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Increased fat mass and insulin resistance in mice lacking pancreatic lipase-related protein 1

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Abstract

Pancreatic triglyceride lipase (PTL) and its cofactor, colipase, are required for efficient dietary triglyceride digestion. In addition to PTL, pancreatic acinar cells synthesize two pancreatic lipase-related proteins (PLRP1 and PLRP2), which have a high degree of sequence and structural homology with PTL. The lipase activity of PLRP2 has been confirmed, whereas no known triglyceride lipase activity has been detected with PLRP1 up to now. To explore the biological functions of PLRP1 *in vivo*, we generated *Plrp1* knockout (KO) mice in our laboratory. Here we show that the *Plrp1* KO mice displayed mature-onset obesity with increased fat mass, impaired glucose clearance and the resultant insulin resistance. When fed on high-fat (HF) diet, the *Plrp1* KO mice exhibited an increased weight gain, fat mass and severe insulin resistance compared with wild-type mice. Pancreatic juice extracted from *Plrp1* KO mice had greater ability to hydrolyze triglyceride than that from the wild-type littermates. We propose that PLRP1 may function as a metabolic inhibitor *in vivo* of PLT–colipase-mediated dietary triglyceride digestion and provides potential anti-obesity targets for developing new drugs.

Keywords: Fat mass; Insulin resistance; Obesity; Pancreatic lipase-related protein 1; Knockout

1. Introduction

Obesity is a risk factor for a variety of diseases, particularly heart disease and type 2 diabetes [1,2]. The cause of obesity is excess of dietary calories relative to energy expenditure. Fat contains much higher calories in comparison to carbohydrate and protein. Accordingly, digestion and absorption of dietary triglycerides in the digestive tract have been extensively explored for controlling obesity [3]. Gastric and pancreatic lipases are the main enzymes that digest dietary triglycerides [4]. Up to 20% of the hydrolysis of dietary lipids occurs in the stomach; the rest is completed in the small intestine by pancreatic lipases [5,6]. Bile salts contribute to this process by emulsifying dietary fats. In addition, they also bind to the oil–water interface and prevent PTL adsorption and thus lipolysis [7,8]. This inhibition can be reversed by colipase [9–12], a specific pancreatic protein which is co-secreted with lipase, *via* the formation of a specific lipase–colipase complex [13].

Screening of pancreatic cDNA libraries from different species revealed the presence of two lipase-related proteins (PLRP1 and PLRP2) besides PTL [14–17]. Sequence alignment showed that the two proteins possess all the amino acids that were essential for colipase binding. Recombinant PLRP2 is able to hydrolyze both phospholipids and triacylglycerols [16–18]. Mice deficient in PLRP2 showed that PLRP2 contributes to fat digestion in suckling animals [19]. PLRP1 showed few activity against triglycerides and no measurable activity against phospholipids, galactolipids or cholesterol esters [20,21]. Neither bile salts nor colipase activates PLRPl, although indirect measures indicated that PLRP1 could interact with colipase [21]. Kinetic studies also supported binding between colipase and PLRP1 [22], yet the exact physiological roles of PLRP1 still remain unknown.

In the present study, *PLRP1* KO mice were prepared and studied to explore the biological function of PLRP1 *in vivo*. These *Plrp1* KO mice were viable and fertile. No statistical difference was found between *Plrp1* KO mice and wild-type littermates in body weight before adulthood. However, adult *Plrp1* KO mice displayed increased fat mass and developed impaired glucose tolerance associated with insulin resistance. A high-fat diet exacerbated these conditions in *Plrp1* KO mice. Lipase activity assay revealed higher pancreatic lipase activity in pancreatic juice from the KO mice. These results suggested

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that PLRP1 may influence classic PTL–colipase-mediated dietary triglyceride digestion, and this animal model was useful to investigate PLRP1-mediated physiological role *in vivo*.

2. Materials and methods

2.1. Targeting of the Plrp1 gene in embryonic stem cell (129/SvEv)

KO mice were produced by replacing exons 1–8 of *Plrp1* with a neo cassette (Fig. 1A). The homology arms (1.6 and 4.1 kb) flanking the positive selection marker gene (neo) were amplified from 129/SvEv embryonic stem (ES) cell genomic DNA. Polymerase chain reaction (PCR) analysis of genomic DNA from ES cell clones was carried out with the following primers: F1: 5-GCCCACAAAGCAAAACCAGAG-3; F2: 5-TACATAGACAGCAGATACATACCCCTTCAC-3. Positive clone was identified by two PCR products at 9 and 5 kb (see Fig. 1A–B). The 9-kb band was amplified from the non-recombined allele (the left four lanes in Fig. 1B). The 5-kb band represents the recombined left arm. The recombination was verified by amplification with primers F3 and F4; the generation of the 5.7-kb band represented the recombined right arm (Fig. 1A and C). Primer F3: 5-GCTTGGCGGCGAATGGGCTGAC-3; primer F4: 5-TGGTGGGGGAATAACTACATGGCTTTGAAT-3. The veracity of the recombination was further confirmed by DNA sequencing of the PCR products.

2.2. Generation of Plrp1 knockout mice

Positive ES clone was injected into blastocysts to generate chimeras. The chimeric mice were bred with C57BL/6 to generate F1 progeny. The heterozygotes ($PLRP1^{+/-}$) were used to generate wild-type ($PLRP1^{+/+}$), heterozygotes and homozygous ($PLRP1^{-/-}$) subjects for further experiments. Mice were genotyped by PCR using three primers. Wild-type (WT) mice contained only a 1-kb PCR band, whereas the homozygotes contained only a 1.5-kb PCR product. The presence of both PCR products indicated heterozygosity (HT). Primers were as follows: F, 5-GGGCCCCACCATGCTTGCTCT-3 (in 1.6 kb homology-arm region); R1, 5-CCACCGGGACCTTTTTATGCTC-3 (in exon 1–8 region of *Plrp1*); and R2, 5-TCGGCAGGAGCAAGGTGAGATGACAGGAG-3 (in tk-neo cassette).

Mice were housed at a temperature of 23±1°C under a 12-h light/12-h dark cycle (lights on at 7:00 a.m.). Mice were fed either a normal chow or high-fat (HF) diet (D12492, Research Diets, Inc., New Brunswick, NJ, USA). Age-matched wild-type littermates served as the controls for all experiments. Male mice were used in the studies if not defined. All experiments were conducted in accordance with institutional guidelines.

2.3. RT-PCR analysis and qRT-PCR

For semi-quantitative reverse transcription-PCR (RT-PCR), total RNA was extracted from the pancreas by using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). cDNA was prepared by reverse transcription of 1 μg of total RNA. The resulting cDNAs were amplified using the following primers: 5-ATGCTGATTCTCTGGACAATCCC-3 and 5-GAAGTTTTAGGGGCCTGATAGC-3. The internal 18S RNA control was amplified using 5-GTAACCCGTTGAACCCCATT-3 and 5-CCATCCAATCGGTAGTAGCG-3. Real-time quantitative PCR was conducted using a SYBR Green detection system (Bio-Rad, Hercules, CA, USA). Relative gene expression levels were normalized against GADPH. The primer sequences were as follows: GADPH, 5-TTGCCATCAATGACCCCTTCA-3 and 5-CGCCCCACTTGATTTTGGA-3; PTL, 5-CTGGGAGCAGTAGCTGGAAG-3 and 5-AGCGGGTGTTGATCTGTGC-3; PLRP2,

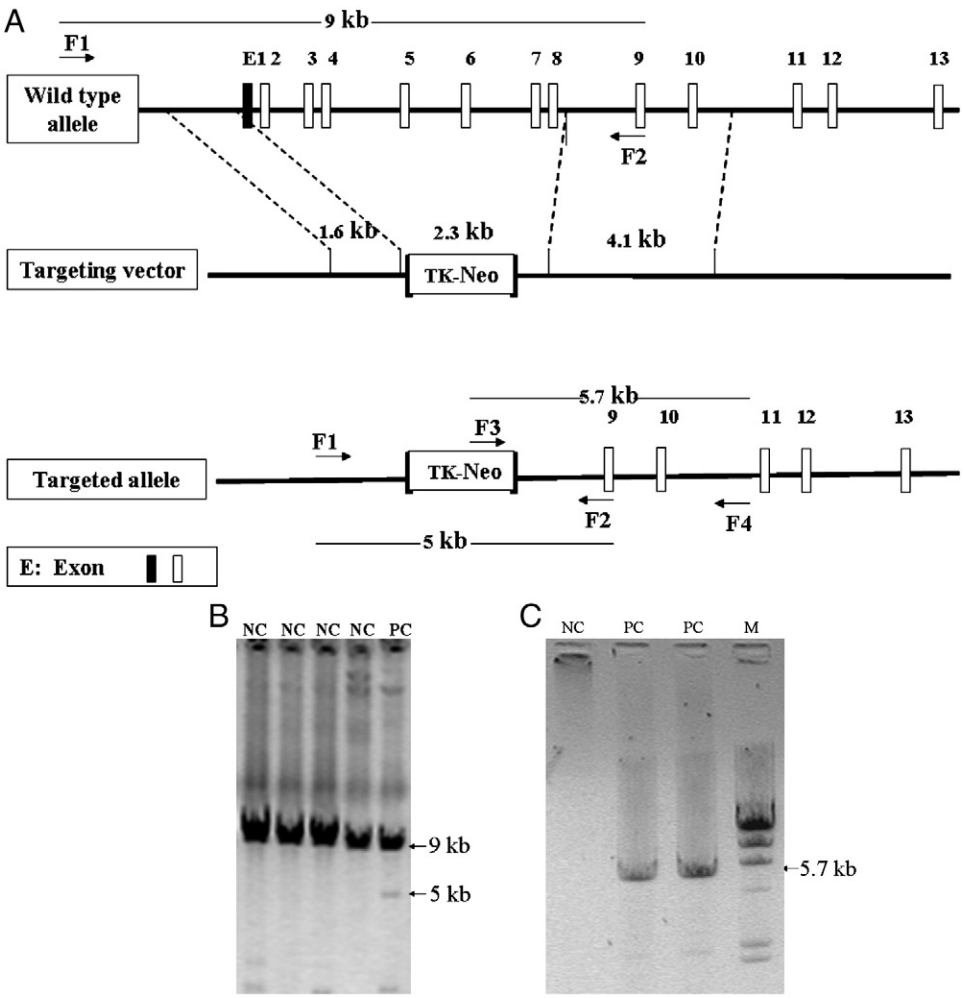

Fig. 1. Targeted disruption of the murine *Plrp1* gene in ES cells. (A) Schematic representation of the *Plrp1* locus and targeting vector. The open boxes in the targeting vector schematics correspond to the tk-neo selectable marker genes. (B–C) Homologous recombination of the positive ES cell clone was verified by PCR analysis. The wild-type allele (negative clone) generated a 9-kb band with primers of F1 and F2 [left four lanes in (B)], while the mutant allele (positive clone) produced a 5-kb band due to the replacement of exons 1–8 with a neo cassette in the targeting vector. The 5.7-kb PCR products with primers of F3 and F4 showed that the right arm had also been appropriately recombined. PC, Positive clone; NC, negative clone; M, DNA marker.

5-ATGCCTATGGATGTCCGTGGA-3 and 5-TGCCCAGGGCTTGTCATTG-3; CLPS (colipase), 5-GCTCTTGCCTTCTGCTGTCTGA-3 and 5-ATGGCGCCGATGATGCTCCTGT-3.

2.4. Western blot analysis

Pancreatic protein extracts were resolved by SDS-PAGE and transferred to a poly (vinylidene difluoride) membrane (Amersham Pharmacia, USA). The membrane was blocked in 1× TBS containing 0.1% Tween (TBST) and 5% nonfat dry milk for 1 h at room temperature. After incubation with a primary antibody at 4°C overnight, the membrane was washed three times with TBST buffer and incubated with goat anti-rabbit or anti-mouse HRP-conjugated IgG for 1 h at room temperature. Proteins of interest were detected by using an enhanced chemiluminescence detection system. Polyclonal anti-PLRP1 antiserum was generated in the laboratory by immunization of rabbits with purified GST fusion protein containing amino acids 65–86 and 465–473 of PLRP1.

2.5. Determination of total fat/lean content by magnetic resonance imaging

Mice were fasted overnight for 16 h prior to analysis of total fat mass, lean mass and body fluid using a Bruker's minispec Lean Fat Analyzer (Bruker Optics, Inc., Billerica, MA, USA).

2.6. White adipose tissue histology

Epididymal fat tissues were dissected from 11-month-old mice. Sections (10 μm) were stained with hematoxylin and eosin. Photographs were taken at ×200 magnification.

2.7. Blood glucose, glucose tolerance tests, insulin tolerance tests, serum insulin assay

Blood glucose was determined by using an automated glucose monitor (Roche Diagnostics Shanghai Ltd., Shanghai, China). Serum insulin levels were measured by enzyme-linked immunosorbent assay (Linco Research Inc., St. Charles, MO, USA). Glucose tolerance test and insulin tolerance test were performed after 16 h of fasting overnight. In the glucose tolerance test, the mice received 2 g/kg glucose intraperitoneally. Glucose level in tail blood was measured immediately before and at 15, 30, 60 and 120 min after the injection. In the insulin tolerance test, mice received 0.6 mU/g insulin (Humulin R, Eli Lilly, Indianapolis, IN, USA) intraperitoneally. Blood sample was collected from the tail vein at 0, 15, 30, 45 and 60 min after the insulin injection.

2.8. Extraction of pancreatic juice and lipase activity assay

Mice were fasted for 6 h and euthanized by cervical dislocation. The pancreas was removed and soaked in a physiological saline solution for 8 h on ice, then centrifuged at 14,000×*g* for 10 min at 4°C. Protein concentration of the supernatant was determined with a BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China).

Lipase activity in pancreatic juice was determined using a lipase assay kit (Nanjing Jiancheng Bioengineering Institute, China). Five microliters of pancreatic juice was added to 95 μl of modified emulsified triglyceride substrate at room temperature. The absorbance at 410 nm (reflecting the remaining emulsified triglyceride) was recorded every 10 s. The decrease in absorbance at 410 nm represents the hydrolysis of the emulsified triglyceride due to lipase activity in pancreatic juice.

2.9. Statistical analysis

Data were analyzed by a two-tailed Student's *t* test or one-way ANOVA. Multifactorial two-way ANOVA was also adopted to assess statistical difference. Values were presented as mean±S.D. Statistical significance was set at $P<.05$.

3. Results

3.1. Generation of Plrp1 knockout mice

The targeting vector was designed as shown in Fig. 1A. The *Plrp1* knockout mice were generated in our laboratory (for details see Materials and Methods). RT-PCR (Fig. 2B) and Western blot analysis (Fig. 2C) indicated that *Plrp1* transcripts and corresponding protein were not detected in the pancreas of KO mice. The *Plrp1* deficient mice were viable, fertile and not significantly different from the wild-type controls in body weight at 4 and 10 weeks after birth (data not shown), although PLRP1 exhibits high neonatal mRNA level in the pancreas [14].

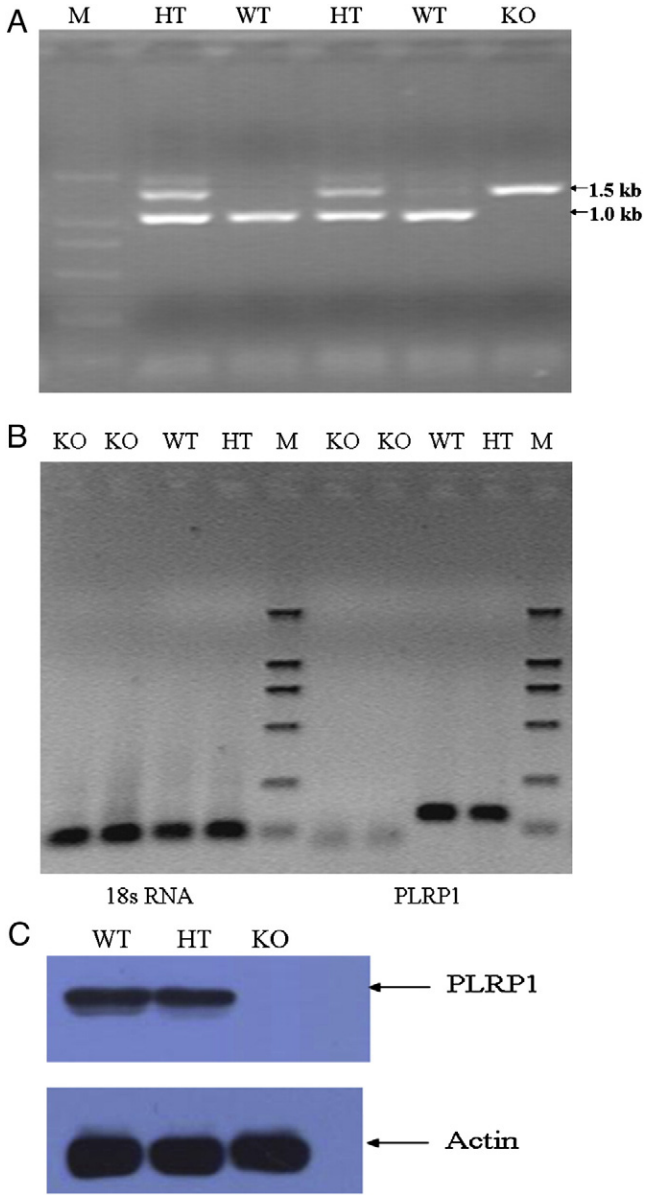

Fig. 2. Confirmation of disruption of the *Plrp1* gene in adult mice. (A) PCR analysis of offspring from heterozygote intercrosses was carried out with the three primers in the reaction system. Arrow shows the location of the knockout (KO) allele band (1.5 kb) and wild-type (WT) allele band (1 kb). The PCR products with both bands indicate heterozygous (HT) mice. M, DNA marker. (B) RT-PCR analysis of *Plrp1*1ex-pression in pancreas from adult WT, HT and KO. The 18s RNA was the internal control (left four panels). (C) Western blot analysis of *Plrp1*1 expression in pancreas from adult WT, HT and KO mice. The blot was probed with rabbit polyclonal antibodies against mouse PLRP1.

3.2. Increased fat accumulation in Plrp1 knockout mice

Body weights of adult KO mice and wild-type littermate controls were measured every 2 weeks as shown in Fig. 3A. The mean body weight of KO mice was higher than that of wild-type littermates, although there was no statistically significant difference. Furthermore, magnetic resonance imaging (MRI) showed that the KO mice had body fat contents at 24.4%, 23.6% and 22.7% of their respective body weights, compared with 13.2%, 10.0% and 8.6% of body weights from WT littermates at 28, 37 and 45 weeks, respectively. In contrast, the KO mice had lean mass contents at 68.7%, 68.9% and 69.5% compared with 80.1%, 82.9% and 84.5% in

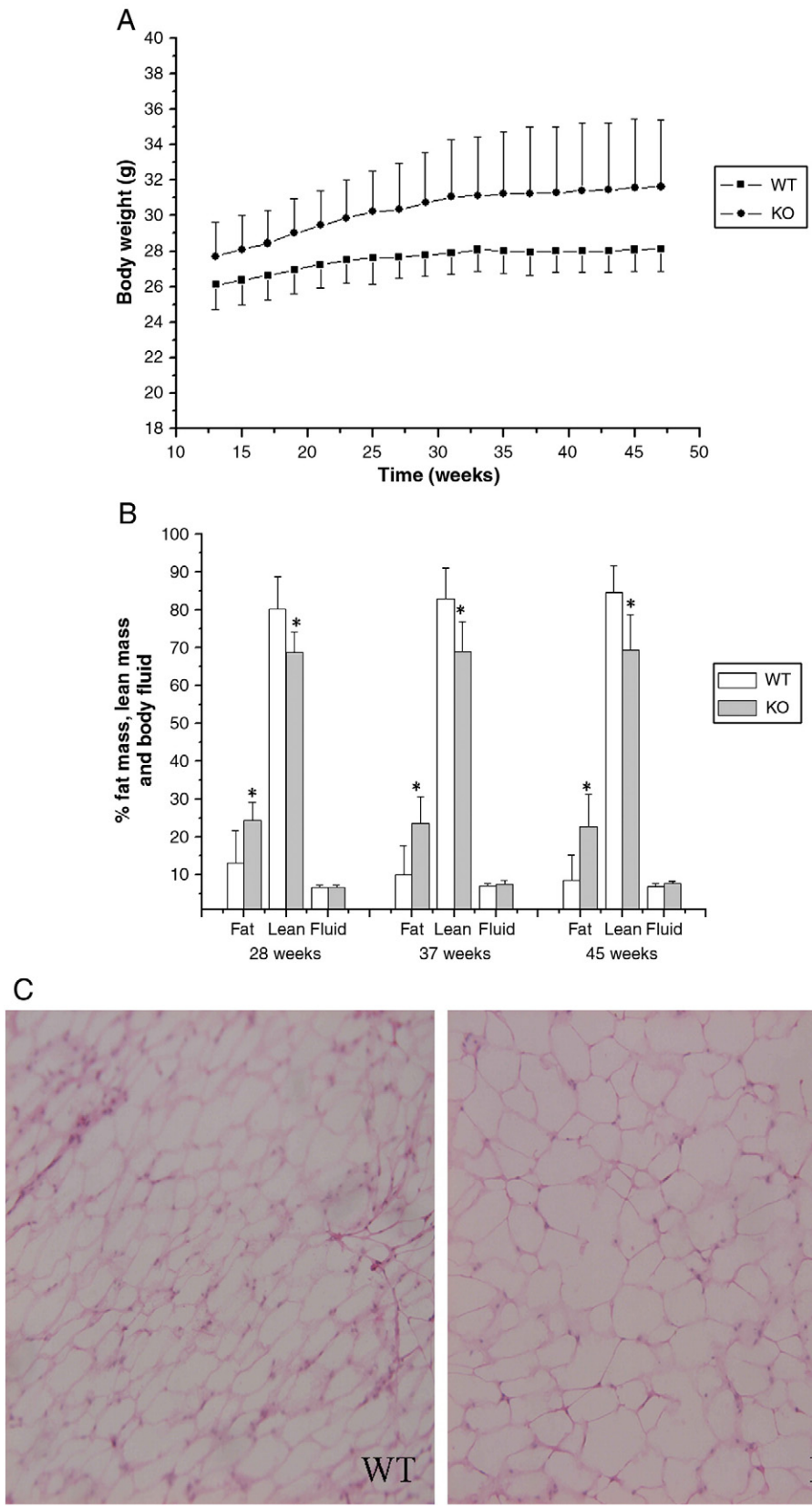

Fig. 3. Deletion of *Plrp1* in male mice may lead to obesity. (A) Body weights of male *Plrp1* KO mice and WT littermates. Data are presented as means±S.D. n=5–6. (B) The percentage of fat, lean mass and body fluid was determined with a Bruker minispec Live Mice Analyzer at the age of 28, 37, 45 weeks. Data are presented as means±S.D. n=5–6. *P<.05. (C) Histology of adipose tissue. Hematoxylin and eosin-stained epididymal white adipose tissues are shown. Magnification, ×200.

wild-type mice, respectively (Fig. 3B). These results suggested that the KO mice had become mildly obese with higher fat content and lower lean mass relative to the wild-type littermates on a normal chow diet.

Macroscopic analyses showed that the subcutaneous, visceral and epididymal fat depots were increased in *Plrp1* KO mice in comparison to those in wild-type mice (Supplementary Fig. 1). The size of adipocytes in white adipose tissue was also larger than that in wild-type controls (Fig. 3C). These results showed that deletion of *Plrp1* in mice led to mature-onset obesity.

3.3. Impaired glucose tolerance in Plrp1 null mice

Plrp1 KO mice showed mild hyperglycemia and hyperinsulinemia at 7 months (Fig. 4A–B). To investigate this point further, we performed glucose and insulin tolerance tests (GTT and ITT). In the GTT, KO mice displayed impaired glucose tolerance with higher blood glucose at 30, 60 and 120 min after glucose administration compared with that from WT littermates (Fig. 4C). Insulin sensitivity was also significantly decreased in KO mice (Fig. 4D).

3.4. Plrp1 KO mice developed more severe obesity and insulin resistance on high-fat diet

We next challenged mice with high-fat diet at the age of 10 weeks. The *Plrp1* KO mice had significantly higher body weight than WT littermates upon a high-fat diet for 6 weeks (Fig. 5A). Ten weeks of high-fat diet increased fat content in KO mice to 27.8%, compared with WT littermates at 17.8%. There was also a tendency for KO mice to have a higher fat content at the age of 20 weeks when mice were fed on a normal chow diet (Fig. 5B). Furthermore, *Plrp1* KO mice displayed more severe glucose intolerance and insulin resistance than wild-type mice after feeding high-fat diet for 14 weeks (Fig. 5C and D).

3.5. Increased lipase activity in pancreatic juice extracted from Plrp1 KO mice

In vitro studies indicated that PLRP1 could bind to colipase and inhibited PTL activity in the presence of colipase, and increasing the colipase concentration could restore the PTL activity [23], which suggested that PLRP1 could compete with PTL for colipase. To further test the hypothesis that PLRP1 inhibits dietary triglyceride digestion

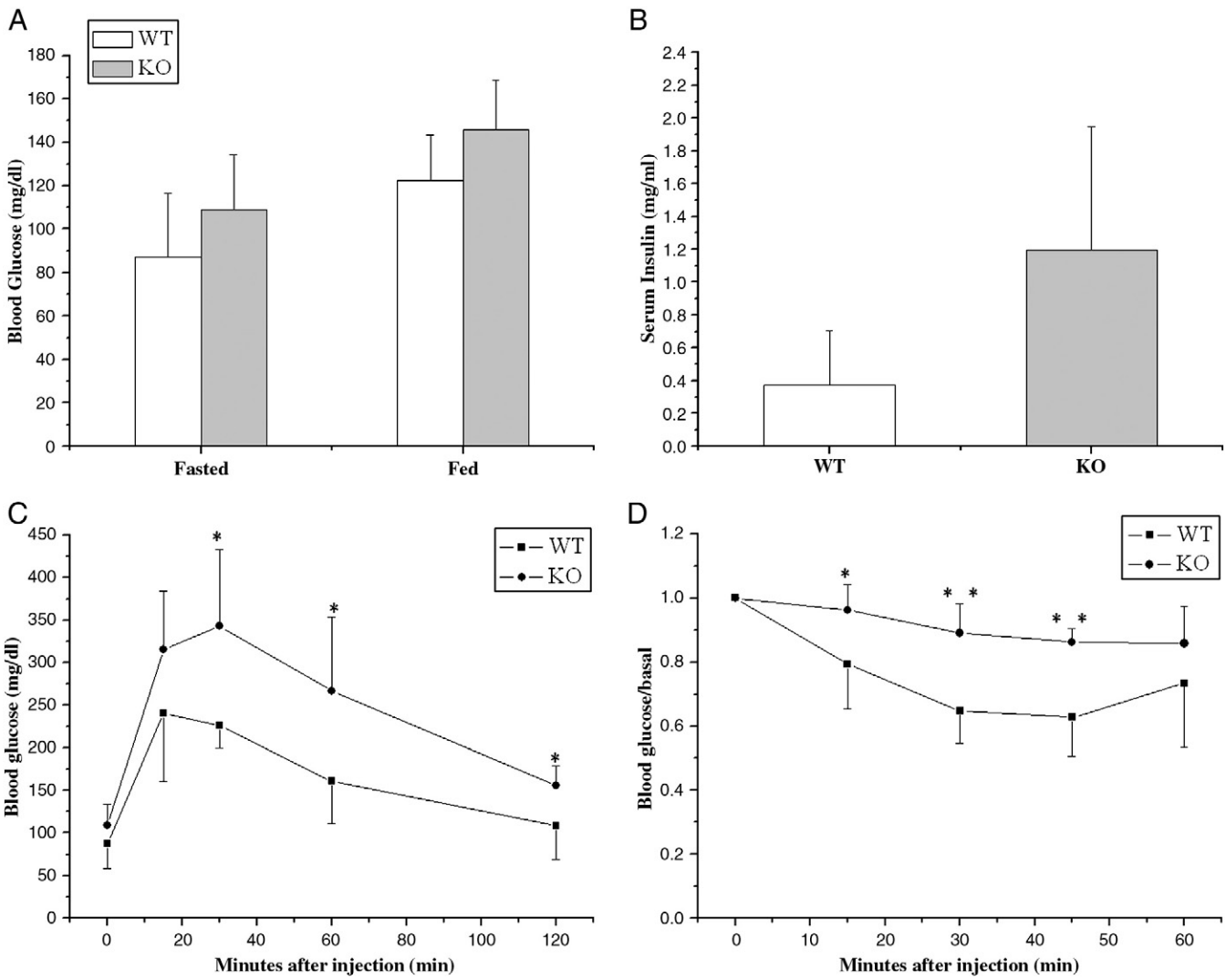

Fig. 4. *Plrp1* KO mice display insulin resistance. (A) Blood glucose levels of KO mice and WT littermates under fasted (16 h) and fed conditions at the age of 7 months. (B) Serum insulin concentration for 7-month-old KO mice and WT littermates. (C–D) Glucose and insulin tolerance tests in 9-month-old KO mice and WT littermates. Data are presented as means±S.D. n=5. *P<.05; **P<.01.

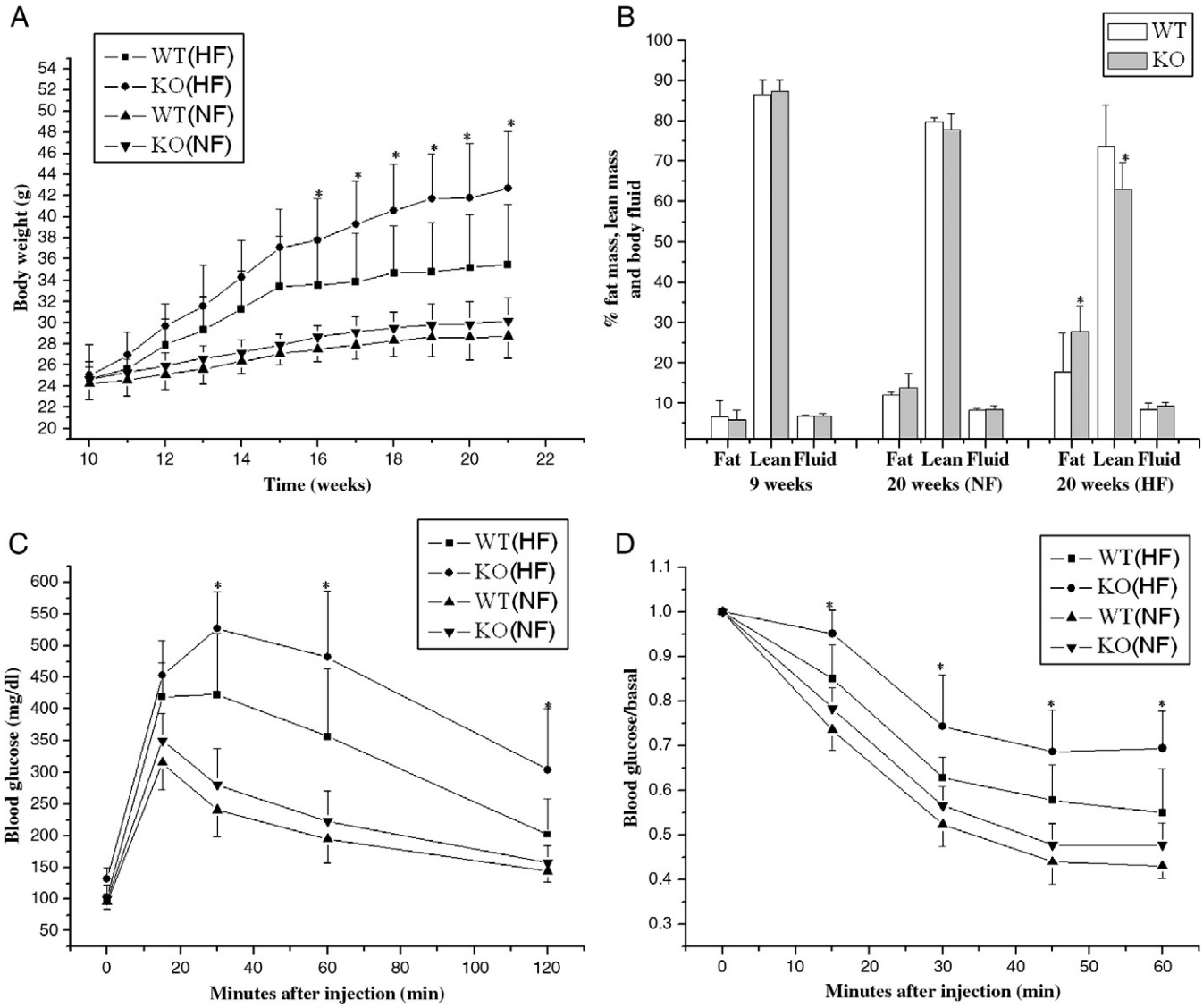

Fig. 5. Mice deficient in *Plrp1* develop obesity and insulin resistance on high-fat (HF) diet. (A) Body weights of *Plrp1* KO mice and WT littermates when mice were on HF diet from age of 10 weeks or fed normal food (NF). Data are presented as means±S.D. n=7–10. (B) NMR determination of fat contents of KO mice and WT littermates. Mice were on NF or HF diet at age of 10 weeks. Data are presented as means±S.D. n=7–10. (C–D) Glucose and insulin tolerance tests in 6-month-old KO mice and WT littermates fed NF or HF diet for 14 weeks. Data are presented as means±S.D. n=7. *P<.05.

in vivo, we assessed the lipase activity of pancreatic juice from KO mice and WT littermates. Pancreatic juice extracted from KO mice had higher lipase activity in comparison to WT littermates (Fig. 6A). Furthermore, real-time PCR showed that the expression of PTL, PLRP2 and CLPS (colipase) was not different between the KO and WT mice (Fig. 6B–D).

4. Discussion

It is now widely accepted that PTL performs a key function in dietary fat digestion and absorption by hydrolyzing triglycerides into diglycerides and subsequently into monoglycerides and free fatty acids. PLRP1 has a 68% amino acid sequence identity with PTL, but several investigators have revealed that native or recombinant PLRP1 showed no lipolytic activity in a standard pancreatic lipase assay using a large variety of substrates and condition, although PLRP1 was shown to possess the same affinity as PTL for colipase. Substituting two residues (V179 and A181) in PLRP1 for those found in PTL (A179 and P181) restores a significant lipolytic activity [20,21]. Thus, the lack of lipase activity of PLRP1 is likely to result mainly from particular features of the N-terminal domain. Until now, the physiological function of PLRP1 has not been established, although it has been demonstrated in a restricted expression in the pancreas and PLRP1 is secreted with PTL and colipase.

We generated and characterized mice deficient in PLRP1. The *Plrp1* KO mice were viable, fertile and had no significant difference from the wild-type controls in body weight at 4 and 10 weeks after birth. However, they displayed increased fat mass content and insulin resistance after adulthood. Obesity and insulin resistance were exacerbated by high-fat diet in KO mice. A previous *in vitro* study showed that PLRP1 could reduce PTL lipase activity in the presence of colipase and bile salts, and this activity was recovered upon further colipase addition [23], indicating that PLRP1 is a metabolic inhibitor of PLT–colipase-mediated dietary triglyceride digestion. PLRP1 and PLT share a homolog colipase binding domain and the same affinity to colipase; hence, it may be that PLRP1 competes with PTL for colipase *in vivo*. This

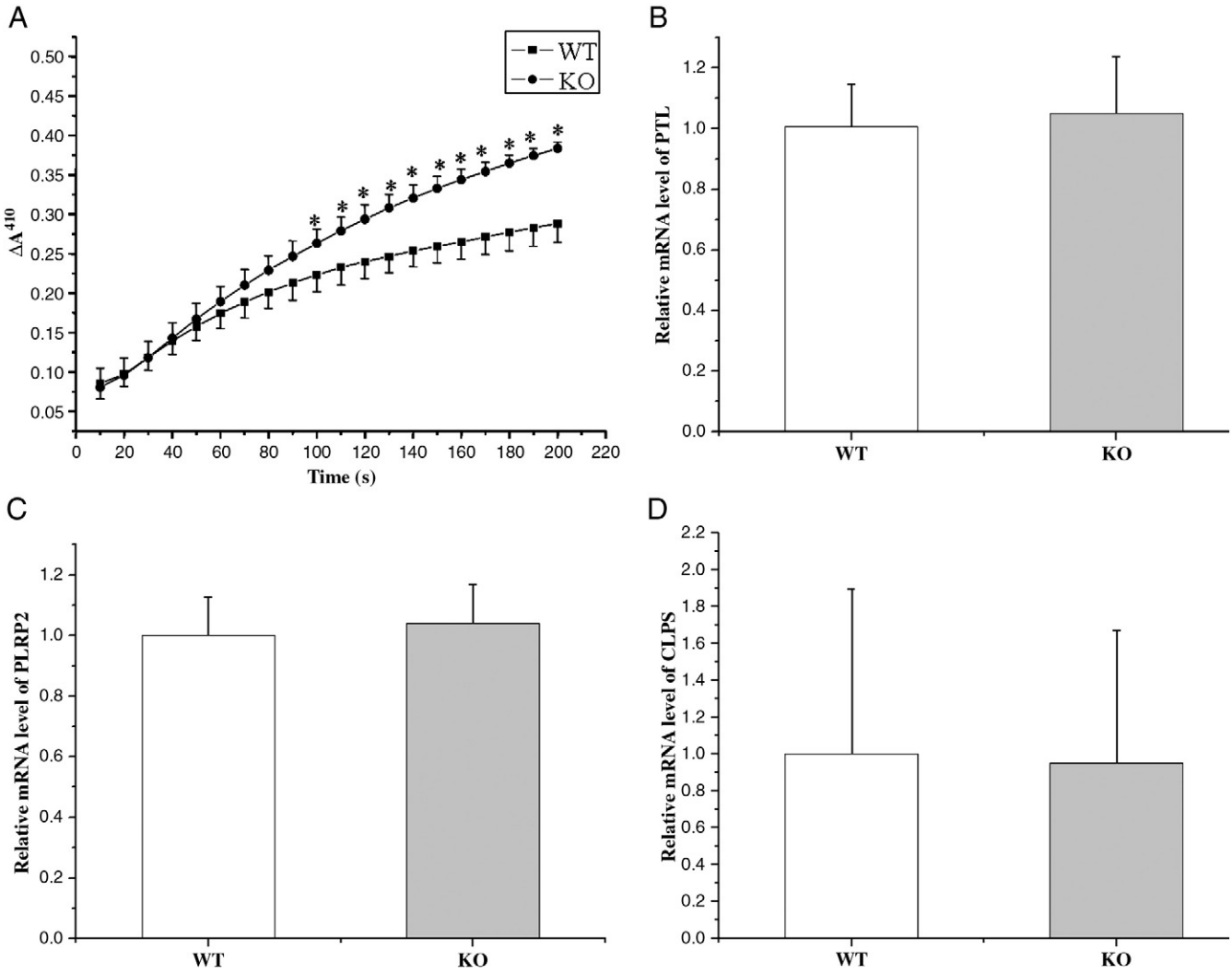

Fig. 6. Determination of lipase activity of pancreatic juice and expression levels of PTL and PLRP2 in KO and WT mice. (A) The pancreatic lipase activity was measured using a spectrophotometric assay with emulsified triglyceride as the substrate, and the decrease of absorbance at 410 nm represents the hydrolysis of triglyceride. Data are presented as means±S.D. $n=4$. (B–D) Expressions of PTL, PLRP2 and CLPS in *Plrp1* KO mice were not significantly different from WT controls by comparing the relative mRNA levels in pancreas from WT and KO mice. Data are shown as relative values. $n=4$.

hypothesis is in agreement with our studies showing that pancreatic juice extracted from KO mice had higher lipase activity in comparison to WT littermates. When KO mice are placed on a normal chow diet, the higher lipase activity in their digestive system is helpful for digestion of dietary triglycerides, although the process is very effective in the intestine and may contribute to increased fat mass content in KO mice over a long period of time. As for high-fat diet, the high-fat content in food causes incomplete digestion and absorption of dietary triglycerides, so the lack of PLRP1 would improve the process and the KO mice deficient in *Plrp1* developed obesity early and significantly compared to wild-type mice.

In newborns, the mRNA levels of PLRP1 and PLRP2 are high, while PTL is not expressed at detected levels [14]. It has been reported that PLRP2 was critical for efficient fat digestion in this period. Whether or not PLRP1 has a role in suckling animals remains unclear since no significant difference in body weight at 4 weeks has been observed. PTL exhibits maximal expression level in adulthood, and colipase is an obligatory cofactor for PTL [14]. It is reasonable to speculate that colipase deficiency due to competition by PLRP1 may influence PTL activity in adulthood. This provides a potential explanation for our observations that pancreatic juice extracted from KO mice had higher lipase activity. Although no significant difference in expression of PTL, PLRP2 and CLPS (colipase) was observed, it cannot exclude the potential possibility that expression levels of other pancreatic lipases may change. Thus, further investigations are required to elucidate the detailed mechanisms.

It is well accepted that adipose tissue has a substantial influence on systemic glucose homeostasis through secretion of adipocytokines [24,25]. Our findings showed that adult *Plrp1* KO mice displayed impaired glucose clearance associated with insulin resistance. These results may be due to increased fat mass and the development of obesity in *Plrp1* KO mice.

In summary, we provide the first evidence *in vivo* that mice lacking PLRP1 may develop obesity and the symptom of glucose intolerance and insulin resistance. Although the detailed mechanism and the significance of the physiological regulation *in vivo* are still waiting for exploration, the PLRP1 itself or compounds regulating its expression and activity may have a great potential for developing attractive pharmacological interventions counteracting obesity and related metabolic diseases.

Supplementary material related to this article can be found online at doi:10.1016/j.jnutbio.2010.06.002.

Acknowledgments

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