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GRK5 ablation contributes to insulin resistance

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ABSTRACT

The G-protein-coupled receptor kinase 5 (GRK5) is an important member of the threonine/serine kinase family that phosphorylates and regulates the G-protein-coupled receptor (GPCR) signaling pathway. GRK5 is highly expressed in adipose tissue and may act as an adipogenetic factor under high-fat load [1]. Insulin resistance is associated with the pathogenesis of metabolic disorders such as type 2 diabetes and obesity; however, the potential role of GRK5 in insulin resistance is unknown. We characterized the biochemical and molecular alterations related to metabolic complications observed in GRK5$^{-/-}$ mice. These mice, which are partially resistant to obesity induced by a high-fat diet, had impaired glucose tolerance and insulin sensitivity, as well as disruption of AKT signaling transduction compared with their wild-type littermates. Further study showed that the decreased insulin sensitivity was not attributable to alterations in inflammatory status such as the NF-κB signaling pathway or inflammatory gene expression. Instead, hepatic steatosis and changes of mRNA in genes involved in hepatic glucose and lipid homeostasis were found. Overall, our data identified GRK5 as a positive regulator of insulin sensitivity. Our results showed that this protein is a potential therapeutic target in the treatment of insulin resistance and related disorders.

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1. Introduction

Insulin resistance, a condition in which cells fail to respond to the action of insulin, is a key feature in the pathogenesis of metabolic disorders such as type 2 diabetes and obesity [2]. Adipocytes are insulin-sensitive cells that take up glucose and store energy in the form of triglycerides. In addition to their storage function, adipocytes have recently been shown to be dynamic endocrine cells that produce and secrete various adipocytokines, such as TNF-α, IL-6, leptin, resistin, and adiponectin [3]. The insulin resistance that accompanies obesity is attributable, at least in part, to changes in adipokine secretion. Excess accumulation of adipose tissue is detrimental to many systems, and involved in the pathologies including insulin resistance, type 2 diabetes (T2D) and fatty infiltration of the liver [4,5]. Lipodystrophy, the lack of adipose tissue, is also associated with insulin resistance and abnormal lipid metabolism [6]. However, the precise mechanisms regulating insulin resistance in physiopathological conditions are not fully understood.

G-protein-coupled receptor (GPCR) kinase 5 (GRK5) is an important member of the threonine/serine kinase family that phosphorylates GPCRs as part of feedback inhibition of GPCRs in signal transduction [7,8]. The *in vivo* physiological functions of GRK5 have been ascribed to its kinase activity, phosphorylating and desensitizing specific GPCRs [9], as well as its kinase independent function and targeting to non-GPCR substrates. GRK5 interacts with IκBα and inhibits NFκB-mediated transcriptional responses [10]. GRK5 also phosphorylates p53 and regulates p53-mediated apoptosis in response to DNA damage [11]. Chen et al. recently identified GRK5 as a critical mediator of dendritic development that coordinates the actin cytoskeleton and membrane remodeling [12]. These results suggest that GRK5 might regulate many aspects of physiology by multiple molecular mechanisms. Our previous studies found that GRK5-deficient mice fed on a high-fat diet (HFD) show reduced gain in body weight and white adipose tissue (WAT). This is not due to changes in food consumption and energy expenditure induced by GRK5 ablation; instead, GRK5 deficiency decreased the transcription of adipogenic genes and inhibited adipocyte differentiation [1]. A recently performed genome-wide association study has found that the rs10886471, a T2D risk-increasing allele, was associated with higher *GRK5* mRNA expression level, higher fasting insulin, but not with higher fasting

Abbreviations: ANOVA, analysis of variance; GPCR, G-protein-coupled receptor; GRK, GPCR kinase; KO, knockout; SD, standard diet; HFD, high-fat diet; WAT, white adipose tissue; PBS, phosphate buffered saline; EDTA, ethylene diamine tetraacetic acid; TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

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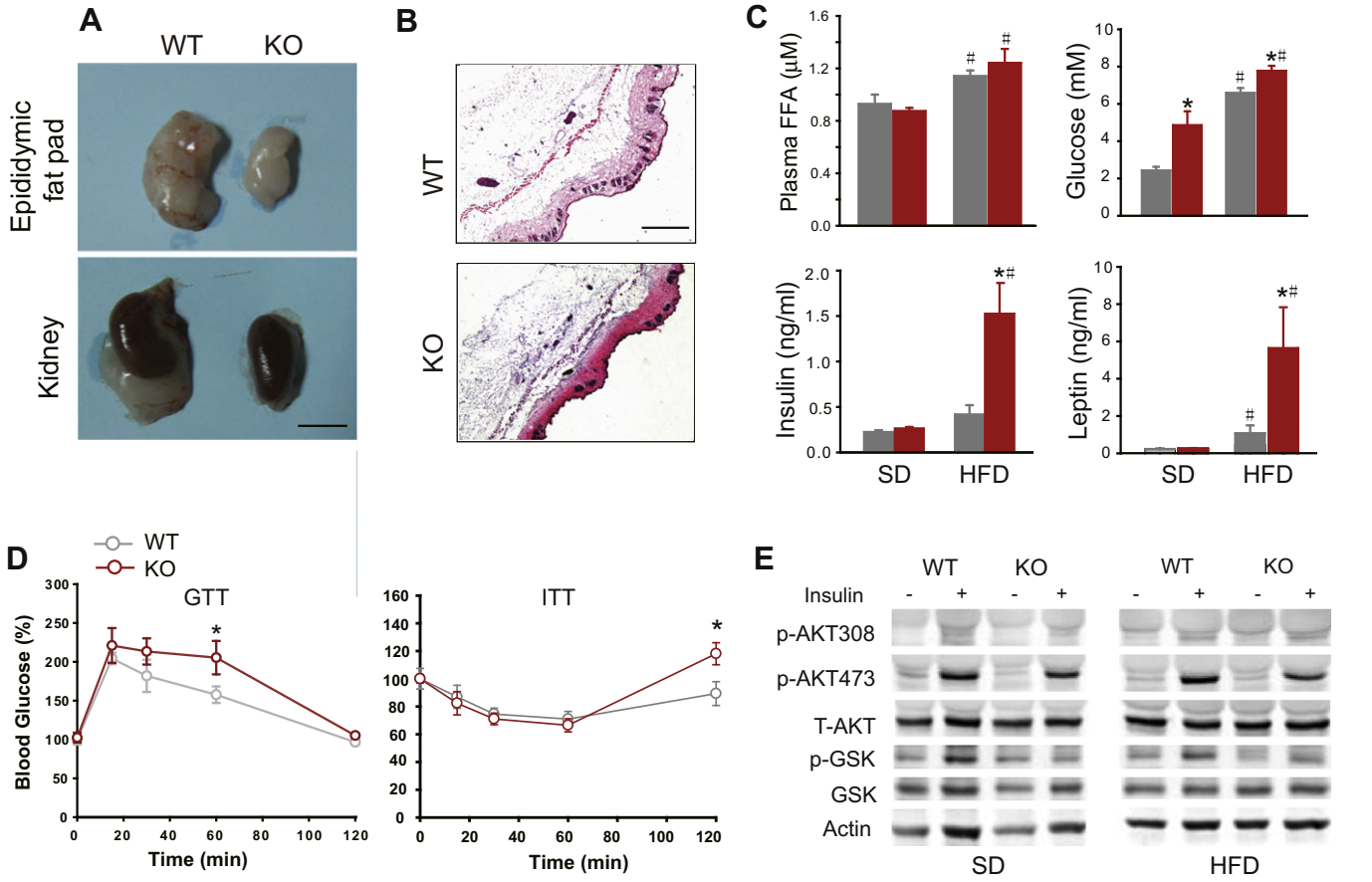

Fig. 1. GRK5$^{-/-}$ mice had decreased glucose tolerance and insulin sensitivity. (A) Representative photographs of epididymic fat pads and kidneys of 19-week-old male WT and GRK5$^{-/-}$ mice fed on a HFD. Scale bar, 1 cm. (B) Representative HE-staining photographs of cross-sections of skin from WT and GRK5$^{-/-}$ mice fed a HFD. Scale bar, 100 μm. (C) Plasma parameters from 6–12 fasted animals. $^{*}P < 0.05$ vs. WT mice of the corresponding diet group; $^{\#}P < 0.05$ vs. SD group of the corresponding genotype. (D) GTTs were performed on animals fasted for 24 h and given an injection of glucose. ITTs were performed on animals fasted for 4 h that received insulin. Glucose concentration was determined in tail vein blood samples. Results are means ± SEM for each group of 6–8 animals fed a HFD. (E) WT and GRK5$^{-/-}$ male mice were untreated or treated with insulin (5 mU/g body weight) for 15 min, and WAT was removed. Lysates were subjected to western blot with the indicated antibodies. Representative immunoblots of four independent experiments are shown.

glucose [13]. These results indicated GRK5 might act as a regulator in diet-induced obesity and T2D. Given the emerging role of signal transduction and adipocyte development in insulin resistance and metabolic disorders, we investigated the possibility that GRK5 might act as a modulator of metabolic complications such as insulin resistance.

2. Materials and methods

2.1. Mouse maintenance

GRK5 heterozygous C57BL/6 mice were provided by R.J. Lefkowitz and R.T. Premont (Duke University Medical Center, Durham, NC, USA). GRK5 knockout (GRK5$^{-/-}$) mice and their wild-type (WT) littermates were obtained by crossing GRK5 heterozygous mice. Genotyping was carried out by PCR amplification using tail tip DNA as described previously [14]. Mice were housed in groups and maintained on a 12 h light/dark cycle with food and water available *ad libitum*. Mice were fed on a standard diet (SD, 10% fat, 70% carbohydrates, and 20% proteins) or a high fat diet (HFD; 45% fat, 35% carbohydrates, and 20% proteins), for 12–16 weeks. All animal treatments were strictly in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All studies received approval from the University of Fudan Animal Care and Use Committee.

2.2. Signaling pathway analysis

Epididymal fat, liver and skeletal muscle tissues collected from GRK5$^{-/-}$ and WT mice were suspended in lysis buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 250 mM sucrose, 0.5% Triton, 2 mM DTT, 1 mM sodium vanadate, 100 mM NaF) with freshly added protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Basel, Switzerland). Crude lysates were centrifuged at 10,000*g* for 10 min and the protein concentration was determined using Pierce BCA Reagents (Pierce Biotechnology, Rockford, IL. USA) Protein samples of 40 μg were resolved by 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Rabbit anti-phospho-IκBα, phospho-NFκB, phospho-AKT (473), phospho-AKT (308), phospho-GSK3β, AKT, GSK3β, NFκB, or mouse anti-IκBα antibodies (Cell Signaling Technology, Beverly, MA, USA) were used to detect proteins. For Western analysis, blots were incubated with IRDye 800CW-conjugated or 700CW-conjugated antibody (Rockland Biosciences, Gilbertsville, PA, USA). Infrared fluorescence images were obtained with the Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE, USA).

2.3. Immunohistochemistry, oil red O staining and confocal microscopy

Tissues fixed in 4% paraformaldehyde were processed and subjected to dehydration in increasing sucrose solutions (20–30%).

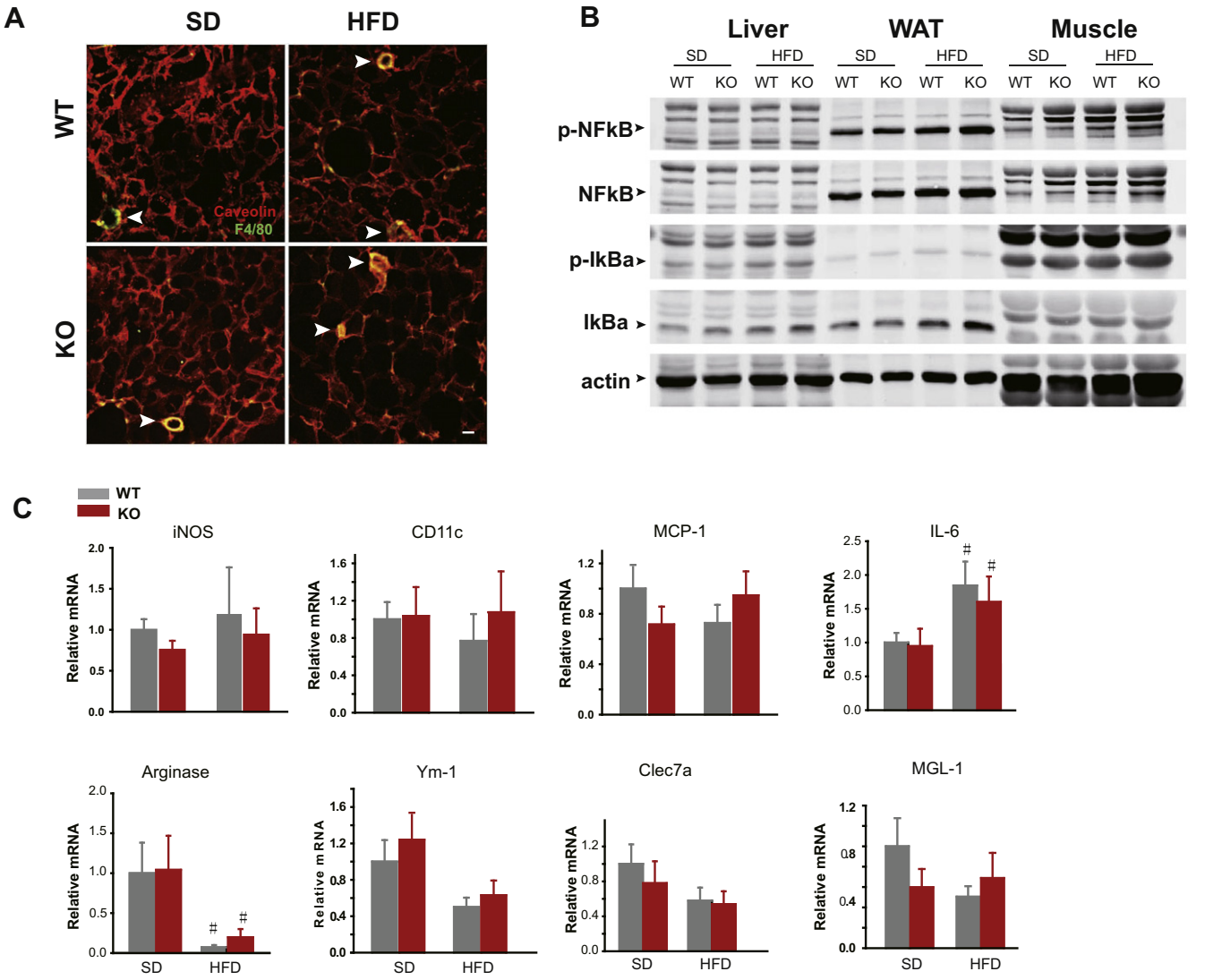

Fig. 2. GRK5 ablation did not affect inflammatory signaling in WAT. (A) Confocal images of cells from epididymal fat pads of SD and HFD-fed WT and GRK5$^{-/-}$ mice. Fluorescence signals for F4/80 (green) and caveolin1 (red) were merged. Scale bar, 100 μm. Images are representative of similar results from 3–4 independent experiments. (B) Representative immunoblots of phospho-IκB, IκB, phospho-NFκB, and NFκB from WAT lysates of SD- or HFD-fed WT and GRK5$^{-/-}$ mice. Each lane represents an individual mouse. (C) Quantitative RT-PCR of the expression of M1 pro-inflammatory and M2 anti-inflammatory genes in WAT of WT and GRK5$^{-/-}$ mice fed a SD or HFD. Each group contained 6–8 animals. $^{\#}P < 0.05$ vs. SD group of the corresponding genotype. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Sections (20 μm) prepared from tissue frozen in O.C.T. compound were mounted on coverslips for staining with haematoxylin-eosin (Vector Laboratories, Burlingame, CA, USA), or co-staining with an ALEXA FLUOR 488-conjugated macrophage antigen F4/80 antibody (Jackson Immunoresearch, West Grove, PA, USA) and rabbit anti-cavolin-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Sections were washed and mounted on slides, and examined by confocal fluorescence microscopy (Zeiss 510; Carl Zeiss, Jena, Germany).

2.4. RNA extraction and real-time PCR analysis

Mouse tissues were isolated and rinsed in PBS. Total RNA after a DNAse digestion step was extracted from tissues using the RNeasy Lipid Tissue Kit (Qiagen GmbH, Hilden, German) according to the manufacturer's instructions. The Superscript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Gaithersburg, MD, USA) was used with random primers for reverse transcription. Quantative RT-PCR was performed on duplicate samples with Power SYBR Green PCR Master Mix (TAKARA, Shiga, Japan) using the Applied Biosystems 7900HT Fast Realtime PCR System (Applied Biosystems, Foster City, CA, USA). mRNA expression of the target gene was normalized to the internal control GAPDH in Fig. 2C and β-actin in Table 1.

2.5. Intraperitonal glucose tolerance test, insulin tolerance test and plasma measurements

Glucose and insulin tolerance tests (GTT and ITT) were performed on 18- to 20-week-old animals at 14 weeks after the beginning of the HFD. For the GTT, mice were intraperitoneally injected with D-glucose (2 mg/kg i.p.) after an overnight fast. For ITT, mice were injected with insulin (1 mU/g i.p.). Blood samples were withdrawn from the tail vein at the indicated time. Plasma glucose was measured using OneTouch Ultra Glucometer (Johnson & Johnson, Milpitas, CA, USA). Whole blood was collected into heparin tubes.

Table 1
Profile of gene expression in livers of GRK5$^{+/+}$ (WT) and GRK5$^{-/-}$ (KO) Mice.

Category	Gene	SD		HFD	
		WT	KO	WT	KO
Fatty acid and TG synthesis	Agpat2	1.00 ± 0.13	0.81 ± 0.06	1.41 ± 0.26	1.32 ± 0.15
	Dgat1	1.00 ± 0.14	1.32 ± 0.19	1.50 ± 0.20	1.00 ± 0.17
	Mgat1	1.00 ± 0.05	0.30 ± 0.02*	2.74 ± 0.16#	1.10 ± 0.16*,#
	Scd2	1.00 ± 0.16	1.20 ± 0.13	1.20 ± 0.20	0.99 ± 0.07
	FAS	1.00 ± 0.15	0.66 ± 0.12	0.41 ± 0.15#	0.39 ± 0.11
	Pgc-1b	1.00 ± 0.18	0.61 ± 0.10	0.97 ± 0.20	0.68 ± 0.05
Fatty acid oxidation	Acc2	1.00 ± 0.13	0.51 ± 0.09*	0.40 ± 0.06#	0.48 ± 0.04
	Lcad	1.00 ± 0.09	0.53 ± 0.07	1.06 ± 0.18	0.86 ± 0.04
	Mcad	1.00 ± 0.15	0.68 ± 0.06	1.49 ± 0.33	1.11 ± 0.06
	Ucp3	1.00 ± 0.08	0.30 ± 0.10*	1.65 ± 0.56#	1.07 ± 0.37*,#
Lipid metabolism	Lipin1	1.00 ± 0.27	0.54 ± 0.23	0.41 ± 0.14	0.29 ± 0.11
	Lipin2	1.00 ± 0.06	1.78 ± 0.13*	1.36 ± 0.21	2.19 ± 0.26*
	Lipin3	1.00 ± 0.12	1.44 ± 0.19	0.79 ± 0.09	0.93 ± 0.07
	Ppap2b	1.00 ± 0.03	0.79 ± 0.09	0.83 ± 0.08	0.85 ± 0.09
	Ppap2a-v1	1.00 ± 0.13	1.27 ± 0.09	1.07 ± 0.11	1.20 ± 0.10
Glucose metabolism	G6pase	1.00 ± 0.20	0.76 ± 0.34	1.46 ± 0.16	1.64 ± 0.08
	Glucokinase	1.00 ± 0.13	0.33 ± 0.12*	0.82 ± 0.30	0.09 ± 0.01*,#
	PDK4	1.00 ± 0.38	1.23 ± 0.24	2.04 ± 0.58#	1.71 ± 0.59#
	GLUT4	1.00 ± 0.29	0.76 ± 0.35	0.43 ± 0.17	0.77 ± 0.29
	Pyruvatekinase	1.00 ± 0.25	0.38 ± 0.11	0.73 ± 0.18	0.27 ± 0.02

Data are means ± SE of measurements obtained from 6–8 animals fed on SD or HFD for 14 weeks.
* $P < 0.05$ vs. WT mice of the corresponding diet group.
# $P < 0.05$ vs. SD group of the corresponding genotype.

Plasma insulin and leptin concentrations were measured using insulin and leptin ELISA kit (Linco Research, Charles, MO, USA). Plasma free fatty acids (FFA) were measured by colorimetric assay (Wako, Osaka, Japan). Cholesterol and triglyceride were determined using Hitachi 747 Analyzer (Roche Diagnostics, Mannheim, Germany).

2.6. Statistical analysis

Data are presented as means ± standard error (SE). We assessed the results by Student's *t*-test to compare two groups or by two-way analysis of variance with Bonferroni *post hoc* test for multiple comparisons using Sigma Stat (Systat Software, San Jose, CA, USA). All significant differences are given in the figures and figure legends.

3. Results

3.1. GRK5 ablation increased insulin resistance

GRK5 is abundantly expressed in white adipose tissue (WAT), and GRK5 deficiency prevents HFD-induced obesity and decreases mRNA from lipogenic genes in WAT [1]. The lower body weight of GRK5$^{-/-}$ mice was largely accounted for by a reduction in WAT, such as in the epididymal and renal fat pads (Fig. 1A), as well as in subcutaneous fat (Fig. 1B). To better understand the metabolic complications of GRK5 deficiency, we characterized the biochemical and molecular alterations related to the metabolic complications observed in these mice. We analyzed plasma parameters in fasting mice. On a SD, circulating levels of plasma FFA, insulin and leptin were not significantly difference between GRK5$^{-/-}$ mice and their WT counterparts, although glucose levels were higher in GRK5$^{-/-}$ mice (Fig. 1C). However, after exposure to a HFD, circulating levels of glucose, insulin, and leptin were significantly higher in GRK5$^{-/-}$ mice compared with WT mice (Fig. 1C). We performed GTTs and ITTs on HFD-fed GRK5$^{-/-}$ and WT mice. As shown in Fig. 1D, after injection with glucose, GRK5$^{-/-}$ mice exhibited higher plasma glucose levels than WT mice. GRK5$^{-/-}$ mice also showed decreased sensitivity to injection of insulin than WT mice. These data indicated that GRK5 ablation impaired glucose homeostasis.

Insulin resistance results from defects in insulin signaling in peripheral tissues. Mice were sacrificed after injection with or without insulin. WAT extracts were prepared, and the activation of Akt and GSK3β was determined by immunoblotting. Insulin-stimulated phosphorylation of Akt-473 and GSK3β was slightly reduced in the WAT of GRK5$^{-/-}$ mice fed either a SD or HFD (Fig. 1E). These data indicated that the loss of GRK5 led to impaired insulin signaling transduction.

3.2. GRK5 ablation did not affect inflammatory status of WAT

Inflammation plays a pivotal role in the development of metabolic diseases. Knockout of inflammatory genes such as JNK1 and NF-κB disrupts the link between dietary or genetic obesity and insulin resistance [15,16]. GRK5 is reported to interact with IκBα and to regulate the NF-κB signaling pathway [10,17,18]. To assess the effect of GRK5 on macrophage infiltration of WAT, adipose tissue macrophages (ATMs) from WT and GRK5$^{-/-}$ mice were examined by immunostaining with the M1 macrophage marker F4/80. As shown in Fig. 2A, HFD induced an increase in F4/80-positive ATMs, which form crown-like structures around adipocytes in WT and GRK5$^{-/-}$ mice; however, no significant difference was observed in the HFD-induced increase of F4/80-positive ATMs between WT and GRK5$^{-/-}$ mice. A change in NF-κB signal transduction in the liver, WAT, and skeletal muscle tissues are thought to be associated with chronic inflammation and insulin resistance [19]. However, as shown in Fig. 2B, a HFD increased NF-κB and I-κBα phosphorylation in these three tissues from WT and GRK5$^{-/-}$ mice were not significantly different. The expression level of several M1 inflammatory genes (such as *iNOS*, *CD11c*, *MCP-1* and *IL-6*) and M2 anti-inflammatory genes (Such as *Arginase 1*, *Ym-1*, *Clec7a* and *MGL1*) was not significantly changed in GRK5$^{-/-}$ mice compared to WT on either a SD or HFD (Fig. 2C). Taken together, these results indicated that the chronic inflammatory status of WAT and NF-κB signal transduction might not be a major target of GRK5-mediated insulin resistance.

3.3. Effects of GRK5 on hepatic steatosis and expression of genes involved in hepatic glucose and lipid homeostasis

Our previous study showed a significant increase in GRK5 mRNA in the liver with a HFD, even if GRK5 itself was not highly expressed [1]. This suggests a potential impact of this increase on insulin resistance. Low WAT accumulation in the presence of high insulin and glucose levels in GRK5$^{-/-}$ mice on a HFD suggested that lipids and fatty acids might accumulate in the liver, since hepatic steatosis is strongly associated with insulin resistance in animals and humans [20,21]. To assess this possibility, histological analysis of liver sections by oil red-O staining was performed. The results revealed an increase in liver lipid deposition in GRK5$^{-/-}$ mice on a SD or a HFD (Fig. 3A). Although circulating levels of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) were comparable between GRK5$^{-/-}$ mice on a SD or a HFD, plasma TG concentrations were significantly elevated in GRK5$^{-/-}$ mice compared to WT on a HFD (Fig. 3B).

Considering the role of hepatic fat accumulation in insulin resistance, we examined the expression of hepatic genes that regulate lipid synthesis and metabolism in GRK5$^{-/-}$ and WT mice. Although the profiles of hepatic mRNA levels for enzymes in lipid and glucose metabolism were mainly comparable, the expression of lipogenic genes (such as *Mgat1*) and fatty acid oxidation genes (such as *Ucp3*) was decreased while the mRNA level of *Lipin2*, which promotes hepatic insulin resistance [22] when overexpressed, was significantly upregulated in GRK5$^{-/-}$ mice either on a SD or a HFD (Table 1). *glucokinase*, which is critical for glucose homeostasis, was significantly decreased in GRK5$^{-/-}$ mice (Table 1). A reduction in glucokinase could decrease the flux of glucose through glycolysis, possibly accounting for the impairment in glucose tolerance. Taken together, these data suggested that GRK5 was critically involved in regulation of hepatic glucose and lipid homeostasis.

4. Discussion

GRKs are important kinases that phosphorylate serine/threonine residues of GPCRs and promote the high-affinity binding of arrestins to induce receptor internalization. This feedback inhibits GPCR responses to extracellular signals. Using GRK5 knockout mice, we demonstrated for the first time that GRK5 protein deficiency leads to insulin resistance and hepatic steatosis under a HFD. Taken together, these results indicated that GRK5 is a potential therapeutic target in the treatment of insulin resistance and other metabolic disorders.

GRK5$^{-/-}$ mice showed a higher plasma leptin level in HFD conditions (Fig. 1C). The adipose-derived hormone leptin is thought to promote energy expenditure through increased sympathetic nerve activity that enhances catecholamine signaling in WAT and brown adipose tissue (BAT) [14,23]. Triggering of β-adrenergic receptors appears important for subsequent increases in lipolysis and fatty-acid oxidation [24]. Overexpression of GRK5 in the heart revealed that exogenous GRK5 augmented desensitization of β-adrenergic-mediate contractile responses [25]. However, we did not observe any significant change in lipolysis in WAT from GRK5$^{-/-}$ mice [1]. This indicated that the physiological role of GRK5 in WAT function and insulin resistance might not be attributable to desensitization of the classic β-adrenergic GPCR-signaling pathway.

Insulin is a key signal of plasma glucose levels. High level of insulin leads organs and tissues to take up glucose from the bloodstream [26]. The major cellular mechanism for disposal of an exogenous glucose load is insulin-stimulated glucose transport into skeletal muscle and the GLUT4 glucose transporter is a key regulator of whole-body glucose homeostasis. A recent study suggested that GRK2, another important member of the GRKs, inhibits insulin-stimulated glucose uptake and signaling by a mechanism independent of kinase activity that involves forming a dynamic GRK2/IRS1 (insulin receptor substrate 1) complex [27]. Previous studies

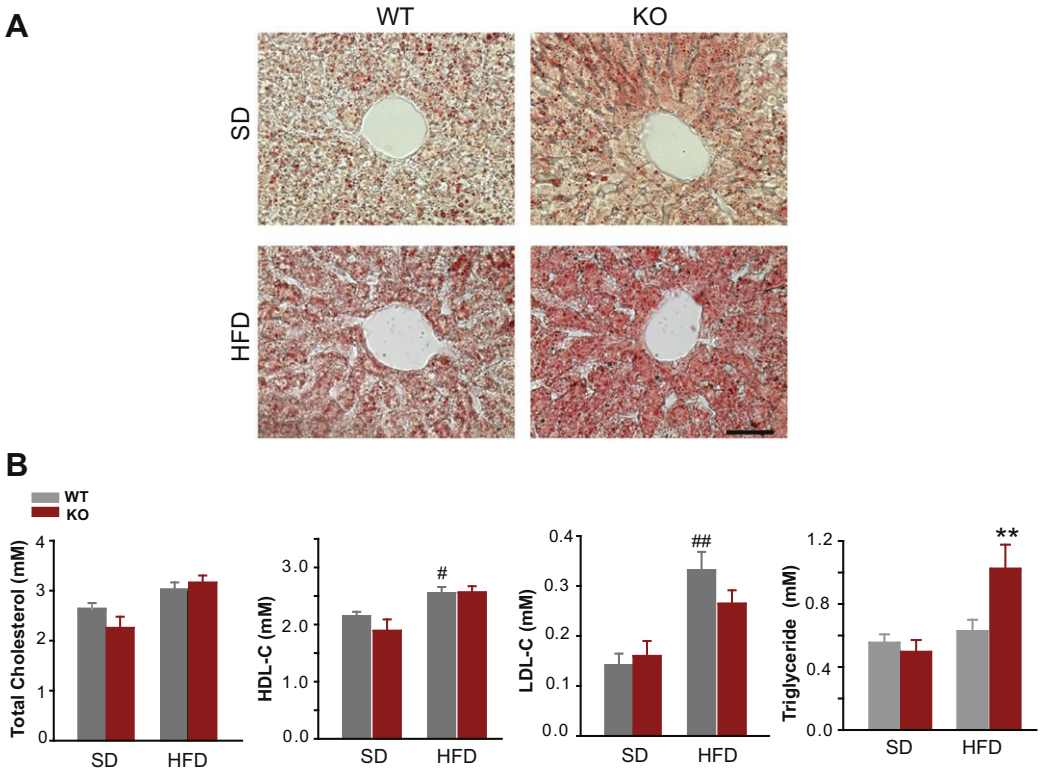

Fig. 3. GRK5$^{-/-}$ mice displayed hepatic steatosis. (A) Histological analysis of liver sections from WT and GRK5$^{-/-}$ mice by oil red-O staining. Representative images are shown. Scale bar, 100 μm. (B) Plasma parameters were monitored from 6–12 fasted animals after a SD or HFD for 14 weeks. $^{**}P < 0.01$ vs. WT mice of the corresponding diet group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. SD group of the corresponding genotype.

demonstrated that endogenous GRK2 is a negative regulator of insulin-stimulated glucose transport, interfering with Gαq/11 signaling to GLUT4 translocation in 3T3-L1 adipocytes. However, the regulation of GLUT4 translocation by GRK5 was not observed [28], suggesting that GRK5 may regulate insulin resistance via an alternative pathway.

Upon insulin stimulation, the insulin receptor phosphorylates and activates the PI(3)K-Akt pathway. Activated Akt phosphorylates downstream signaling transduction, mediating most of the metabolic actions of insulin. β-arrestin2, another important regulator of the GPCR signal transduction, positively regulates insulin sensitivity by serving as a scaffold of AKT and Src to the insulin receptor [29]. β-Arrestin2 levels are downregulated in liver and muscle in animal models with insulin resistance. Zhang et al. demonstrated that lowering GRK5/6 abolishes activation of ERK and AKT mediated by insulin-like growth factor-1 receptor (IGF-1R), whereas GRK2 inhibition increases ERK activation and partially inhibits AKT signaling. They also showed that GRK2 decreases while GRK6 enhances ligand-induced degradation of IGF-1R [30]. In our study, we also found that GRK5 regulated insulin-mediated AKT activation in WAT. However, how this pathway is regulated by GRK5 needs to be further assessed.

Activation of the immune response in obesity and insulin resistance is mediated by specific signaling pathways. Numerous genetic and pharmacological approaches indicate that the NF-κB pathway may be crucial in this response [5,31]. Even though GRK5 is reported to be a mediator of IκBα/NF-κB signaling pathway, results of NF-κB activation are controversial. GRK5 interacts with IκBα by its RGS homology domain, leading to the stabilization and accumulation of the IκBα/NF-κB complex in the nucleus and inhibition of NF-κB-mediated transcription [10]. However, in our study, GRK5 ablation did not affect the NF-κB signaling pathway or the expression of inflammatory or anti-inflammatory genes in WAT. This suggested that GRK5 does not function in insulin resistance by regulating the NF-κB pathway.

Our studies defined the metabolic effects of GRK5. GRK5 deficiency led to insulin resistance and hepatic steatosis. Whether catalytic activity, nuclear transcription function, or specific interaction with differential signaling pathway components of GRK5 are involved in insulin resistance regulation remains to be established. Additionally, the underlying molecular mechanism of GRK5 kinase activity remains to be further explored using tissue-specific knockout mice.

Acknowledgments

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