VLDL particles is impaired due, for instance, to hepatitis C virus infection (Gupte, Dudhade, & Desai, 2006; Perlemuter et al., 2002) or genetic mutations in apolipoprotein B (ApoB), and microsomal triglyceride transfer protein (MTTP) (Berriot-Varoqueaux, Aggerbeck, Samson-Bouma, & Wetterau, 2000; Tanoli, Yue, Yablonskiy, & Schonfeld, 2004), the important component of VLDL and key enzyme catalyzing VLDL synthesis, respectively. Notably, high fat diet (HFD) also inhibits the VLDL secretion in rodents (Cahova, Dankova, Palenickova, Papackova, & Kazdova, 2012; Francone, Griffaton, & Kalopissis, 1992; Oussadou, Griffaton, & Kalopissis, 1996; Yang et al., 2015). Over-consumption of fat is the more widespread in the development of NAFLD compare to hepatitis C virus infection or genetic mutation. However, how HFD affects hepatic VLDL production remains to be elucidated.

Hepatocyte nuclear factor 4α (HNF4α, NR2A1) is a highly conserved member of the nuclear receptor superfamily and plays important roles in both liver early development and regulating hepatocyte proliferation in adults (Alder et al., 2014; Bonzo, Ferry, Matsubara, Kim, & Gonzalez, 2012; Walesky et al., 2013). HNF4α is a master regulator of liver gene expression, and critically regulates multiple hepatic metabolic pathways (Martinez-Jimenez, Kyrmizi, Cardot, Gonzalez, & Talianidis, 2010; Odom et al., 2004). Liver-specific conditional knockout of HNF4α in adult mice led to severe steatosis in association with disruption of VLDL secretion (Hayhurst, Lee, Lambert, Ward, & Gonzalez, 2001; Yin et al., 2011; Yin, Ma, Ge, Edwards, & Zhang, 2011), and misregulation of ApoB and MTTP expression. Although HNF4α has important functions in maintaining hepatic lipid homeostasis, its roles in the development of HFD-induced NAFLD remain to be elucidated.

In this study, we analyzed the fatty liver induced by HFD in mice, demonstrating that the cytoplasmic retention of hepatic HNF4α is caused by the enhanced oxidative stress in liver, which mediates phosphorylation of HNF4α at Ser78 by activating PKCs. The phosphorylative modification prevents the transportation of HNF4α into nucleus via importin. Thus, the impaired transcriptional activity of HNF4α reduces expression of ApoB and subsequently decreases the VLDL secretion. As the result, more TAG accumulates in liver and deteriorates the NAFLD. The present investigation reveals a new mechanism that oxidative stress promotes NAFLD development.

2 | MATERIALS AND METHODS

2.1 | Animals

Male C57BL/6 and ob/ob mice were purchased from Model Animal Research Center of Nanjing University, and housed in a pathogen-free barrier facility with a 12 hr light/dark cycle and free access to food, and water. At the age of 8 weeks, WT mice were fed with either a standard laboratory rodent chow diet (Chow) providing 3.85 kcal/g of energy (10% fat, 20% proteins, and 70% carbohydrates) or a HFD providing 5.24 kcal/g of energy (60% fat, 20% carbohydrate, 20% protein; supplemented with 1% cholesterol; FBSH Biopharmaceutical Co., Ltd., Shanghai, China) for 12 weeks. Mice in other groups were fed HFD for 4 or 8 weeks until the age of 20 weeks, ob/ob mice were always fed with chow diet. Mice were sacrificed during the light phase without food deprivation. Serum and livers were isolated and stored in liquid nitrogen. All animal husbandry and procedures for experiments were approved by the Animal Experiment Committee of the Nanjing Medical University and complied with the guidelines of the Nanjing Medical University's Regulations of Animal Experiments.

2.2 | Assay for TAG, VLDL, and VLDL-TAGs in serum, liver, and medium of hepatocytes

VLDL levels in serum and medium were measured using mouse VLDL ELISA kit (Luyuan Bode Biological technology, Beijing, China). VLDL and low-density lipoprotein (LDL) in serum were precipitated by phosphotungstic acid/Mg^{2+} reagent (Demacker, Hessels, Toenhake-Dijkstra, & Baadenhuijsen, 1997), whereas LDL was selectively precipitated by polyvinylsulphate/polyethyleneglycol methyl ether (Demacker, Hijmans, Brenninkmeijer, Jansen, & Van't Laar, 1984). The content of TAG in serum and corresponding supernatant lack of LDL or LDL plus VLDL was determined enzymatically with commercial TAG assay kits (GPO-PAP; Dong'ou Bioengineering Co. Ltd, Wenzhou, China), and subsequently the content of TAG in VLDL could be calculated. VLDL-TAGs in medium secreted by cultured hepatocytes were directly measured by TAG assay kits, for VLDL was the unique carrier of TAG in culture medium of hepatocytes. For liver TAG assay, liver samples were homogenized in 1% acetic acid and extracted by chloroform-methanol (2:1). The organic phase was evaporated in a new tube. Lipid residues were dissolved in isopropanol and measured using the TAG assay kit.

2.3 | Reactive oxygen species (ROS) assays

Liver samples were homogenized in a lysis buffer, mixed with dihydroethidium fluorescent probe (DHE, 37291, Sigma-Aldrich, Shanghai, China) to a final probe concentration of 5 μM for 30 min at 37°C. DHE fluorescence was measured using a BioTek Synergy 2 Multi-Mode Microplate Reader (358 nm excitation and 461 nm emission) (Kalyanaraman et al., 2012).

2.4 | Hepatic superoxide dismutase (SOD), catalase (CAT) activity, and lipid peroxidation content assay

Total hepatic SOD and CAT activities were measured on homogenized liver tissue using commercial assay kits according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Levels of malondialdehyde (MDA), a product of lipid peroxidation, were measured based on reaction between MDA and thiobarbituric acid (TBA) with commercial kit from Nanjing Jiancheng Bioengineering Institute (Lv et al., 2017). Briefly, 500 μl of tissue sample was mixed with 2 ml of 0.6% TBA in acetic acid, after 40 min incubation at 95°C, the final reaction mixture was centrifugated at 4,000 rpm for 10 min. MDA levels were determined at wavelengths of 532 nm. The enzymatic activities were recorded as units per milligram of protein (U/mg protein). Values obtained were the average of three independent measurements.

2.5 | Cell culture

The human hepatoblastoma/hepatocellular carcinoma HepG2 cell line (ATCC HB-8065) and COS-7 cells line (Institute of Biochemistry and Cell Biology, GNO-2, Shanghai, China) were maintained at 37°C in 5% CO_2 in Dulbecco's modified eagle medium (DMEM) containing 10% fetal bovine serum (FBS) plus penicillin (100 U/ml) and streptomycin (100 μg/ml). Hepatocytes were isolated from 10-week old male mice and cultured in Williams' medium E (WME) supplemented with 6% FBS as previously described (Sheng et al., 2012). After seeded in plates (5×10^6 cells/10 cm dishes) for 5 hr, hepatocytes were serum deprived and treated by stimuli including TNFα (10 ng/ml), palmitate (PA, 250 μM), lipopolysaccharide (LPS, 1 μg/ml), hydrogen peroxide (H_2O_2, 1 μM), glucose (30 mM) or insulin (100 nM) for further 24 hr. Dexamethasone (100 nM) was added 6 hr before harvesting. Pan-PKC inhibitor Go6983 and proteasome inhibitor MG132 were purchased from Selleck Chemical (Houston, TX), nuclear transport receptor importin-β inhibitor importazole (#SML0341) was obtained from Sigma–Aldrich.

2.6 | Immunoprecipitation and immunoblotting

COS-7 cells, hepatocytes or frozen liver samples were homogenized in ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na_3VO_4, 100 mM NaF, 10 mM $Na_4P_2O_7$, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin). Cell or tissue extracts were incubated with primary antibody of HNF4α at 4°C for 2 hr and with protein A-sepharose beads (P001-3, 7-Sea Biotech, Shanghai, China) for an additional hour at 4°C. The immunocomplexes adsorbed on the protein A-sepharose beads were washed three times with lysis buffer and boiled at 95°C for 5 min in loading buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 2% mercaptoethanol, 10% glycerol, 0.005% bromphenol blue). The proteins in cytoplasm and nucleus were extracted by kit from Beyotime Institute of Biotechnology (Shanghai, China). Serum samples were directly mixed with loading buffer for boiling. Protein was separated by SDS–PAGE, immunoblotted with indicated primary antibodies and horseradish peroxidase-conjugated secondary antibodies (1:5000 dilution; Anti-Rabbit IgG, A21020; Anti-mouse IgG, A21010; Abbkine, Wuhan, China) and visualized using Tanon-5200 Chemiluminescence Imager (Tanon, Shanghai, China) with ECL Western blotting substrate (Biovision, Milpitas, CA). Rabbit polyclonal antibodies used were as follows: phospho-serine antibody (1:1000 dilution; ab9332, Abcam, Shanghai, China), HNF4α (1:1000 dilution; ab199431, Abcam), ApoB (1:1000 dilution; ab31992, Abcam), MTTP (1:1000 dilution; ab75316, Abcam), acetylated-lysine (1:2000 dilution; 9441S, Cell Signaling Technology, Shanghai, China). Mouse monoclonal antibodies were as follows: β-actin (1:4000 dilution; 200068-8F10, Zen BioScience, Chengdu, China) and tubulin (1:4000 dilution; 4466, Cell Signaling Technology).

2.7 | Quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted from livers or primary hepatocytes and used to measure the mRNA abundance using Absolute TM QPCR SYBRGreen kits (Thermo Scientific, Waltham, MA) and a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific Inc.). The primer sequences were as follows: ApoB, 5′-CCAGAGTGTGGAGCTGAATGT-3′ (forward) and 5′-TTGCTTTTTAGGGAGCCTAGC-3′ (reverse); MTTP, 5′-AGCCAGTGGGCATAGAAAATC-3′ (forward) and 5′-GGTCACTTTACAATCCCCAGAG-3′ (reverse); HNF4α, 5′-ATGCGACTCTCTAAAACCCTTG-3′ (forward) and 5′-ACCTTCAGATGGGGACGTGT-3′(reverse); SOD1, 5′-AACCAGTTGTGTTGTCAGGAC-3′ (forward) and 5′-CCACCATGTTTCTTAGAGTGAGG-3′ (reverse); CAT, 5′-AGCGACCAGATGAAGCAGTG-3′ (forward) and 5′-TCCGCTCT CTGTCAAAGTGTG-3′ (reverse); NRF2, 5′-TCTTGGAGTAAGTCGAGAAGTGT-3′ (forward) and 5′-GTTGAAACTGAGCGAAAAAGGC-3′ (reverse); 36B4, 5′-AAGCGCGTCCTGGCATTG TCT-3′ (forward) and 5′-CCGCAGGGGCAGCAGTGGT-3′(reverse);

2.8 | Vectors used

pGL3-ApoB containing ApoB promoter was kindly provided by Wei, Dongping (The First Hospital of Nanjing, Nanjing, Jiangsu, China). pcDNA3.0-HNF4α was a present from Rui, Liangyou (University of Michigan, MI), which is used to construct HNF4α mutant (S78A and S78D) by Shanghai Generay Biotech Co., Ltd. A PCR-generated EcoR1/Xho1 fragment encoding mouse PKCδ and

PKCε from mouse cDNA were inserted into the EcoR1/Xho1-digested pcDNA3.1(+) (Invitrogen, Shanghai, China). The primers for cloning are as follows: PKCδ, 5′-GTCCAGTGTGGTGGAATTCGCCACCATGGCACCCTTCCTGCGCATCT-3′ (forward) and 5′-CGGGCCCTCTAGACTCGAGTTAAATGTCCAGGAATTGCTCA-3′ (reverse); PKCε, 5′-GTCCAGTGTGGTGGAATTCGCCACCATGGTAGTGTTCAATGGCCTTC-3′ (forward) and 5′-CGGGCCCTCTAGACTCGAGTCAGGGCATCAGGTCTTCACCA-3′(reverse).

2.9 | Luciferase assays

HepG2 cells were seeded onto 24-well plates, grown to 40–50% confluency, and transfected with luciferase reporter plasmids using polyethylenimine (1 mg/ml). Briefly, cells were incubated for 4 hr in 250 µl of serum-free DMEM containing pGL3-ApoB (200 ng), pRL-TK-Renilla (10 ng), pcDNA3.0-Flag-HNF4α (40 ng), and polyethylenimine (3 µl) and then grown in DMEM with 10% FBS for 24 hr. The cells were subsequently incubated for 24 hr in the complete medium described above supplemented with H_2O_2 (1 µM) or Go6983 (1 µM). Luciferase activity was measured using the Dual-luciferase reporter assay System (Promega, Madison, WI) and normalized to Renilla luciferase values. Measurements for three biological replicates were taken in triplicate and averaged.

2.10 | Immunofluorescence staining

Primary hepatocytes were seeded on 18-mm glass coverslips in 12-well plates at a density of 5×10^4 cells/well and treated as described above. After treatment, cells were fixed in 4% paraformaldehyde, and stained for indirect immunofluorescence using an anti-HNF4α primary antibody (1:200 dilution), followed by an Alexa Fluor 488 goat anti-rabbit secondary antibody (1:200 dilution, Abcam). HNF4α proteins and 4,6-diamidino-2-phenylindole (DAPI)-stained nuclei were visualized using an Olympus IX81 inverted fluorescence microscope. Combined DAPI/Alexa Fluor 488 images were generated using an Olympus DP70 digital camera with DP Controller and DP Manager software.

2.11 | Statistical analysis

Data were presented as mean ± S.E. Data between groups were analyzed by using unpaired two-sided Student's *t*-test or one-way ANOVA (GraphPad Prisim, La Jolla, CA). $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | HFD induces hepatic TAG accumulation but decreases VLDL secretion in mice

The earliest stage of NAFLD is hepatic steatosis, characterized by the deposition of TAG in lipid droplets of hepatocytes (Cohen et al., 2011). Consistent to previous reports (Cahova et al., 2012; Francone et al., 1992; Oussadou et al., 1996), hepatic TAG level in C57BL/6 mice was significantly increased after 4-week HFD-feeding and further elevated (12-fold) with 12-week HFD-feeding (Figure 1a). Serum TAG level was not significantly increased until 12-week HFD-feeding (Figure 1b). Since VLDL secretion is the major export pathway for hepatic TAGs (Cohen et al., 2011), we next examined the profiles of serum level of VLDL under HFD-feeding. Interestingly, the serum level of VLDL was significantly reduced up to 12-week HFD-feeding (Figure 1c). To determine VLDL production rate from the liver, we measured serum VLDL, and VLDL-TAG following intravenous injection of tyloxapol, the inhibitor of lipoprotein lipase preventing the metabolism, and removal of serum lipoprotein transporting TAG (VLDL and chylomicron). Notably, serum VLDL and VLDL-TAG levels were declined in mice fed with HFD for 12 weeks by around 35% and 25%, respectively, compared to the chow diet-fed mice within 2 hr after tyloxapol injection (Figures 1d and 1e). It is consistent with the previous studies showing attenuated VLDL secretion after HFD-feeding (Cahova et al., 2012; Francone et al., 1992; Oussadou et al., 1996; Yang et al., 2015). In the present investigation, HFD-induced increase in serum TAG should be due to the elevated chylomicron concentration, the lipoprotein mediating TAG absorption from food, since the secretion of Apo-48, the typical component in chylomicron, was increased after 12-week HFD-feeding (data not shown). Our data suggest that the decline of VLDL secretion may be an important driver in the development of hepatic seatosis.

3.2 | The expression of hepatic ApoB is reduced in hepatic steatosis

Since HFD-feeding attenuated VLDL secretion, we hypothesize that it may result from the misregulation of ApoB, the key component of VLDL, or MTTP, the enzyme mediating the assembling, and secretion of VLDL. Although hepatic mRNA expression of MTTP was up-regulated by HFD-feeding (Figure 2a), the protein expression remains normal (Figure 2b, upper panel). However, both hepatic mRNA and protein levels of ApoB-100, the subtype of ApoB synthesized in liver, were reduced under HFD-feeding (Figures 2a and 2b, middle panel). Furthermore, HFD-fed mice had lower serum level of ApoB-100 (Figure 2c, upper panel), probably caused by less secretion of ApoB-100 compared to those fed with normal chow. It is evidenced by the attenuated accumulation of serum ApoB-100 under the treatment of tyloxapol in HFD-fed mice (Figure 2c, lower panel). Together, these results suggest that HFD-reduced hepatic VLDL secretion is correlated to the inhibition of ApoB expression in liver.

3.3 | HFD induces cytoplasmic retention of HNF4α in liver

HNF4α is a master regulator for ApoB expression (Hayhurst et al., 2001; Yin et al., 2011), we reason that HFD leads to malfunction of HNF4α, which disturbs ApoB transcription. To this end, we examined the expression of hepatic HNF4α, and found that both mRNA and total protein levels of hepatic HNF4α of HFD-fed mice is comparable to

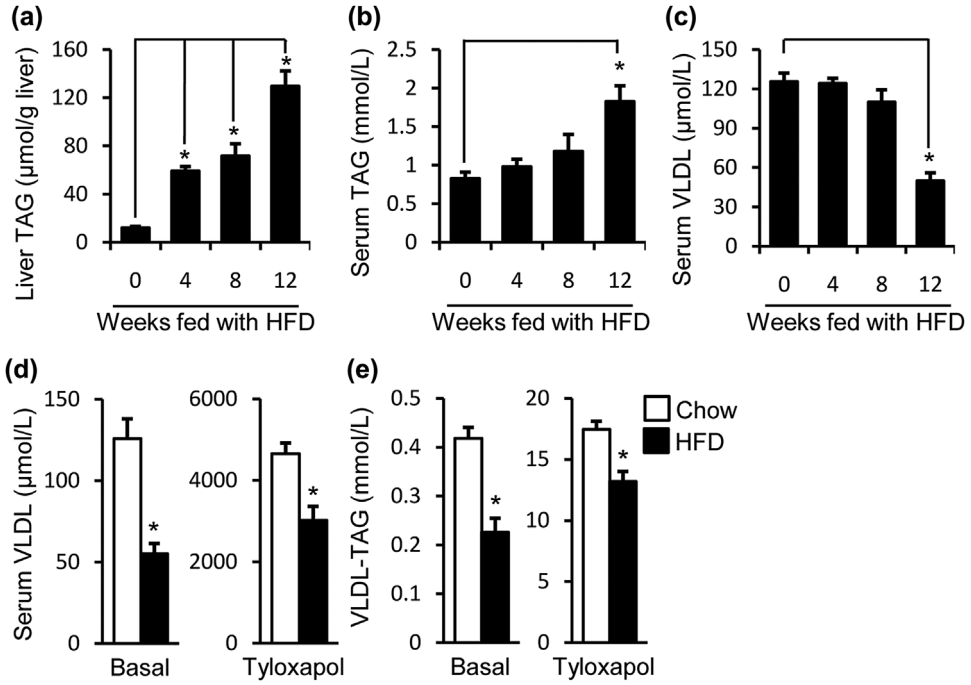

FIGURE 1 HFD induces hepatic TAG accumulation with reduction of VLDL secretion in mice. Mice, fed with HFD for 0, 4, 8, or 12 weeks until 20 weeks old, were executed in fed states ($n = 6$ per group). (a) Liver TAG assay; (b) serum TAG assay; (c) serum VLDL assay. (d and e) Mice, fed with normal chow or HFD for 12 weeks until 20 weeks old were administrated by tyloxapol (600 mg/kg) i.v.; serum was taken for VLDL assay (d) and VLDL-TAG assay (e). The data are expressed as the mean ± S.E., $^*p < 0.05$

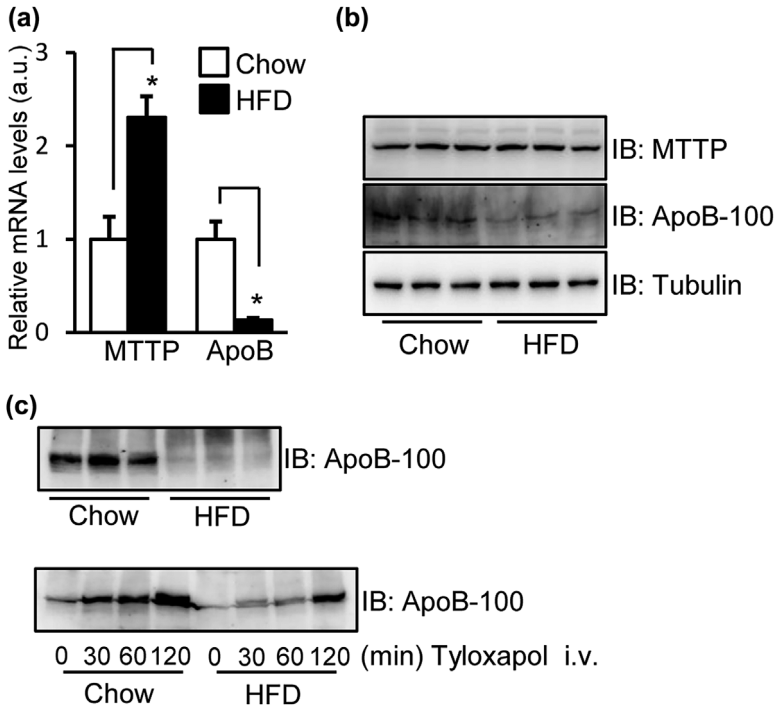

FIGURE 2 Hepatic ApoB expression is reduced in hepatic steatosis. Male mice were fed with normal chow or HFD for 12 weeks until 20 weeks old. (a) Total RNA was isolated from livers for mRNA level assay detected by RT-qPCR and normalized to 36B4 mRNA level. Data are expressed as fold-change relative to the level of chow-fed mice, $n = 6$ per group. The data are expressed as the mean ± S.E., $^*p < 0.05$. (b) Liver extracts were immunoblotted by antibodies against MTTP, ApoB-100 or tubulin. (c) Upper panel, serum isolated from three mice were individually immunoblotted by ApoB-100 antibody; lower panel, mixed serum isolated from three mice at different time point after the treatment of tyloxapol were immunoblotted by ApoB-100 antibody. a.u., arbitrary units

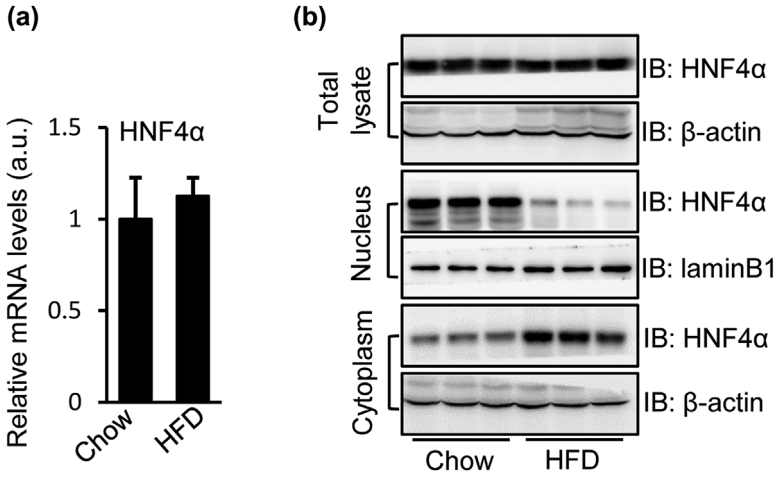

FIGURE 3 HFD induces cytoplasmic retention of HNF4α in liver. Male mice were fed with normal chow or HFD for 12 weeks until 20 weeks old. (a) Total RNA was isolated from livers to detect mRNA level of HNF4α by RT-qPCR and normalized to 36B4 mRNA level. Data are expressed as fold-change relative to the level of chow-fed mice, $n = 6$ per group. The data are expressed as the mean ± S.E., $^*p < 0.05$. (b) Proteins from nucleus, cytoplasm, and liver extracts were immunoblotted by antibodies of HNF4α, laminB1 or β-actin. a.u., arbitrary units

those fed with normal chow (Figures 3a and 3b, upper panel). However, further examination in nucleus/cytoplasm distribution of HNF4α revealed that HFD-feeding reduced HNF4α protein content in the nucleus, but increased its cytoplasmic content (Figure 3b, middle and lower panel), suggesting that the abnormal subcellular localization of HNF4α reduces the regulating capacity of HNF4α on ApoB expression.

3.4 | Oxidative stress-induced phosphorylation and cytoplasmic retention of HNF4α are regulated by PKCs in hepatocytes

We have shown above that the HFD-feeding induced the cytoplasmic retention of HNF4α. To dissect which factor dominates the subcellular localization of HNF4α in hepatic steatosis, we treated primary hepatocytes with H_2O_2, PA, LPS, TNFα, insulin, and glucose to mimic hepatic internal milieu, such as oxidative stress, hyperlipidemia, inflammation, hyperinsulinemia, and hyperglycemia, the typical risk factors related to hepatic steatosis. It is noteworthy that only H_2O_2 treatment resulted in striking cytoplasmic retention of HNF4α in the hepatocytes (Figure 4a). To determine whether HFD-feeding leads to liver oxidative stress, we examined the hepatic levels of two oxidant markers, ROS, and MDA. As shown in Figures 4b and 4c, 12-week HFD feeding significantly increased the levels of liver ROS and MDA, which is consistent to the previous reports (Dutta et al., 2014; Rincón-Cervera et al., 2016). Furthermore, we found that HFD reduced the activity and mRNA levels of some important antioxidant enzymes, SOD, and CAT (Figures 4d and 4e). NRF2, an essential regulator promoting the transcription of a broad range of antioxidant genes and usually activated by oxidative stress (Kensler, Wakabayashi, & Biswal, 2007), was significantly increased in mRNA level (Figure 4e). All the data indicated that the oxidative stress affects throughout the development of NAFLD.

It had been reported that phosphorylation on Ser78 by PKC leads to the cytoplasmic localization of HNF4α (Sun et al., 2007) and acetylation of HNF4α by CREB-binding protein increased the HNF4α DNA binding and its transcriptional activity (Soutoglou, Katrakili, & Talianidis, 2000). Thus, we examined the phosphorylation and acetylation status of HNF4α in the liver of HFD-induced obese mice. Interestingly, immunoprecipitation of HNF4α followed by immunoblot against either serine phosphorylation or lysine acetylation sites revealed that HFD-feeding increased the serine phosphorylation of HNF4α without affecting its acetylation in liver (Figure 5a). Given that H_2O_2 treatment activates PKC (Konishi et al., 1997; Lee et al., 2009), and HNF4α is a downstream substrate of PKC (Sun et al., 2007), we next test whether oxidative stress by H_2O_2 regulates serine phosphorylation of HNF4α. As shown in Figure 5b, H_2O_2 treatment increased the serine phosphorylation of HNF4α by 60% in hepatocytes, while the combination with Go6983 (denoted as Go), a PKC inhibitor, partially reversed the phosphorylation by 30%. Correspondingly, H_2O_2 treatment increased cytoplasmic protein fraction of HNF4α and decreased its nuclear protein content, while Go6983 partially reversed H_2O_2 mediated cytoplasmic retention of HNF4α (Figure 5c). Furthermore, immunofluorescence data also confirmed the H_2O_2-regulated nucleus/cytoplasm distribution of HNF4α via PKC (Figure 5d).

3.5 | Oxidative stress inhibits HNF4α mediated transcription of ApoB and reduces VLDL and TAG secretion from primary hepatocytes

Given that H_2O_2 promotes cytoplasmic retention of HNF4α, this urge us to test whether H_2O_2 affects transcriptional activity of HNF4α on its target gene. As expect, our reporter gene assay performed in HepG2 cells demonstrated that H_2O_2 treatment significantly inhibited the ApoB promoter-driven luciferase when co-transfecting HNF4α,

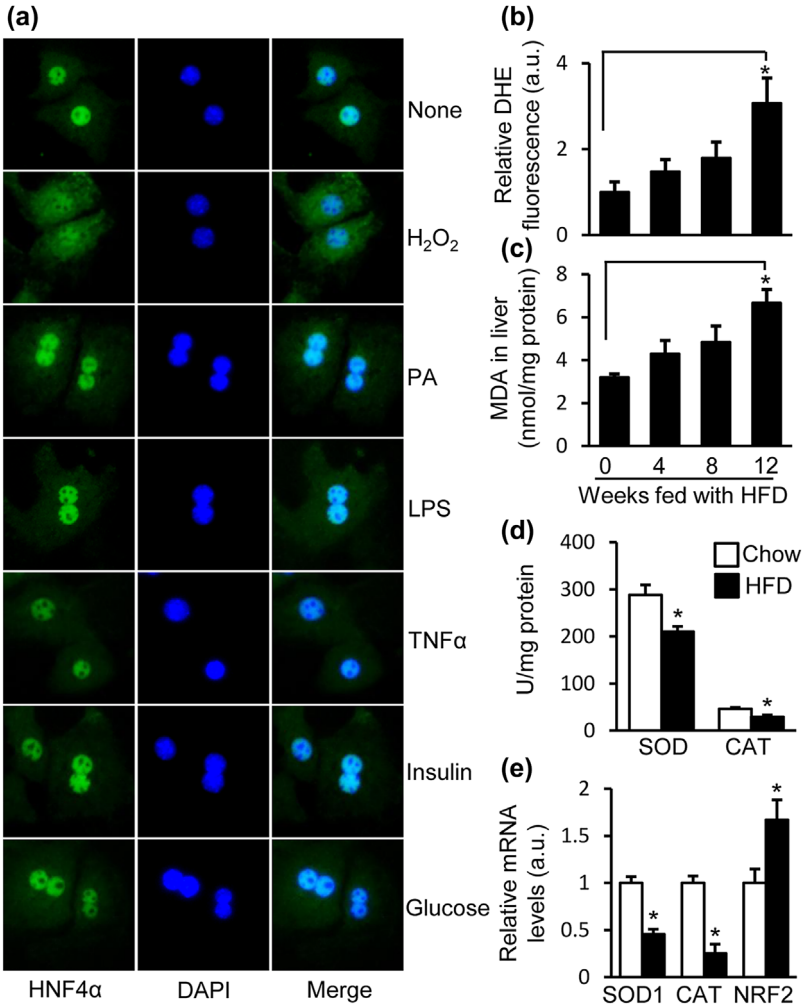

FIGURE 4 Oxidative stress induces the cytoplasmic retention of HNF4α in hepatocyte. (a) Primary hepatocytes were isolated and cultured in WME supplemented with 6% FBS for 5 hr and then grown in serum free medium plus stimuli, including H_2O_2 (1 μM), PA (250 μM), LPS (1 μg/ml), TNFα (10 ng/ml), insulin (100 nM), glucose (30 mM) for further 24 hr. Dexamethasone (100 nM) was added 6 hr before harvesting. Fixed cells were stained with HNF4α primary antibody and Alexa Fluor 488 goat anti-rabbit secondary antibody (Green), DAPI (blue) was incorporated in the mounting solution. (b–e) Mice fed with HFD for 0, 4, 8, or 12 weeks until 20 weeks old. ROS (b), MDA (c), SOD and CAT activity (d) in liver were assayed ($n = 6$ per group). (e) Hepatic mRNA levels of SOD1, CAT, and NRF2 were determined using RT-qPCR and normalized to 36B4 mRNA level. The data are expressed as the mean ± S.E., $^*p < 0.05$ versus group without HFD-feeding. a.u., arbitrary units

while addition of Go6983 eliminated the inhibitory effect of H_2O_2 (Figure 6a). In addition, the similar pattern was found in the regulation of endogenous mRNA level of ApoB by H_2O_2 or plus Go6983 treatment in primary hepatocytes (Figure 6b). Subsequently, whether the reduced transcriptional activity of HNF4α by H_2O_2 treatment affects the VLDL secretion was tested. As expected, H_2O_2 treatment inhibited the secretion of VLDL and TAG from the primary hepatocytes, while Go6983 almost eliminated this effect (Figures 6c and 6d).

3.6 | PKC δ and ε phosphorylate HNF4α specifically at Ser78 and blocks HNF4α from transferring into nucleus

PKC includes various isotypes, therefore it is necessary to make out which one mediates the phosphorylation and malfunction of HNF4α. PKC δ and ε were chosen to be tested, for they are over-activated in livers developing steatosis or by oxidative stress (Birkenfeld & Shulman, 2014; Greene et al., 2010; Jung et al., 2004; Majumder et al., 2001). The luciferase assay indicated that both isotypes suppressed HNF4α-mediated transcription of ApoB (Figure 7a). Actually, we also tested the effect of PKC α, β, and ζ, and found that they were all inhibitory to HNF4α (data not shown), which implies that the inhibitory effect on HNF4α may be prevail among PKC isotypes.

Utilizing in vitro kinase assay, Sun et al. (2007) revealed that PKC phosphorylated HNF4α at multiple sites at least including Ser78 and Ser304. But this in vitro zymological method could not precisely demonstrate the physiologic interaction between PKC and HNF4α in cell. In the present study, we expressed the WT HNF4α or S78A (the Ser78-nonphophorylated HNF4α mutant) with PKCδ in COS-7 cells (the cell line with low endogenous HNF4α expression, Supplemental

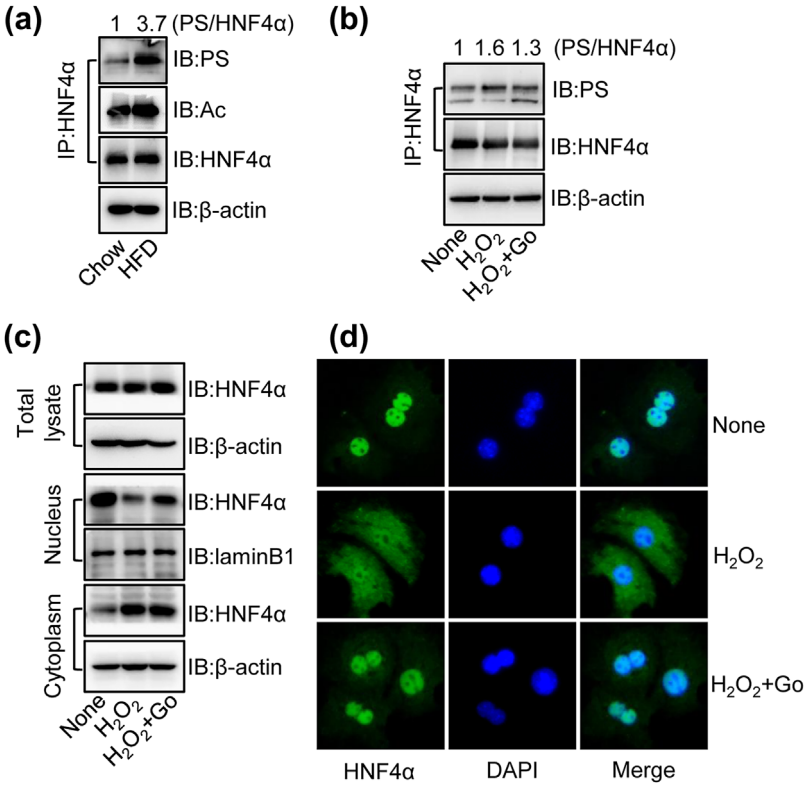

FIGURE 5 Oxidative stress induces the cytoplasmic retention of HNF4α via PKC mediated phosphorylation on HNF4α. (a) Extracts, from livers of mice fed with normal chow or HFD for 12 weeks, were immunoprecipitated by anti-HNF4α antibody; the blots were immunoblotted by antibody against phospho-serine (denoted as PS), acetylated-lysine (denoted as Ac), or HNF4α. β-actin in extracts was immunoblotted as input. (b–d) Primary hepatocytes were cultured in WME supplemented with 6 % FBS for 5 hr and then grown in serum-free medium with/without H_2O_2 (1 μM) or plus Go6983 (1 μM, denoted as Go) for further 24 hr. Dexamethasone (100 nM) was added 6 hr before harvesting. (b) Hepatocyte extracts were immunoprecipitated by HNF4α antibody; the blots were immunoblotted by phospho-serine (PS) and HNF4α antibody. β-actin in extracts was immunoblotted as input. Bands were quantified from immunoblot digital images using ImageJ software. Relative intensity of phospho-serine bands was normalized by that of HNF4α bands, and results are presented above the immunoblot image. (c) Proteins from hepatocyte extracts, nucleus and cytoplasm were immunoblotted by antibody of HNF4α, laminB1 or β-actin. (d) Fixed cells were stained with HNF4α antibody and Alexa Fluor 488 goat anti-rabbit secondary antibody (Green), DAPI (blue) was incorporated in the mounting solution

Figure S1A). Via immunoprecipitation-immunoblotting, we found that PKCδ enhanced serine phosphorylation of WT HNF4α by 50%, but no similar enhancement in phosphorylation of S78A was observed (Figure 7b), implying that, in the physiologic state, PKCδ mainly acts on Ser78 of HNF4α at cellular level. Interestingly, S78A got higher basal level in serine-phosphorylation than WT HNF4α. We reason that Ser78 affects the phosphorylation of other serine residues in HNF4α, which need to be further explored.

Previous study reported that the protein nuclear exporting system had no effect on the subcellular localization of HNF4α (Sun et al., 2007). In the present study, we focus on the protein nuclear importing mediator, importin. Its inhibitor, importazole, was used to treat COS-7 cells transfected with the expressing vectors of WT HNF4α, S78A, and S78D (the constitutively Ser78-phophorylated HNF4α mutant). MG132 was utilized to prevent the potential proteasome-induced protein degradation. The Figure 7c showed that WT and S78A could be transported into nucleas and their nuclear transportation was inhibited by importazole. S78D was dominantly localized in cytoplasm and not affected by importazole. This result infers that the nuclear transportation of HNF4α relays on importin, but, once phosphorylated at Ser78, HNF4α is not transportable and thus retains in the cytoplasm.

4 | DISCUSSION

Hepatic VLDL secretion is the main pathway to transport the TAG from liver to peripheral tissues and also an important contributor to the hepatic lipid homeostasis (Cohen et al., 2011). Disruption of VLDL secretion would generate severe hepatic steatosis, for instance, in hepatitis C virus infected patients (Gupte, Dudhade, & Desai, 2006), who got acquired deficiency in ApoB, a key component of VLDL (Gruffat, Durand, Graulet, & Bauchart, 1996). Interestingly, the malfunction of VLDL secretion also occurs in HFD-induced NAFLD (Cahova et al., 2012; Francone et al., 1992; Kalopissis, Griglio, Malewiak, & Rozen, 1980; Oussadou et al., 1996; Yang et al., 2015), and the underlying mechanism is rather complicated. The present investigation reveals that HFD-induced oxidative stress prevents the

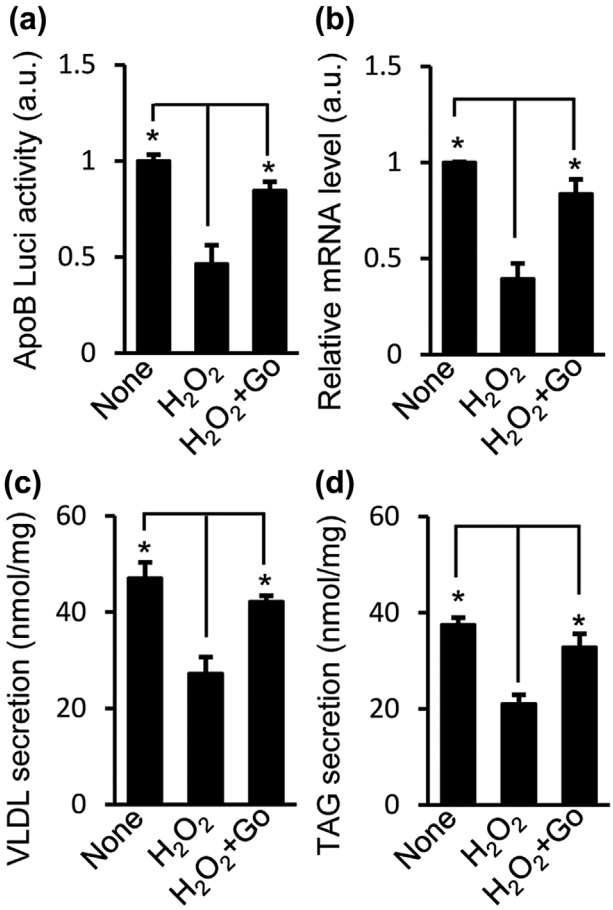

FIGURE 6 Oxidative stress inhibits VLDL secretion by reducing ApoB transcription in hepatocytes. (a) HNF4α expression vectors (50 ng), and pGL3-ApoB (ApoB promoter driven luciferase) construct (200 ng) plus pRL-TK-Renilla (10 ng) were co-transducted into HepG2 cells. Thirty-six hours later, cells were serum starved and treated with or without H_2O_2 (1 μM) or plus Go6983 (1 μM, denoted as Go) for further 24 hr culture. Luciferase (Luci) activity was normalized by Renilla luciferase values. Data are expressed as fold-change relative to the level of control. (b–d) Primary hepatocytes were cultured in WME supplemented with 6% FBS for 5 hr and then grown in serum free medium with dexamethasone (100 nM) for further 24 hr. H_2O_2 (1 μM) or plus Go6983 (1 μM) was added at the same time. (b) Total RNA was isolated from hepatocytes to detect mRNA level of HNF4α by RT-qPCR and normalized to 36B4 mRNA level. (c and d) After 24-hr culture in serum-free WME, medium was switched to serum free, phenol free DMEM with 11 mM glucose for further 24-hr culture. H_2O_2 (1 μM) or plus Go6983 (1 μM) was added at the same time. Then, supernatants were used for VLDL (c) and TAG (d) secretion assay. All these data were normalized by cell protein levels. Data were presented as mean ± S.E., $^*p < 0.05$. a.u., arbitrary units

nuclear translocation of HNF4α due to PKC-mediated phosphorylation, and thus reduced the transcription of ApoB, the downstream of HNF4α, as well as the subsequent VLDL secretion.

The NAFLD model was replicated in C57BL/6 mice by HFD-feeding, and showed similar reduction in hepatic VLDL secretion as in previous studies (Figure 1). Hepatic VLDL secretion depends on ApoB, the key component of VLDL, and MTTP, the enzyme mediating the assembling and secretion of VLDL. The down-regulation in mRNA and protein levels of hepatic ApoB should induce the disruption of VLDL secretion in HFD-fed mice, in terms of no change found in MTTP protein level (Figure 2).

ApoB expression is regulated by several transcriptional factors, including HNF4α, C/EBPα, and PPARα (Hayhurst et al., 2001; Nóvak, Dantas, Charbel, & Bydlowski, 1998; Yin et al., 2011), of which HNF4α may dominate this regulatory process. First, previous data indicated that the level of ApoB was closely related to the expression of HNF4α in liver, that is, the adenovirus vector-delivered over-expression of HNF4α in liver enhanced the expression of hepatic ApoB (Yin et al., 2011), and the liver-specific knockout of HNF4α or short hairpin RNA-induced knockdown of HNF4α reduced ApoB expression (Hayhurst et al., 2001; Yin et al., 2011). Moreover, HNF4α is required in the promotion of ApoB transcription via C/EBPα, that is, HNF4α binding to DNA induces a DNA helix bend, thus facilitating the communication with a C/EBPα located one helix turn from this HNF4α in the ApoB promoter (Nóvak et al., 1998). Finally, PPARα just functions as a guard against the overexpression of ApoB but would not reduce the hepatic ApoB transcription, for PPARα deficiency increases but PPARα overactivation fails to reduce hepatic ApoB mRNA level (Linden et al., 2002; Su et al., 2014).

Strikingly, we observed the HFD-induced abnormal cytoplasmic retention of HNF4α (Figure 3b) with normal mRNA and protein levels (Figure 3), instead of reduction in mRNA and protein levels of

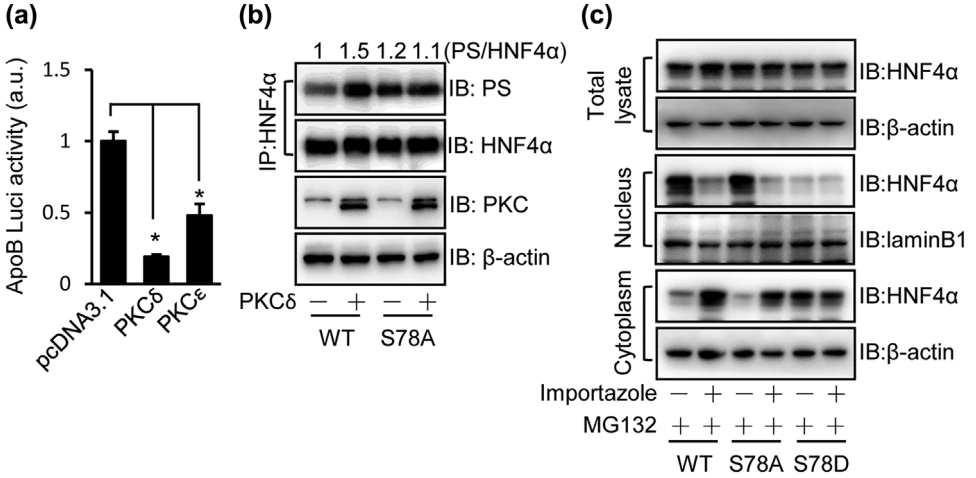

FIGURE 7 PKC δ and ε phophorylate HNF4α specifically at Ser78 and inhibits the translocation of HNF4α into nucleus. (a) The expression vectors of PKC isotypes (100 ng) were co-transfected with HNF4α (50 ng), pGL3-ApoB construct (200 ng), and pRL-TK-Renilla (10 ng) into HepG2 cells. Thirty-six hours later, cells were harvested for luciferase activity assay. Data represent average of three biological replicates assayed in technical triplicates and are presented as mean ± S.E., $^*p < 0.05$. a.u., arbitrary units. (b) COS-7 cells were transfected with expressing vectors of WT HNF4α or S78A mutant with PKCδ. 48 hr later, cells were harvested and cell extracts were immunoprecipitated by HNF4α antibody; the blots were immunoblotted by phospho-serine (PS) and HNF4α antibody. β-actin (as input) and PKCδ in extracts was immunoblotted. Bands were quantified from immunoblot digital images using Image J software. Relative intensity of phospho-serine bands was normalized by that of HNF4α bands, and results are presented above the immunoblot image. (c) COS-7 cells were transfected with expressing vectors of WT HNF4α, S78A and S78D mutants. Twelve hours later, cells were treated by importazole (40 μM) and MG132 (50 μM) for another 12 hr. Proteins from nucleus, cytoplasm, and total cell extracts were immunoblotted by antibodies of HNF4α, laminB1 or β-actin

hepatic HNF4α after HFD feeding, as reported by Yang et al (2015), although both ways could block the transcriptional function of HNF4α, and consequently result in the attenuated expression of ApoB. The different phenotypes were possibly caused by the different HFD-feeding duration, since HFD with similar nutritional composition was fed for 12 weeks in present study and 16 weeks in the previous one. It implies that before the reduction in expression level, hepatic HNF4α already got functional impairment in the early stage of HFD-feeding.

However, the protein level of MTTP, another target gene of HNF4α, did not manifest reduction in HFD-fed mice (Figure 2b). Probably, other regulators of MTTP, PPARα, (Améen et al., 2005) and Foxo1 (Kamagate et al., 2008), with increased transcriptional activity in hepatic steatosis (Sambasiva Rao & Reddy, 2004; Valenti et al., 2008), would up-regulate MTTP transcription, suggesting that PPARα, and Foxo1 may exert compensatory effects on MTTP expression under the malfunction of HNF4α.

To explore the subcellular misallocation of HNF4α in hepatocytes, we screened potential factors affecting HNF4α localization in cellular level, and found that H_2O_2 (imitating oxidative stress) rather than other treatments, induced the cytoplasmic retention of HNF4α (Figure 4a). Consistently, H_2O_2 blocks HNF4α mediated ApoB transcription in HepG2 cells and primary hepatocytes, evidenced by the decrease in ApoB promoter driven luciferase, endogenous ApoB mRNA level, and hepatic secretion of VLDL and TAG (Figure 6). In agree with those ex vivo results, in vivo data indicated that HFD-fed mice suffered from oxidative stress in liver, evidenced by the elevated hepatic ROS, and MDA levels (Figures b and 4c), as well as the abnormality in activity or mRNA levels of antioxidative genes, including SOD, CAT, and NRF-2 (Figures 4d and 4e). Notably, it is reported that HNF4α promotes the anti-oxidative capacity of cells via stimulating NRF-2 to transcribe its downstream anti-oxidative genes, such as SOD, and CAT (Marcil et al., 2010). We reason that oxidative stress inhibiting HNF4α activity would enhance itself via impairing HNF4α/NRF-2/anti-oxidative genes pathway. However, this hypothesis needs to be tested in additional study.

Another NAFLD model, leptin-deficient mice (ob/ob) confirmed that oxidative stress rather than hepatic steatosis that directly induces the misallocation and malfunction of HNF4α, since no significant reduction in nuclear HNF4α protein level or ApoB mRNA level occurred in liver of ob/ob mice (Supplemental Figure S2A and S2B), who got relatively normal hepatic ROS and MDA levels (Supplemental Figures S2C). Therefore, oxidative stress is likely the main cause inhibiting transcriptional activity of HNF4α, which subsequently results in the lower ApoB expression, decreased secretion of VLDL, and eventually develops NAFLD. "Multiple hit" hypothesis considers that oxidative stress evoked by mitochondrial dysfunction under over-accumulation of lipids in liver plays an important role in NASH pathogenesis (Sumida, Niki, Naito, & Yoshikawa, 2013; Takaki, Kawai, & Yamamoto, 2013). However, our data suggest that oxidative stress may also aggravate the hepatic steatosis, therefore the anti-oxidative therapy, such as activation of antioxidant system, is a feasible way to prevent hepatic steatosis, and NASH (Okada et al., 2012; Wheeler et al., 2001).

The mechanism how H_2O_2 modulates HNF4α was investigated next. It is interesting to note that phosphorylation on Ser78 by PKC

leads to cytoplasmic localization of HNF4α (Sun et al., 2007). Thus, we presume that PKC could mediate H_2O_2-induced HNF4α misallocation since H_2O_2 is a PKC activator (Konishi et al., 1997; Lee et al., 2009). Indeed, H_2O_2 treatment promoted the serine phosphorylation of HNF4α in hepatocytes, which could be reversed by a specific PKC inhibitor, Go6983 (Figure 5b). Besides, Go6983 also prevent the H_2O_2-induced HNF4α misallocation (Figures 5c and 5d) as well as reduction in ApoB transcription (Figure 6). Consistent with the ex vivo data, the serine phosphorylation level of HNF4α was significantly enhanced by HFD (Figure 5a) under the elevated hepatic oxidative stress (Figures 4b and 4c). Therefore, the misregulation of HNF4α cellular localization by H_2O_2 is through PKC-mediated serine phosphorylation of HNF4α (Figures 5–6). It is a pity that we are lack of specific antibody against phospho-HNF4α (Ser78) currently to directly value the phosphorylation level of Ser78 in HNF4α, which is still under preparation. But the failure to enhance serine-phosphorylation level of HNF4α mutant (S78A) by PKC (Figure 7b) confirms that Ser78 is the primary site acted on by PKC, and thus total serine-phosphorylation level could generally reflect the phosphorylation level of Ser78 in HNF4α.

PKC has various isotypes, but only PKCδ and PKCε are over-activated in livers when steatosis develops or by oxidative stress (Birkenfeld & Shulman, 2014; Greene et al., 2010; Jung et al., 2004; Majumder et al., 2001). The luciferase assay indicated that these two PKC isotypes inhibited the transcriptional activity of HNF4α on ApoB expression (Figure 7a), implying that PKCδ and PKCε are likely responsible for the malfunction of hepatic HNF4α in HFD-fed mice. The specific phosphorylation sites in HNF4α recognized by PKC had been studied by Sun et al. (2007), but their ex vivo zymological method was defective to precisely reflect the physiologic protein-interaction in alive cells. Thus, we tested the protein modification of HNF4α by PKCs at the cellular level, and found that Ser78 was the primary site acted on by PKC, since the phosphorylation level of S78A mutants could not be enhanced by co-transfected PKCδ (Figure 7b). Ser78 is functional in HNF4α subcellular localization. Previous study reported that the protein nuclear exporting system had no effect on the subcellular localization of HNF4α (Sun et al., 2007). Thus, the present study focuses on the role of the protein nuclear importing mediator, importin. Results in Figure 7c suggest that once phosphorylated on Ser78, the HNF4α could not transfer into nucleus via importin, and thus retains in the cytoplasm. While, the nonphosphorylated HNF4α is susceptible to importin and mostly localizes in nucleus. Therefore, the high Ser78 phosphorylation rate reduces the nuclear transportation of HNF4α and subsequently suppresses its transcriptional activity.

In summary, our study confirms that the hepatic oxidative stress provoked by HFD induces cytoplasmic retention of HNF4α in hepatocytes is mediated by the activation of PKC. For the first time, we find that HNF4α is prevented from transferring into nucleus once phosphorylated at Ser78 by PKC, which consequently blocks transcription of ApoB, decreases the hepatic secretion of VLDL and promotes hepatic TAG accumulation (Figure 8). Thus, oxidative stress/PKC/HNF4α/ApoB pathway may be a novel target to treat HFD-induced fatty liver.

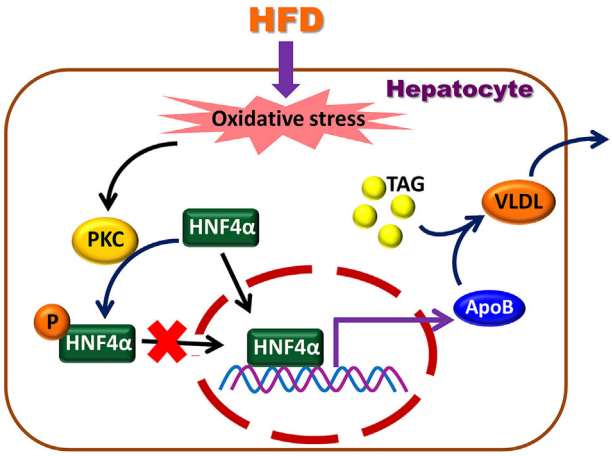

FIGURE 8 A scheme demonstrating the function of HFD-evoked oxidative stress in hepatocytes, modulation of the transcriptional activity of HNF4α, impairment of VLDL secretion, and contribute in the development of hepatic steatosis

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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