Vitamin D deficiency induces insulin resistance and re-supplementation attenuates hepatic glucose output via the PI3K-AKT-FOXO1 mediated pathway

Shivaprakash Jagalur Mutt[1,2,3], Ghulam Shere Raza[1,2], Markus J Mäkinen[3,4], Sirkka Keinänen-Kiukaanniemi [5,6,7], Marjo-Riitta Järvelin[2,5,7,8,9,10], Karl-Heinz Herzig, [1,2,3,11]

[1] Institute of Biomedicine, Department of Physiology, University of Oulu, 90014 Oulu, Finland

[2] Biocenter of Oulu, University of Oulu, 90014 Oulu, Finland

[3] Medical Research Center (MRC), University of Oulu and Oulu University Hospital, 90014 Oulu, Finland

[4] Cancer and Translational Research Unit, Department of Pathology, University of Oulu, 90014 Oulu, Finland

[5] Center for Life Course Health Research, Faculty of Medicine, University of Oulu, 90014 Oulu, Finland

[6] Unit of General Practice, Oulu University Hospital, 90220 Oulu, Finland

[7] Institute of Health Sciences, University of Oulu, 90014 Oulu, Finland

[8] Unit of Primary Care, Oulu University Hospital, 90220 Oulu, Finland

[9] Department of Children, Young People and Families, National Institute for Health and Welfare, 90101 Oulu, Finland

[10] Department of Epidemiology and Biostatistics, and MRC-PHE Center for Environment and Health, School of Public Health, Imperial College London, W2 1PG London, UK

[11] Department of Gastroenterology and Metabolism, Poznan University of Medical Sciences, 61-701 Poznan, Poland

Short title: Vitamin D status and hepatic insulin sensitivity in mice

Corresponding authors:

Professor Karl-Heinz Herzig, MD, PhD, Research Unit of Biomedicine and Biocenter of Oulu,

P.O. Box 5000, FIN-90014 University of Oulu, Finland, E-mail: karl-heinz.herzig@oulu.fi

Received: 25/07/2019; Revised: 11/10/2019; Accepted: 25/11/2019
This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/mnfr.201900728.

Shivaprakash Jagalur Mutt, Research Unit of Biomedicine, Department of Physiology & Biocenter of Oulu, University of Oulu, 90014 Oulu, Finland; E-mail: shivaprakash.jagalur@oulu.fi

Abstract

Background. Pandemic vitamin D deficiency is associated with insulin resistance and type 2 diabetes (T2D). Vitamin D supplementation has been reported to have improved glucose homeostasis. However, its mechanism to improve insulin sensitivity remains unclear.

Methods and Results. Male C57BL/6J mice were fed with/without vitamin D control (CD) or Western (WD) diets for 15 weeks. The Vitamin D deficient lean (CDVDD) and obese (WDVDD) mice were further subdivided into two groups. One group was re-supplemented with vitamin D for 6 weeks and hepatic insulin signaling was examined. Both CD and WD mice with vitamin D deficiency developed insulin resistance. Vitamin D supplementation in CDVDD mice significantly improved insulin sensitivity, hepatic inflammation and antioxidative capacity. The hepatic insulin signals like pAKT, pFOXO1 and pGSK3β were increased and the downstream *Pepck*, *G6pase* and *Pgc1α* were reduced. Furthermore, the lipogenic genes *Srebp1c*, *Acc* and *Fasn* were decreased, indicating that hepatic lipid accumulation was inhibited.

Conclusions. Our results demonstrate that vitamin D deficiency induces insulin resistance. Its supplementation has significant beneficial effects on pathophysiological mechanisms in T2D but only in lean and not in the obese phenotype. The increased subacute inflammation and insulin resistance in obesity could not be significantly alleviated by vitamin D supplementation. This needs to be taken consideration in the design of new clinical trials.

Key words: AKT-FOXO1 pathway; AKT-GSK3β pathway; HOMA; inflammation; insulin resistance; lean mice; liver; obese mice; type 2 diabetes; vitamin D status; vitamin D supplementation; 25OHD.

Abbreviations:

25OHD: 25-hydroxyvitamin D3; ACC: acetyl-CoA carboxylase; AKT: protein kinase B; AUC: area under curve; CAT: catalase; CD: control diet; CDVDD: vitamin D deficient control diet; CDVDD-S: CDVDD mice vitamin D supplemented; CVD: cardiovascular diseases; EIA: enzyme immunoassay; ELISA: Enzyme-linked immunosorbent assay; FASN: fatty acid synthase; FFA: free fatty acids; FOXO1: forkhead box protein 1; G6P: glucose-6-phosphatase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GS: glycogen synthase; GSK3b: glycogen synthase kinase 3b; GTT: glucose tolerance test; HCl: hydrochloric acid; HK: hexokinase; HOMA-IR: homeostasis model for insulin resistance; IL: interleukin; ITT: insulin tolerance test; MAP: mitogen-activated protein; MCP1: monocyte chemoattractant protein 1; MDA: malondialdehyde; NaOH: sodium hydroxide; NFκB: nuclear factor kappa B; PEPCK: phosphoenolpyruvate carboxykinase; PGC1α: peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PI3K: phosphatidylinositol-3 kinase; RANTES: regulated upon activation, normal T cell expressed, and secreted; ROS: reactive oxygen species; SREBP1c: sterol regulatory element-binding protein 1c; T2D: type 2 diabetes; TBARS: thiobarbituric acid reactive substances; TNFα: tumor necrosis factor alpha; WD: western diet; VDRs: vitamin D receptors; WDVDD: vitamin D deficient western diet; WDVDD-S: WDVDD mice vitamin D supplemented.

Introduction

Insulin resistance is the main hallmark in the development of type 2 diabetes which is associated with several diseases such as cardiovascular diseases (CVD), Alzheimer's and some cancers. [1]. It is defined as the reduced inability of insulin to stimulate glucose uptake in metabolic tissues such as liver, adipose and skeletal muscle. However, the sensitivity of these tissues differs. High fat diet feeding in rats has shown that insulin resistance in the liver develops prior to skeletal muscle [2, 3]. Furthermore, insulin resistance seem to affect rat skeletal muscle before adipose tissue [4].

Reduced glycogen synthesis and glucose oxidation, increased free fatty acids (FFA), inflammation and oxidative stress are additional factors in insulin resistance and elevated in obesity [5–7]. The increased FFA causes dysregulated fatty acid oxidation, activation of inflammatory toll-like receptor (TLR) and de novo lipogenesis in the liver. Hepatic accumulation of lipids contributes to the hepatic steatosis and predisposes to increased oxidative stress through production of reactive oxygen species (ROS). Hepatic insulin resistance is marked by increased liver glucose production (gluconeogenesis, glycogenolysis) or reduced liver glucose storage (glycogen synthesis) [8]. The downregulation of the protein kinase B (AKT) activation in the liver induces the transcriptional activation of rate limiting enzymes of gluconeogenesis, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) expressions via forkhead box protein 1 (FOXO1) phosphorylation inhibition. AKT inactivation inhibits the phosphorylation of glycogen synthase kinase 3β (GSK3β) and reduces glycogen synthesis by inactivating glycogen synthase (GS) [9].

Vitamin D is an essential hormone produced mainly by exposure to sunlight, but also available in certain foods like fatty fish, egg yolk and mushrooms. The 25-hydroxyvitamin D3 (25OHD) is the inactive circulating metabolite and maker of vitamin D status, which

undergoes hydroxylation to form 1α,25-dihydroxyvitamin D3 or calcitriol [10]. Calcitriol exerts its biological effects on gene regulation through vitamin D receptors (VDRs) and non-genomic actions via cellular signaling molecules like phosphatidylinositol-3 kinase (PI3K), AKT, mitogen-activated protein (MAP) kinases [11].

Pandemic vitamin D deficiency [12, 13] has been increasingly recognized for its association with many metabolic diseases such as type 2 diabetes, CVD, obesity and cancer [14–17]. Hypovitaminosis D has been related to the impaired glucose homeostasis, defective insulin secretions and contribute to the development of insulin resistance [16, 18–20]. Animal experiments demonstrated that vitamin D deficiency causes hepatic insulin resistance mediated by the upregulation of inflammation [20] and oxidative stress [21]. Observational and pre-clinical studies showed that vitamin D supplementation improved insulin sensitivity [22–26]. On the contrary, randomized control studies (RCTs) failed to show effects of vitamin D supplementation on insulin sensitivity in overweight or obese subjects [27, 28]. Nevertheless, the responses to vitamin D supplementation in humans are inversely associated with increasing adiposity. Moreover, the optimal dosage of vitamin D to achieve diverse health benefits is still under debate [29]. Previously, we have demonstrated that vitamin D inhibited pro-inflammatory cytokine secretion via down regulating nuclear factor kappa B (NFκB) signaling in human adipocytes [30]. In addition, vitamin D has been shown to have protective effects on diabetes induced liver complications in a type 2 diabetes rat model by modulating inflammation [31].

Our aim was to investigate the effects of vitamin D deficiency on control and high fat western diet induced insulin resistance and signaling pathways in the liver using vitamin D deficient and replenishment lean and obese mice.

Material and Methods

Animals, experimental protocol and dosage information: Inbred 6 weeks old C57BL/6 male mice from the laboratory animal center of Oulu University, Finland were housed in individual cages with 12 h/12 h light/dark cycles, ad libitum food and water. All animal experiments were conducted in accordance with the guidelines set by the European Community Council Directives 86/609/EEC and were approved by Institutional Animal Care and Use Committee of the Provincial Government.

Incandescent bulbs were used to avoid the cutaneous vitamin D synthesis throughout the acclimatization experimentation for the light period and with lights on at 6 pm. After 1 week of acclimatization, mice were fed different diets: a control low fat diet (CD; n=10) or high fat western diet D12079B (WD; n=10) or corresponding vitamin D deficient CD (CDVDD; n=20) and vitamin D deficient WD (WDVDD; n=20) diets (Research Diet Inc; New Brunswick, NJ, USA) for 15 weeks (Supplementary Table1), representing a total four groups with 60 animals. After 15 weeks of feeding with the respective diets, CDVDD (n=20) and WDVDD (n=20) fed mice were each further subdivided into two groups (n=10): one was supplemented (S) with intraperitoneal injections of the vitamin D3 (cholecalciferol, Sigma-Aldrich, St. Louis, MO, USA), 50ng/mouse/3 times a week (CDVDD-S, WDVDD-S) for 6 weeks, while the other received saline injections (Supplementary Figure 1). The supplemented dose was selected on the basis of previous rodent studies [31, 32] to achieve the sufficient 25OHD concentrations (50-75 nmol/l) to exert the health benefits as outlined by Heaney et al.[33, 34].

Intraperitoneal glucose and insulin tolerance tests: Glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed at week 12, 19 and 21 after the start of the

experimental diet. Mice were food deprived 6 hours prior to the procedures. For GTT mice were challenged with intraperitoneal glucose injections (1g/kg lean body mass) and for ITT mice were injected with human insulin intraperitoneal (0.5U/kg, Insulin, Actrapid, Novo Nordisk, Bagsværd, Denmark) followed by blood samples were collected at 0, 15, 30, 45, 60, 90 and 120 minutes. Blood glucose concentration were measured using One Touch Ultra glucometer (LifeScan, Johnson & Johnson, New Brunswick, NJ, U.S.A.) and plasma was separated and used for insulin measurements. The homeostasis model for insulin resistance (HOMA-IR) scores were calculated from the fasting blood glucose (mmol/L) × fasting plasma insulin (μU/ml) divided by 22.5.

Plasma 25OHD, insulin and inflammatory cytokines: Plasma 25OHD levels was measured by using an enzyme immunoassay (EIA) kit (Immunodiagnostic systems Plc (IDS), East Boldon, UK) [35]. Plasma insulin levels were measured using Enzyme-linked immunosorbent assay (ELISA) kit (Merck Millipore, Burlington, MA, USA). Plasma inflammatory cytokines levels were determined using the multiplex kit (Merck Millipore) on a Bioplex 200 platform [36].

Hepatic glycogen: The amount of glycogen was determined by an acid-hydrolytic method [37] with slight modifications. The frozen liver tissue samples (20-40 mg) were hydrolyzed using 2M hydrochloric acid (HCl) and 2M sodium hydroxide (NaOH) on a boiling water bath. Glucose concentrations in the supernatants of hydrolyzed liver glycogen was measured using a glucose hexokinase (HK) assay kit (Sigma-Aldrich). Glucose converted into glucose-6-phosphate (G6P) in the presence of ATP and NADP by enzyme HK. Liver glycogen content was expressed as micromoles glucosyl units/ per gram wet liver weight.

Measurement of oxidative stress markers: Mouse liver were rapidly homogenized in 100μl ice-cold radio immunoprecipitation assay buffer containing protease inhibitors. Oxidative

stress was assessed via changes in malondialdehyde (MDA) using the thiobarbituric acid reactive substances kit (TBARS), and anti-oxidant catalase (CAT) activity was assessed using catalase assay kit (Cayman chemical company, Ann Arbor, MI, USA) by following the manufacturer's instructions and absorbance was measured on VarioskanFlash plate reader (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of MDA was expressed in μM and CAT activity was expressed as nmol/min/ml.

Real-time quantitative PCR: Total RNA was isolated from mice liver using NucleoSpin RNA kits (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according the manufacturer's protocol. First-strand cDNA was synthesized from 1μg total RNA using SensiFast™ cDNA Synthesis kit (Bioline, London, UK). The gene expressions were analyzed by real-time quantitative PCR using *Pepck, G6pase,* peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*Pgc1α*), interleukin (*Il1β* and *Il6*), regulated upon activation, normal T cell expressed, and secreted (*Rantes*), tumor necrosis factor alpha (*Tnfα*), monocyte chemoattractant protein 1 (*Mcp1*), sterol regulatory element-binding protein 1c (*Srebp1c*), fatty acid synthase (*Fasn*), acetyl-CoA carboxylase (*Acc*) and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) primers. Briefly, 10ng of cDNA samples were amplified for 40 cycles in 20μL final reaction volume using FastStart Universal SYBR Green Master reagent (Roche, Basel, Switzerland) gene and *Gapdh* primers. Reactions were run on ABI 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The relative expression was determined by the comparative threshold method (ΔΔCt).

Western blots: Snap frozen mice liver tissue were lysed in RIPA lysis buffer containing protease inhibitors (Roche). Liver protein lysate (40μg) were boiled with loading buffer separated by SDS-PAGE and transferred to PVDF membrane (Merck Millipore). The blots were blocked with 5% nonfat dry milk, probed with primary antibodies at 4°C overnight and

incubated with HRP-conjugated anti-IgG secondary antibody (Thermo Fisher Scientific). The immunoreactive protein bands were visualized using the enhanced chemiluminescence kit (Pierce™ ECL, Thermo Fisher Scientific) and relative intensities of protein bands were quantified using ImageJ. Antibodies against AKT, phospho-AKT (Ser473), FOXO1, phospho-FOXO1 (Ser256), GSK3β, phospho-GSK3β (Ser9), inhibitory kappa B alpha (IκBα), phospho-IκBα and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz Inc, Dallas, Texas, USA).

Statistical analysis: All data presented as the mean ± SEM. GraphPad Prism software, version 7.0 (GraphPad Software, San Diego, CA, USA), was used to perform statistical analysis. The significance of differences was analyzed with Unpaired, 1-tailed t tests or 1-way ANOVA (Tukey's multiple comparison test) and a p value of <0.05 was considered statistically significant.

Results

Effect of vitamin D deficiency on insulin resistance and body weight

Vitamin D deficiency in both WDVDD and CDVDD did not significantly affect body weight gain over 15 weeks in comparison to the respective vitamin D replete CD and WD (Supplementary Figure 2). There were no differences in the average daily food consumptions between CDVDD and WDVDD (Supplementary Figure 3). Vitamin D deficiency in both CDVDD and WDVDD mice caused severe insulin resistance, as assessed by ITT (Figure 1a). WD, WDVDD and CDVDD mice had significantly higher blood glucose levels ($p<0.05$) than CD mice after insulin injections. The area under curve (AUC) for the blood glucose values were significantly higher ($p<0.05$) in all the three groups compared to CD mice (Figure 1b). These results demonstrate that vitamin D deficiency in lean mice induces insulin resistance

(CDVDD: 349±19; CD: 262±14), while in obese mice there were no significant changes (WD: 424±25; WDVDD: 438±29).

Effect of vitamin D supplementation on insulin resistance and vitamin D status

Vitamin D status was assessed by measuring the circulating 25OHD metabolite from plasma samples. CDVDD and WDVDD mice had significantly decreased plasma 25OHD levels in comparison to their respective control groups CD and WD (9±1 nmol/l vs 93±5 nmol/l, $p<0.001$; 23±1 nmol/l vs 113±9 nmol/l, $p<0.001$). After vitamin D supplementation, vitamin D levels increased significantly in CDVDD-S and WDVDD-S mice (73±8 nmol/l and 62±3 nmol/l, $p<0.05$) (Figure 1c). Yet, circulating 25OHD in WDVDD-S mice were lower than CDVDD-S mice, indicating vitamin D sequestration into the adipose tissue.

To further investigate the impact of vitamin D supplementation on vitamin D deficient insulin resistant mice, CDVDD and WDVDD mice were subdivided into two groups: one was re-supplemented with vitamin D injections for 6 weeks, while the other received saline injections. ITT and GTT were performed. After re-supplementation insulin sensitivity (Figure 1e) and glucose tolerance were significantly improved in CDVDD-S compared to CDVDD mice (Figure 1g). Accordingly, ITT and GTT AUC values were significantly lower in CDVDD-S compared to CDVDD mice (Figure 1f and 1h). In line with observed insulin sensitivity and glucose tolerance in CDVDD-S mice, vitamin D supplemented CDVVD mice showed significantly improved HOMA-IR scores in comparison to CDVDD mice (Figure 1d, $p<0.05$). In contrast, WDVDD-S failed to show similar beneficial effects. WD and both vitamin D deficient mice (CDVDD and WDVDD) had a significant increased HOMA-IR values in comparison to CD fed mice ($p<0.05$).

The results indicated that vitamin D supplementation could effectively alleviate the impaired glucose homeostasis and improve insulin sensitivity in lean mice, while in obese mice no difference was observed since these mice were already insulin resistant due to the increased fat mass or to the decreased bioavailability of 25OHD in obese [38].

Vitamin D supplementation on hepatic glycogen and gluconeogenesis

To study the impact of vitamin D deficiency and effects of vitamin D supplementation on hepatic gluconeogenesis and glycogen content, we investigated liver glycogen content, hepatic gluconeogenesis signaling pathway protein expressions and gene expressions of the rate-limiting enzymes of the hepatic gluconeogenesis. The liver glycogen contents were significantly reduced in WD, CDVDD, WDVDD and WDVDD-S ($p<0.05$) in comparison to CD mice (Figure 2a). Vitamin D supplementation attenuated the reduction in hepatic glycogen content in CDVDD-S in comparison to CDVDD mice (13.44±0.4 vs 7.9±0.7, $p=0.003$).

Furthermore, CDVDD mice had significantly reduced AKT induced phosphorylation of GSK3β ($p<0.05$) and subsequent liver glycogen content. In contrast, CDVDD-S mice had an increased GSK3β phosphorylation with an enhanced liver glycogen content ($p<0.05$ vs CDVDD) (Figure 2c and 2d).

In WD, WDVDD and CDVDD mice liver *Pepck* ($p<0.05$) and *G6pase* mRNA expressions were upregulated in comparison to CD mice (Figure 2b). Vitamin D supplementation attenuated the up-regulation of *Pepck* and *G6pase* expressions in CDVDD-S in comparison to CDVDD mice (*Pepck*: 1.3±0.3 vs 4.4±0.1, $p=0.03$; *G6pase*: 1.0±0.1 vs 1.9±0.2, $p=0.2$).

The transcriptional activation of *Pepck* and *G6pase* genes is induced via the AKT/FOXO1 signaling pathway. To investigate the possible role of AKT and its target FOXO1 signaling molecules in the vitamin D induced suppression of liver gluconeogenesis, protein expressions of the AKT/FOXO1 signaling pathway were analyzed. CDVDD-S mice significantly increased AKT phosphorylation and subsequently the AKT target FOXO1 phosphorylation (Figure 2c and 2d). There was a significant inhibition of AKT and FOXO1 phosphorylation in CDVDD, WD, WDVDD and WDVDD-S in comparison to CD mice ($p<0.05$).

These findings suggest that vitamin D supplementation in CDVDD mice prevents hepatic gluconeogenesis, reduced glycogen content and alleviate vitamin D deficiency induced hepatic insulin resistance.

Vitamin D deficiency promotes oxidative stress, liver inflammation and hepatic steatosis

Insulin resistance is often associated with oxidative stress, inflammation and non-alcoholic steatohepatitis (NASH). The oxidative phosphorylation of higher glucose levels and impaired insulin action lead to oxidative stress, ectopic accumulation of fat and activates inflammatory pathways. CAT is an important antioxidant enzyme, scavenging free radicals. On the contrary, increased free radicles or ROS induce lipid peroxidation and elevate MDA levels (lipid peroxidation byproduct). To examine the effect of vitamin D on the antioxidant abilities in the liver, CAT activity and MDA levels were determined. CAT activities in the liver of insulin resistant WD, CDVDD, WDVDD and WDVDD-S mice were significantly reduced (Figure 3a) and MDA contents increased (Figure 3b) when compared to the CD mice ($p< 0.05$). In CDVDD-S mice liver CAT enzyme activities were significantly restored ($p<0.05$ vs CDVDD) to that the levels of CD mice with a remarkable reduction in MDA contents, suggesting that vitamin D supplementation improves antioxidant capacity in CDVDD mice.

The oxidative stress in the liver of insulin resistance mice leads to inflammation. We therefore investigated the inflammatory pathway signaling molecule IκBα, a protein subunit of NFκB, and its phosphorylation. The protein expression levels of pIκBα in the liver of WD, CDVDD, WDVDD and WDVDD-S mice were significantly higher (Figure 3c) in comparison to the CD mice. In contrast, the relative density ratios of pIκBα/IκBα were significantly reduced in CDVDD-S mice in comparison to the CDVDD mice (Figure 3d) ($p<0.05$). The circulatory inflammatory cytokines IL1β (12±2 pg/ml vs 394±186 pg/ml, p=0.03), RANTES (60±10 pg/ml vs 352±121 pg/ml, p=0.002), MCP1 (35±3 pg/ml vs 259±119 pg/ml, p=0.03), IL6 (35±11 pg/ml vs 169±56 pg/ml, p=0.03) and TNFα (8±2 pg/ml vs 38±32 pg/ml, p=0.7) were significantly diminished in CDVDD-S in comparison to CDVDD mice (Figure 4a-e.). In addition, the relative mRNA expression levels of corresponding inflammatory genes in the liver, *Il1β* (1.2±0.2 vs 3.9±0.4, p=0.03), *Rantes* (1.2±0.2 vs 1.7±0.5, p=0.9), *Mcp1* (0.9±0.2 vs 3.9±0.5, p=0.002), *Il6* (1.6±0.3 vs 6.2±0.4, p=0.007) and *Tnfα* (0.5±0.3 vs 2.1±0.8, p=0.005) were significantly lower in CDVDD-S in comparison to CDVDD mice (Figure 4f). These results indicate that the vitamin D supplementation reduced liver inflammation in CDVVD mice.

Vitamin D deficiency and WD feeding significantly increased mRNA expression of lipogenic genes as *Srebp1c*, *Acc*, *Fasn* and *Pgc1α* and fat accumulation in liver in comparison to CD mice ($p<0.05$) (Figure 5a and 5b). Supplementation of vitamin D reduced lipogenic gene expressions as well as hepatic fat accumulation in CDVDD-S mice (Figure 5b and 5c), yet the reductions were not statistically significant.

Overall, our results show that vitamin D deficiency in mice promotes inflammation, oxidative stress and hepatic insulin resistance. The supplementation of vitamin D reduced these adverse effects in lean mice but did not affect the deleterious effects of mice on WD.

Discussion

In the present study, we show that vitamin D deficiency impairs systemic glucose homeostasis and insulin sensitivity in mice fed with CD and WD. Vitamin D supplementation improved insulin sensitivity and ameliorated the harmful metabolic phenotype associated with the hepatic tissue inflammation and oxidative stress in vitamin D deficient lean mice. However, vitamin D supplementation did not ameliorated insulin resistance WD fed mice.

Vitamin D deficiency has been associated with insulin resistance and type 2 diabetes in humans [39]. Several rodent studies reported that dietary vitamin D deficiency exacerbated impaired glucose tolerance and insulin resistance and vitamin D supplementation improved insulin sensitivity [23–25, 31, 40–43]. In our study, we induced vitamin D deficiency by vitamin D depleted diet and blocked cutaneous vitamin D synthesis using incandescent bulbs throughout the experiment. Currently, there are no recommendation for the vitamin D levels in rodents based on the circulating 25OHD concentrations except the recommended vitamin D supplementation of rodent feed (7.5 µg/kg diet (300 IU)) [44]. We used the term vitamin D deficiency in comparison to the dietary vitamin D replete control group mice based on the available literature in rodents [18, 42, 45]. Vitamin D deficiency induced insulin intolerance and disturbed glucose homeostasis in both CDVDD and WDVDD mice (Figure 1e and 1g). The intraperitoneal supplementation of vitamin D for a period of 6 weeks, restored circulating plasma 25OHD levels in both VDD groups to that of control mice (Figure 1c). The supplementation significantly improved insulin sensitivity and HOMA-IR only in CDVDD-S mice (Figure 1d) but showed no effect in WDVDD-S mice.

At the cellular level, increases in both gluconeogenic and lipogenic enzymes, inflammatory and oxidative stress markers, and lipid contents were found in the liver of WD, CDVDD and

WDVDD mice. Interestingly, both WD and vitamin D deficiency induced a pro-inflammatory state with increased cytokine levels [20]. Increased FFA and glycerol are delivered to the peripheral tissues such as liver, leading to an increased hepatic acetyl-CoA content and pyruvate carboxylase (PC) activity, which result in an enhanced gluconeogenesis [46]. The effect of insulin signaling on liver gluconeogenesis and glycogen synthesis is mediated via PI3K/AKT/FOXO1 [8] and PI3K/AKT/GSK3β pathways [47]. FOXO1 is a member of the forkhead family of transcription factors and its transcriptional activity is regulated by phosphorylation. Active AKT (pAKT) inactivates FOXO1 by phosphorylation and inhibits expressions of gluconeogenic enzymes (*Pepck* and *G6pase)*. pAKT inactivates GSK3β by phosphorylation with downstream GS activation and induction of glycogen synthesis. CDVDD-S mice had a reduced gluconeogenesis via AKT/FOXO1 mediated *Pepck* and *G6pase* downregulation and enhanced glycogen synthesis via AKT/ GSK3β mediated GS activation. Previously, Cheng et al.[48] reported that $1,25(OH)_2D_3$ supplementation reduced hepatic triglyceride accumulation and glucose output through activation of Ca2+/CaMKKβ/AMPK (calcium/calmodulin-dependent protein kinase kinase/5'-AMP-activated protein kinase) pathway in HepG2 cells and diabetic db/db mice. In addition, PGC1α transcriptional coactivators are known for their regulation of FOXO1. Increased PGC1α and AKT induce constitutively active phosphorylated FOXO1 via induction of gluconeogenic *Pepck* and *G6pase* expression [49]. We found decreased *Pgc1α* expression (Figure 5a) and reduced AKT mediated FOXO1 phosphorylation in the liver of CDVDD-S mice, indicating that PCG1α/FOXO1 inhibited gluconeogenesis. CDVDD-S mice had increased phosphorylation of pAKT, pFOXO1 and pGSK3β, downstream signaling molecules of hepatic insulin signaling pathway. WDVDD, WDVDD-S and WD mice had reduced phosphorylation of AKT, FOXO1 and GSK3β in hepatocytes (Figure 2c). Thus, we

suggest that vitamin D restores hepatic insulin sensitivity via reduction of hepatic glucose output mediated by IRS/PI3K/AKT and PGC1α/FOXO1.

Excessive glucose toxicity and lipotoxicity in hepatic tissue lead to endoplasmic reticulum (ER) oxidative stress and contribute to the increased inflammation. ER stress acts as a common pathogenic factor on pancreatic β-cells, resulting in reduced insulin secretion and disrupted insulin signaling in liver and other tissues [46]. Vitamin D has been well known for its antioxidant [50] and anti-inflammatory function [30]. Tao S et al. recently reported that vitamin D deficiency induced oxidative stress with insulin resistance in human hepatocytes by silencing 1α-hydroxylase enzyme of the vitamin D biosynthetic pathway [21]. Vitamin D treatment showed reduction in serum MDA levels in a rat model of the metabolic syndrome [43]. Our results show that vitamin D deficiency and WD feeding decreased antioxidant enzyme CAT activities and increased lipid peroxidation (Figure 3a and 3b). In CDVDD-S, CAT activities significantly improved with partially reduced MDA levels.

Oxidative stress increases the production of intracellular ROS, leading to activation of the NFκB signaling pathway with the production of several inflammatory cytokines [51]. Increased proinflammatory cytokines have been associated with the type 2 diabetes risk [36] and the proinflammatory cytokine TNFα is well known for its role in induction of insulin resistance [5]. Vitamin D has been well studied for its immunomodulating effects [30]. Long-term dietary vitamin D deficiency in BALB/c mice and Sprague-Dawley rats elevated expressions of inflammatory cytokines in the liver [20, 52]. In line with these previous findings we found that IκBα phosphorylation was increased in the liver of vitamin D deficiency and WD mice, allowing NFκB to translocate to the nucleus and upregulate a series of proinflammatory genes (Figure 4f). Vitamin D supplementation stabilized IκBα protein levels by reducing its phosphorylation in CDVDD-S mice liver and inhibited the downstream transcription of proinflammatory cytokine genes (Figure 3c.). Our results demonstrate that

vitamin D supplementation can effectively reduce inflammation in the liver of vitamin D deficient insulin resistance mice.

Hepatic insulin resistance is considered to be the result of increased oxidative stress, inflammation and *de novo* lipogenesis and increased the circulating FFA [9, 46]. The vitamin D deficiency in mice increases de novo lipogenesis by the regulation of key lipogenic enzymes. It is well documented that vitamin D deficiency accelerates hepatic steatosis in rodent studies by inducing the lipogenic gene expression such as *Srebp1c, Acc* and *Fasn* [20, 42, 45, 53, 54]. Interestingly, VDR knockout mice had hepatic steatosis similar to that of vitamin D deficient HFD mice [54]. In contrast, Liu et al. [31] reported that vitamin D deficient HFD mice had no change in lipogenic gene expressions. The authors speculated that hepatic lipid accumulation was due to an increased β-oxidation. We found that vitamin D deficiency enhanced hepatic lipid accumulation in WD, WDVDD and CDVDD mice with increased expression of *Srebp1c, Acc* and *Fasn* (Figure 5a). Vitamin D supplementation resulted in a significant reduction in the expression of those lipogenic genes in CDVDD-S mice and a partial reduction on the hepatic lipid accumulation (Figure 5b and 5c). In WDVDD-S mice, the adverse effects of WD feeding subsided the actions of vitamin D on lipogenic genes and fat accumulation. These results suggest that vitamin D regulated the downstream targets of lipogenesis and attenuate the induction of hepatic insulin resistance.

Collectively, our results warrant prospective trails of vitamin D supplementations in insulin resistance in lean rather obese individual. A meta-analysis including 23 RCTs in non-T2D subjects found that vitamin D supplementation reduced fasting plasma glucose and improved insulin sensitivity only in subjects with BMI<25, but not in obese subjects [22]. Recently, Pittas et al. [55] reported that in the Vitamin D intervention trail in Type 2 Diabetes (D2d), consisting 2423 participants and supplemented with either 4000 IU of vitamin D or placebo, failed to show a significant reduction in risk of diabetes. However, subgroup analyses in the

D2d subjects demonstrated that vitamin D supplementation had a lowering effect on the risk of diabetes in the study population with BMI<30 (hazard ratio, 0.71; 95% CI, 0.53 to 0.95) compared to subjects with higher BMI≥30 (hazard ratio, 0.97; 95% CI, 0.80 to 1.17). Our mice study partly addresses these discrepancies from the human RCTs in vitamin D supplementation on insulin resistance and type 2 diabetes incidences [27–29]. Therefore, future vitamin D supplementation trials would need more careful consideration and stratification towards weight in the dosing of vitamin D supplementation and analysis of the results.

In summary, the present study demonstrates that long-term vitamin D deficiency causes insulin resistance in lean mice. Vitamin D supplementation significantly improved insulin sensitivity in vitamin D deficient insulin resistant lean mice (Figure 6b). Our results suggest that vitamin D ameliorates hepatic insulin resistance by increased glycogen synthesis and decreased gluconeogenesis via the activation of the AKT/GSK3β and AKT/FOXO1 signaling pathways along with the reduction of oxidative stress, inflammation (via NFκB) and lipogenesis (Figure 6a). These findings provide a novel mechanistic understanding of the beneficial effects of vitamin D on diabetes treatment in the lean phenotype. In the obese state the beneficial effects of vitamin D are overridden by the pathophysiological consequences of the adiposity. This has profound translational consequences and offers additional explanation for the different outcomes of clinical trials of vitamin D supplementation in type 2 diabetes.

Acknowledgements

We thank Tuula Taskinen for her excellent technical support. The study was funded in part by University of Oulu Hospital Fund and grant support from Finnish cultural foundation – central funds.

Authors' contributions

KHH, SJM designed the study and SKK, MRJ and KHH provided the funding support for the study. SJM and GSR did the animal experiment. SJM analyzed all the parameters, did the data analysis and wrote the first draft of the manuscript. All authors contributed to the writing of the manuscript.

Conflict of interests' statement

All authors declare to have no conflicts of interest relevant to this article.

References

[1] J. S. Skyler, G. L. Bakris, E. Bonifacio, T. Darsow, R. H. Eckel, L. Groop, P.-H. Groop, Y. Handelsman, R. A. Insel, C. Mathieu, A. T. McElvaine, J. P. Palmer, A. Pugliese, D. A. Schatz, J. M. Sosenko, J. P. H. Wilding, R. E. Ratner, *Diabetes* **2017**, *66*, 241.
[2] E. W. Kraegen, P. W. Clark, A. B. Jenkins, E. A. Daley, D. J. Chisholm, L. H. Storlien, *Diabetes* **1991**, *40*, 1397.
[3] R. Kleemann, M. van Erk, L. Verschuren, A. M. van den Hoek, M. Koek, P. Y. Wielinga, A. Jie, L. Pellis, I. Bobeldijk-Pastorova, T. Kelder, K. Toet, S. Wopereis, N. Cnubben, C. Evelo, B. van Ommen, T. Kooistra, *PLoS ONE* **2010**, *5*, e8817.
[4] J. J. Wilkes, A. Bonen, R. C. Bell, *Am. J. Physiol.* **1998**, *275*, E679.
[5] G. S. Hotamisligil, N. S. Shargill, B. M. Spiegelman, *Science* **1993**, *259*, 87.
[6] D. L. Roith, Y. Zick, *Diabetes Care* **2001**, *24*, 588.
[7] B. Sears, M. Perry, *Lipids in Health and Disease* **2015**, *14*, 121.

[8] A. Barthel, D. Schmoll, *American Journal of Physiology-Endocrinology and Metabolism* **2003**, *285*, E685.
[9] V. T. Samuel, G. I. Shulman, *J Clin Invest* **2016**, *126*, 12.
[10] A. Huotari, K.-H. Herzig, *Int J Circumpolar Health* **2008**, *67*, 164.
[11] C. S. Hii, A. Ferrante, *Nutrients* **2016**, *8*, 135.
[12] M. F. Holick, *Rev Endocr Metab Disord* **2017**, *18*, 153.
[13] S. J. Mutt, E. Hyppönen, J. Saarnio, M.-R. Järvelin, K.-H. Herzig, *Front Physiol* **2014**, *5*, 228.
[14] Cheng, J. M. Massaro, C. S. Fox, M. G. Larson, M. J. Keyes, E. L. McCabe, S. J. Robins, C. J. O'Donnell, U. Hoffmann, P. F. Jacques, S. L. Booth, R. S. Vasan, M. Wolf, T. J. Wang, *Diabetes* **2010**, *59*, 242.
[15] W. B. Grant, *Anticancer Res* **2018**, *38*, 1121.
[16] C. Mattila, P. Knekt, S. Männistö, H. Rissanen, M. A. Laaksonen, J. Montonen, A. Reunanen, *Diabetes Care* **2007**, *30*, 2569.
[17] S. J. Mutt, J. Jokelainen, S. Sebert, J. Auvinen, M.-R. Järvelin, S. Keinänen-Kiukaanniemi, K.-H. Herzig, *Nutrients* **2019**, *11*.
[18] C. C. Borges, A. F. Salles, I. Bringhenti, V. Souza-Mello, C. A. Mandarim-de-Lacerda, M. B. Aguila, *Mol Nutr Food Res* **2016**, *60*, 346.
[19] K. C. Chiu, A. Chu, V. L. W. Go, M. F. Saad, *Am. J. Clin. Nutr.* **2004**, *79*, 820.
[20] C. L. Roth, C. T. Elfers, D. P. Figlewicz, S. J. Melhorn, G. J. Morton, A. Hoofnagle, M. M. Yeh, J. E. Nelson, K. V. Kowdley, *Hepatology* **2012**, *55*, 1103.
[21] S. Tao, Q. Yuan, L. Mao, F.-L. Chen, F. Ji, Z.-H. Cui, *Oncotarget* **2017**, *8*, 67605.
[22] S. He, S. Yu, Z. Zhou, C. Wang, Y. Wu, W. Li, *Biomed Rep* **2018**, *8*, 475.
[23] D. Meerza, I. Naseem, J. Ahmed, *Journal of Diabetes and its Complications* **2012**, *26*, 363.
[24] S. R. Sisley, D. M. Arble, A. P. Chambers, R. Gutierrez-Aguilar, Y. He, Y. Xu, D. Gardner, D. D. Moore, R. J. Seeley, D. A. Sandoval, *Diabetes* **2016**, *65*, 2732.
[25] T. Takiishi, L. Ding, F. Baeke, I. Spagnuolo, G. Sebastiani, J. Laureys, A. Verstuyf, G. Carmeliet, F. Dotta, T. L. Van Belle, C. A. Gysemans, C. Mathieu, *Diabetes* **2014**, *63*, 2026.
[26] S. Chen, S. A. Villalta, D. K. Agrawal, *J. Bone Miner. Res.* **2016**, *31*, 585.
[27] A. Mousa, N. Naderpoor, M. P. de Courten, H. Teede, N. Kellow, K. Walker, R. Scragg, B. de Courten, *Am. J. Clin. Nutr.* **2017**, *105*, 1372.
[28] A. Zuk, T. Fitzpatrick, L. C. Rosella, *PLoS ONE* **2016**, *11*, e0154215.
[29] P. J. Ryan, *Int. J. Clin. Pract.* **2007**, *61*, 1894.
[30] S. J. Mutt, T. Karhu, S. Lehtonen, P. Lehenkari, C. Carlberg, J. Saarnio, S. Sebert, E. Hyppönen, M.-R. Järvelin, K.-H. Herzig, *FASEB J.* **2012**, *26*, 4400.
[31] L. Liu, G. Lv, C. Ning, Y. E. Yang, J. Zhu, *Exp Ther Med* **2016**, *11*, 2284.
[32] N. M. Idrus, J. P. Happer, J. D. Thomas, *J. Steroid Biochem. Mol. Biol.* **2013**, *136*, 146.
[33] R. P. Heaney, *Nutr. Rev.* **2014**, *72*, 48.
[34] W. B. Grant, B. J. Boucher, H. P. Bhattoa, H. Lahore, *J. Steroid Biochem. Mol. Biol.* **2018**, *177*, 266.
[35] T. I. Ala-Kokko, S. J. Mutt, S. Nisula, J. Koskenkari, J. Liisanantti, P. Ohtonen, M. Poukkanen, J. J. Laurila, V. Pettilä, K.-H. Herzig, FINNAKI Study Group, *Ann. Med.* **2016**, *48*, 67.
[36] T. Saukkonen, S. J. Mutt, J. Jokelainen, A.-M. Saukkonen, G. S. Raza, T. Karhu, P. Härkönen, J. Eckel, K.-H. Herzig, U. Rajala, S. Keinänen-Kiukaanniemi, *Sci Rep* **2018**, *8*, 12816.
[37] P. Zhang, *Bio-protocol* **2012**, *2*, e186.

[38] J. Wortsman, L. Y. Matsuoka, T. C. Chen, Z. Lu, M. F. Holick, *Am. J. Clin. Nutr.* **2000**, *72*, 690.
[39] N. G. Forouhi, Z. Ye, A. P. Rickard, K. T. Khaw, R. Luben, C. Langenberg, N. J. Wareham, *Diabetologia* **2012**, *55*, 2173.
[40] E. Benetti, R. Mastrocola, F. Chiazza, D. Nigro, G. D'Antona, V. Bordano, R. Fantozzi, M. Aragno, M. Collino, M. A. Minetto, *PLoS ONE* **2018**, *13*, e0189707.
[41] R. Kheder, J. Hobkirk, Z. Saeed, J. Janus, S. Carroll, M. J. Browning, C. Stover, *Immun Inflamm Dis* **2017**, *5*, 151.
[42] M. Kong, L. Zhu, L. Bai, X. Zhang, Y. Chen, S. Liu, S. Zheng, S. J. Pandol, Y.-P. Han, Z. Duan, *Am. J. Physiol. Gastrointest. Liver Physiol.* **2014**, *307*, G883.
[43] D. K. Mostafa, R. A. Nasra, N. Zahran, M. T. Ghoneim, *European Journal of Pharmacology* **2016**, *792*, 38.
[44] H. E. Clarke, M. E. Coates, J. K. Eva, D. J. Ford, C. K. Milner, P. N. O'Donoghue, P. P. Scott, R. J. Ward, *Lab. Anim.* **1977**, *11*, 1.
[45] C. C. Borges, A. F. Salles, I. Bringhenti, C. A. Mandarim-De-Lacerda, M. B. Aguila, *Journal of Nutritional Science and Vitaminology* **2018**, *64*, 106.
[46] R. J. Perry, J.-P. G. Camporez, R. Kursawe, P. M. Titchenell, D. Zhang, C. J. Perry, M. J. Jurczak, A. Abudukadier, M. S. Han, X.-M. Zhang, H.-B. Ruan, X. Yang, S. Caprio, S. M. Kaech, H. S. Sul, M. J. Birnbaum, R. J. Davis, G. W. Cline, K. F. Petersen, G. I. Shulman, *Cell* **2015**, *160*, 745.
[47] J. Lee, M.-S. Kim, *Diabetes Res. Clin. Pract.* **2007**, *77 Suppl 1*, S49.
[48] S. Cheng, W. Y. So, D. Zhang, Q. Cheng, B. J. Boucher, P. S. Leung, *Curr. Mol. Med.* **2016**, *16*, 747.
[49] P. Puigserver, J. Rhee, J. Donovan, C. J. Walkey, J. C. Yoon, F. Oriente, Y. Kitamura, J. Altomonte, H. Dong, D. Accili, B. M. Spiegelman, *Nature* **2003**, *423*, 550.
[50] C.-Y. Ke, F.-L. Yang, W.-T. Wu, C.-H. Chung, R.-P. Lee, W.-T. Yang, Y.-M. Subeq, K.-W. Liao, *Int J Med Sci* **2016**, *13*, 147.
[51] A. Bowie, L. A. O'Neill, *Biochem. Pharmacol.* **2000**, *59*, 13.
[52] L. Zhu, M. Kong, Y.-P. Han, L. Bai, X. Zhang, Y. Chen, S. Zheng, H. Yuan, Z. Duan, *Can. J. Physiol. Pharmacol.* **2015**, *93*, 385.
[53] E.-J. Kang, J.-E. Lee, S.-M. An, J. H. Lee, H. S. Kwon, B. C. Kim, S. J. Kim, J. M. Kim, D. Y. Hwang, Y.-J. Jung, S. Y. Yang, S. C. Kim, B.-S. An, *Int. J. Mol. Med.* **2015**, *36*, 1151.
[54] D. Su, Y. Nie, A. Zhu, Z. Chen, P. Wu, L. Zhang, M. Luo, Q. Sun, L. Cai, Y. Lai, Z. Xiao, Z. Duan, S. Zheng, G. Wu, R. Hu, H. Tsukamoto, A. Lugea, Z. Liu, S. J. Pandol, Y.-P. Han, *Front Physiol* **2016**, *7*, 498.
[55] A. G. Pittas, B. Dawson-Hughes, P. Sheehan, J. H. Ware, W. C. Knowler, V. R. Aroda, I. Brodsky, L. Ceglia, C. Chadha, R. Chatterjee, C. Desouza, R. Dolor, J. Foreyt, P. Fuss, A. Ghazi, D. S. Hsia, K. C. Johnson, S. R. Kashyap, S. Kim, E. S. LeBlanc, M. R. Lewis, E. Liao, L. M. Neff, J. Nelson, P. O'Neil, J. Park, A. Peters, L. S. Phillips, R. Pratley, P. Raskin, N. Rasouli, D. Robbins, C. Rosen, E. M. Vickery, M. Staten, D2d Research Group, *N. Engl. J. Med.* **2019**, *381*, 520.

Figure legends

Figure 1. Vitamin D deficiency and supplementation on insulin resistance in both lean and obese C57BL/6J mice. a,b, Blood glucose levels and AUC during the ITT before vitamin D supplementation. c, Plasma 25OHD levels in mice. d, Insulin resistance index (HOMA-IR). e,f, Blood glucose levels and AUC during the ITT after 4 weeks of vitamin D supplementation. g,h, Blood glucose levels and AUC during the GTT after 6 weeks of vitamin D supplementation. Data are expressed as mean ± SEM (n = 6-10). $/*p < 0.05, **p < 0.01 and ***p < 0.001 vs CD mice; # p < 0.05 vs CDVDD mice.

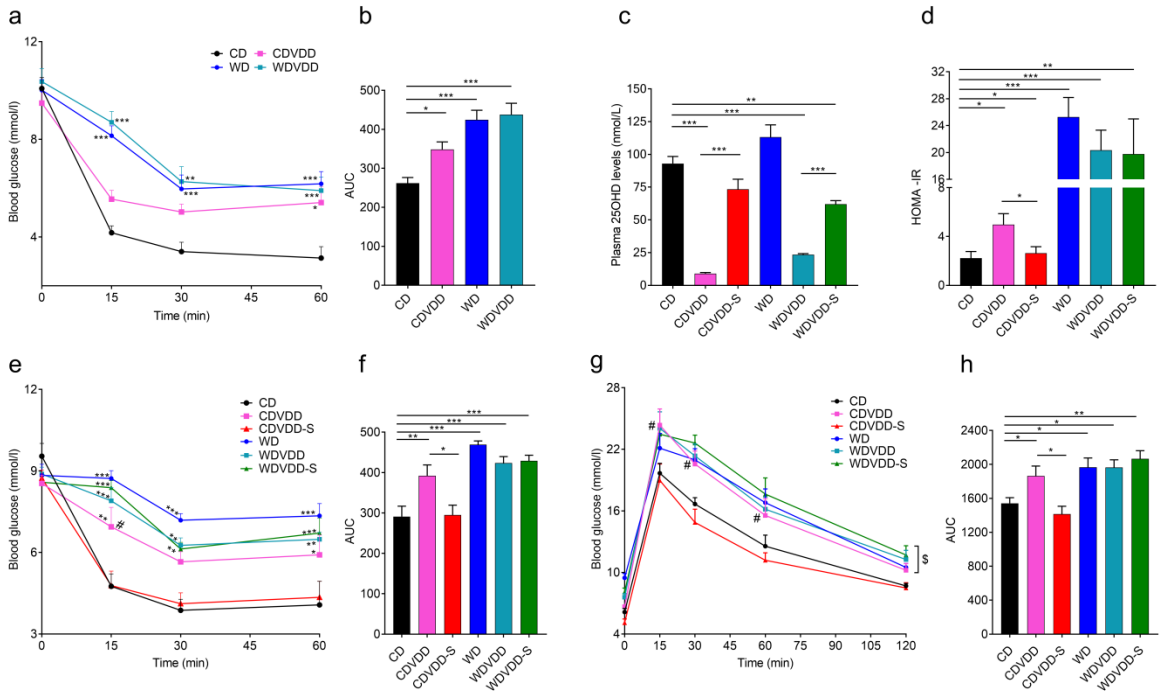


Figure 2. Down regulation of hepatic gluconeogenesis in vitamin D supplemented mice. a, Liver glycogen content. b, The relative expression of *Pepck* and *G6pase* mRNA in mice liver. c,d, Representative images of western blots of pAKT, AKT, pFOXO1, FOXO1, pGSK3β, GSK3β and GAPDH protein expressions and their relative density ratios. Values are expressed as mean ± SEM. *p < 0.05, **p < 0.01 and ***p < 0.001; n = 4-6 for each group.

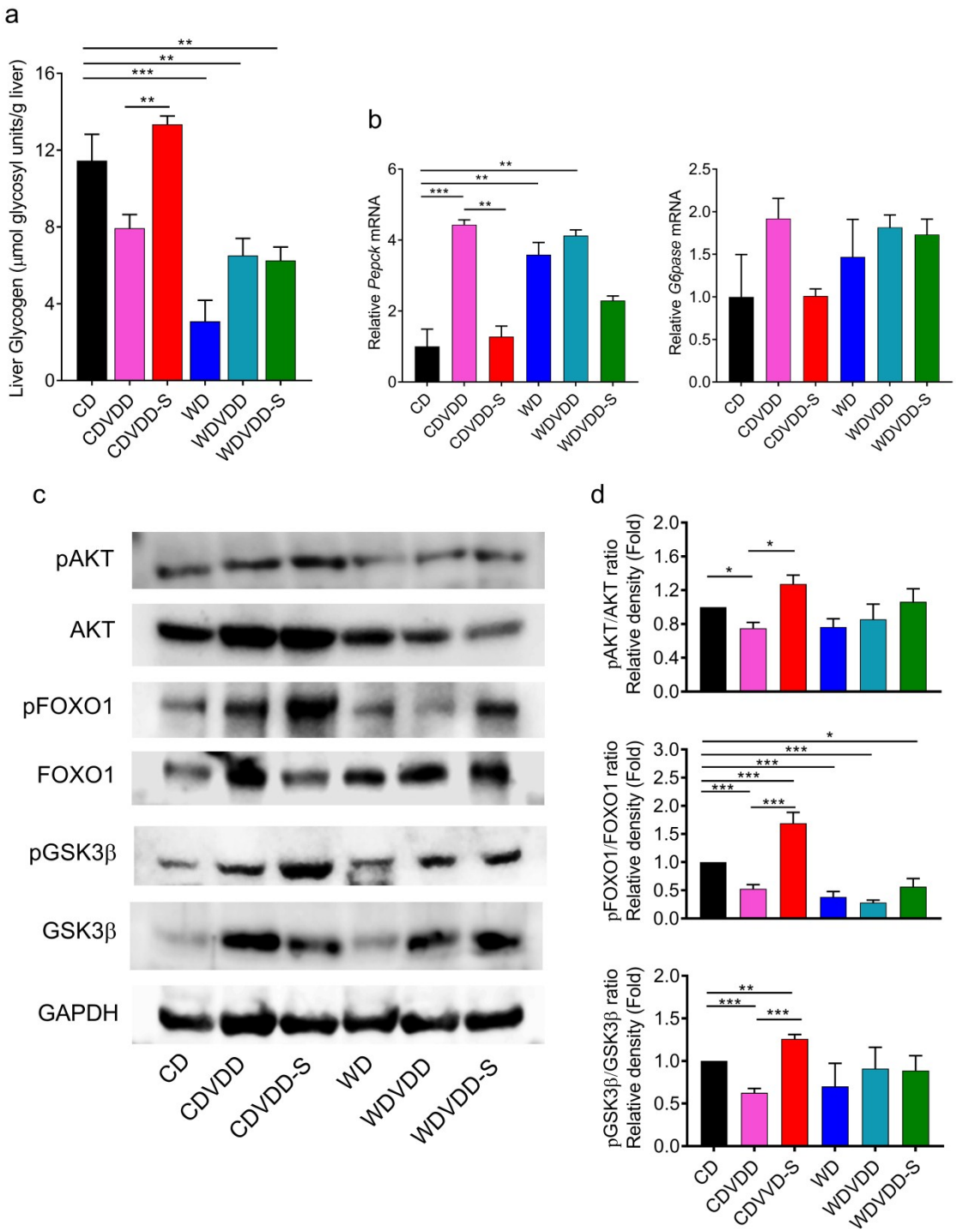


Figure 3. Vitamin D supplementation inhibit oxidative stress and inflammatory markers in mice liver. a,b, Anti-oxidant catalase and oxidative stress marker MDA levels in mice liver. c,d, Representative images of western blot showing IκBα, pIκBα and GAPDH protein expressions from mice liver and their relative density ratios. The values presented are the means ± SEM (n = 4-8), *p < 0.05, **p < 0.01 and ***p < 0.001.

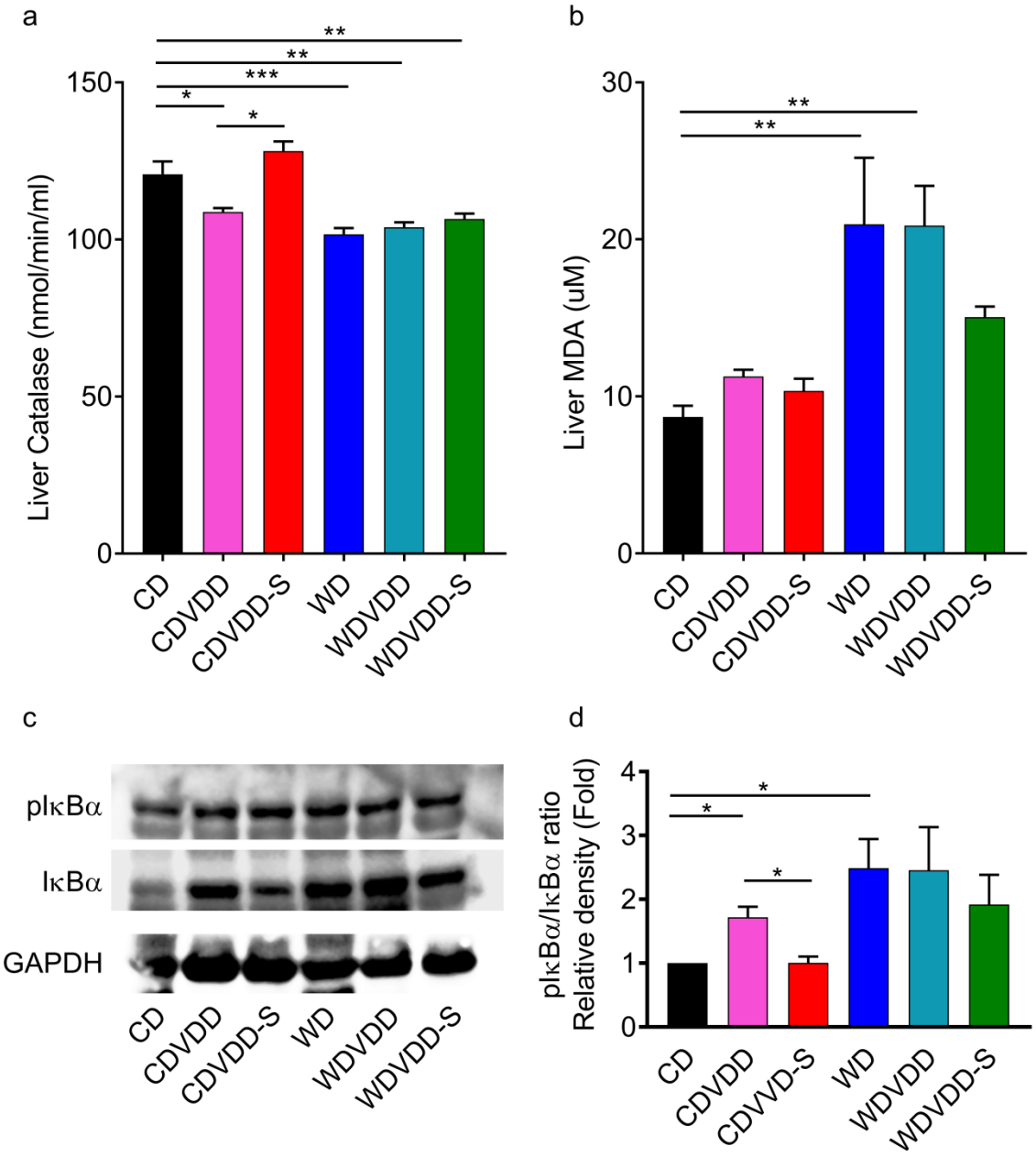


Figure 4. Vitamin D supplementation reduced circulating inflammatory cytokines and gene expression in mice liver. a-e, The protein expression of circulating cytokines IL1β, RANTES, MCP1, IL6 and TNFα levels. f, RT-PCR analysis of the mRNA expression of *Il1β, Rantes, Mcp1, Tnfα* and *Il6*. Gene expression was normalized to housekeeping gene *Gapdh*, the values presented are the means ± SEM (n = 6-8), *p < 0.05, **p < 0.01 and ***p < 0.001.

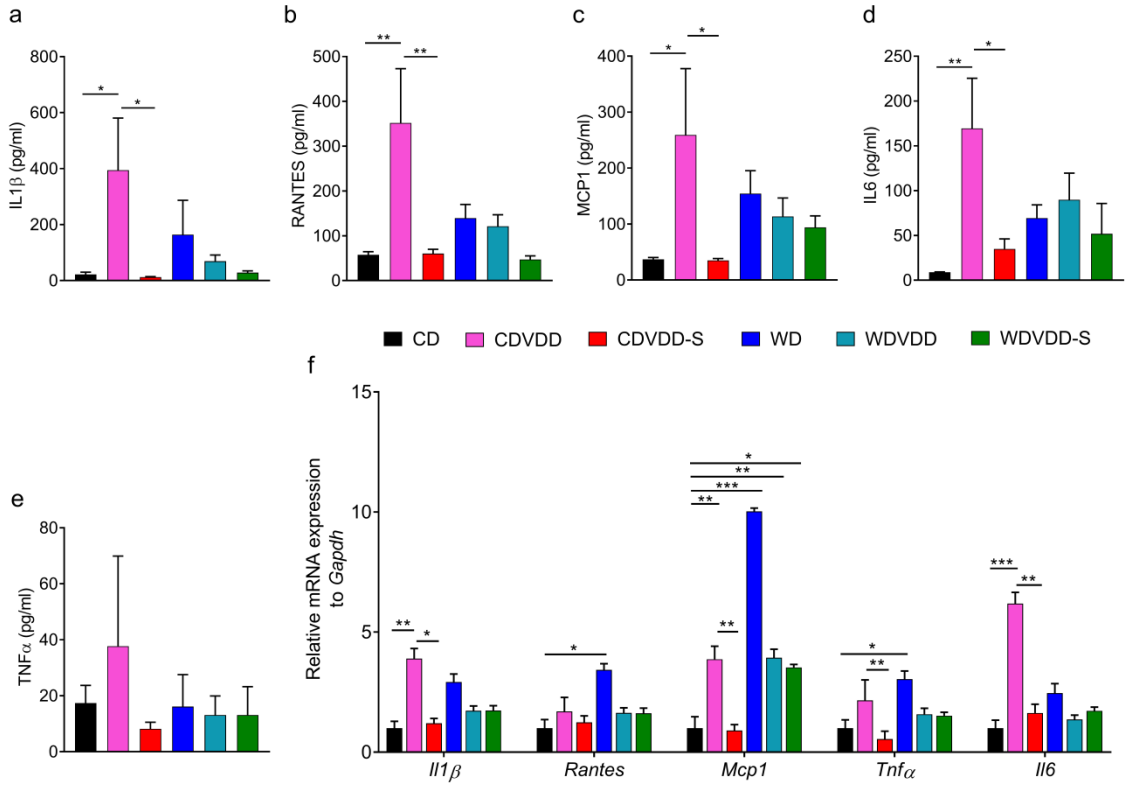


Figure 5. Effect of vitamin D supplementation on lipogenic genes and hepatic steatosis in mice. a, mRNA levels of sterol regulatory element-binding protein 1c (*Srebp1c*), acetyl-CoA carboxylase (*Acc*), fatty acid synthase (*Fasn*) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*Pgc1α*) were analyzed by RT-qPCR. b,c, H&E stained histological analysis of liver sections from mice (magnification, x40) and relative hepatic fat area scores. Data are expressed as the means ± SEM. Gene expression was normalized to housekeeping gene *Gapdh*. *p < 0.05, **p < 0.01 and ***p < 0.001 (n=6).

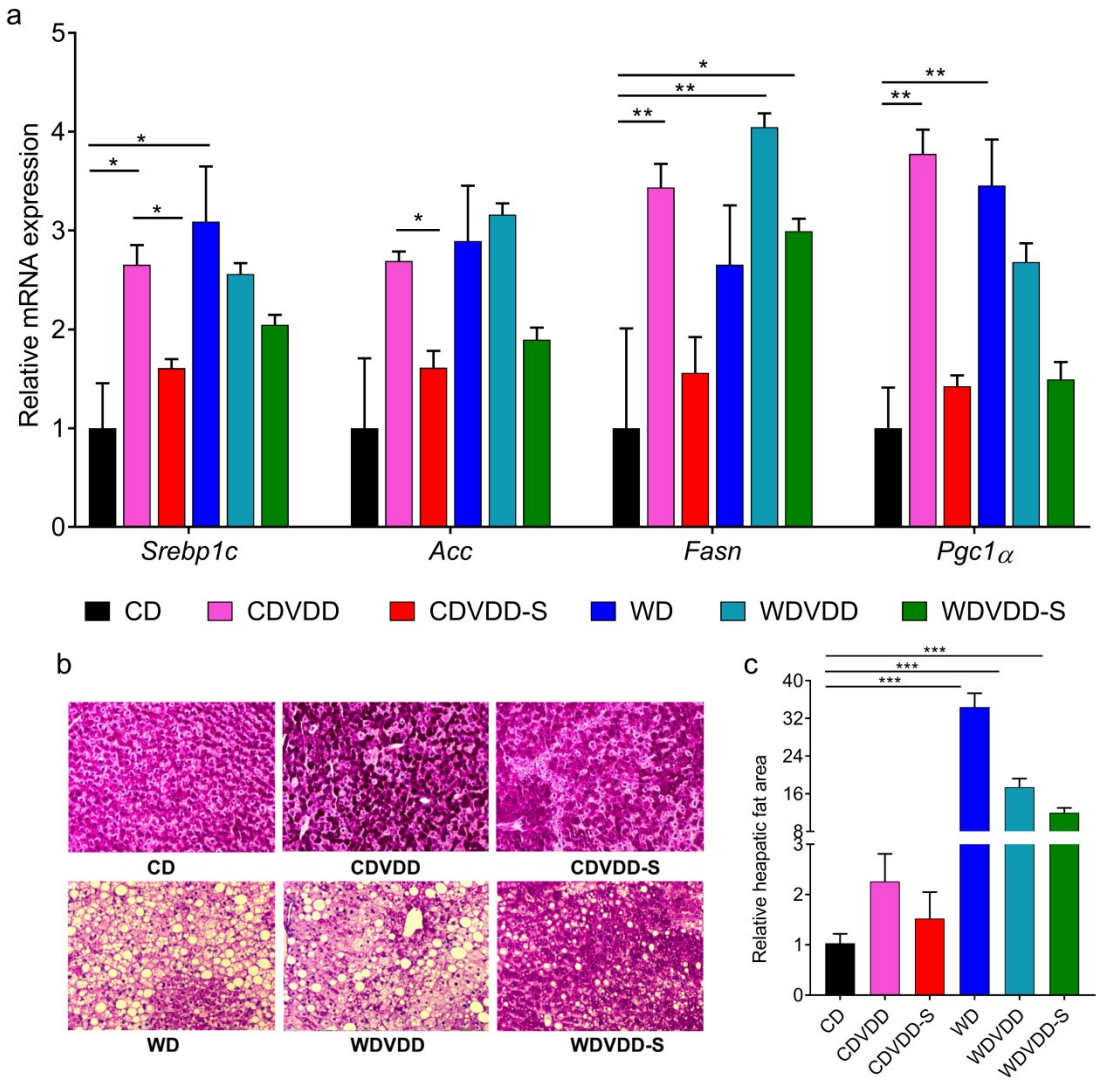
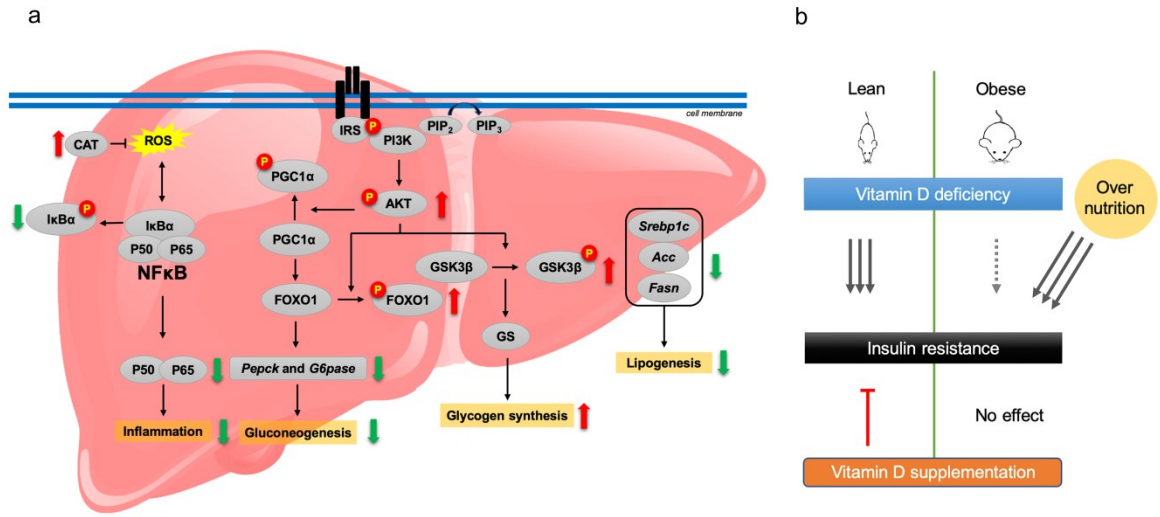


Figure 6. Vitamin D and insulin resistance in mice. a, Schematic diagram of vitamin D regulating hepatic downstream signaling pathways. Red arrows indicate increase and green arrows indicate inhibitory effects by vitamin D. IRS, insulin receptor substrate; P, phosphorylate; PI3K, phosphatidylinositol-3 kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; AKT, protein kinase B; FOXO1, forkhead box protein 1; GSK3β, glycogen synthase kinase 3β; GS, glycogen synthase; PGC1α, PPARγ coactivator 1 alpha; CAT, catalase; ROS, reactive oxygen species; IκBα, inhibitory kappa B alpha; NFκB, nuclear factor kappa B; P50, nuclear factor kappa B p50 subunit; P65, nuclear factor kappa B p65 subunit; *Pepck*, phosphoenolpyruvate carboxykinase; *G6pase*, glucose-6-phosphatase; *Srebp1c*, sterol regulatory element-binding protein 1c; *Acc*, acetyl-CoA carboxylase; *Fasn*, fatty acid synthase. b, Summary of vitamin D effects on insulin resistance in mice. Solid arrows indicate major contributing effect. Dashed arrows indicate minor contributing effect. Blunt line indicate inhibitory effects.



Graphical Abstract