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Integrated Remodeling of Gut–Liver Metabolism Induced by Moderate Protein Restriction Contributes to Improvement of Insulin Sensitivity

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Scope: Protein restriction (PR) is beneficial for relieving metabolic disorders and aging-related diseases. However, extreme PR could result in malnutrition due to severe deficiency of essential amino acids. Therefore, the effect of moderate PR on insulin sensitivity is investigated.

Methods and results: The growing and adult pigs are subjected to moderate PR by 15–30%. Plasma insulin concentration and insulin resistance index HOMA-IR are significantly decreased upon moderate PR. Furthermore, IRS1/PI3K/AKT pathway in the basal state is enhanced in both liver and skeletal muscle. The adapted metabolism in the liver upon moderate PR is in support of improving insulin sensitivity. The liver shares a coordinated metabolic adaption in terms of energy metabolism and amino acid metabolism with the small intestine. Particularly, alteration of the metabolic footprint appeared in the portal venous blood, representing metabolites to be absorbed into liver after intestinal metabolism, is also in favor of improvement of insulin sensitivity.

Conclusion: In summary, the study proves that moderate PR could improve insulin sensitivity from childhood to adulthood in a pig model, and sheds a new light on the role of integrated remodeling of gut and liver metabolism in the improved insulin sensitivity induced by moderate PR.

1. Introduction

Insulin resistance develops with ageing or long-term consumption of excess-energy diets, and leads to metabolic diseases, such as type 2 diabetes (T2D).[1] Recently, T2D has become more common among children and adolescents, in parallel with the increasing prevalence of obesity in pediatrics, which has become an issue of public concern.[2]

Dietary protein restriction (PR), as a feasible dietary intervention, has been shown to extend lifespan,[3] improve human health,[4] especially insulin sensitivity[5] through nutrient sensing pathways to fine tune the metabolic responses in a conserved manner.[6,7] Dietary protein is digested into small peptides and free amino acids (AA), and undergoes a critical metabolism in enterocytes, which reconstitutes absorbed AA profile.[8] Remodeled nitrogen-containing metabolites subsequently enter the liver via the portal vein and transform the following metabolic pathways. Sensors that distribute on intestinal epithelium can initiate crucial negative feedback through a gut–brain axis to mediate appetite, glucose homeostasis, and energy metabolism according to nutritional status.[9–11] Therefore, intestinal metabolism of protein should play a vital role in improving insulin sensitivity induced by PR.

Most previous studies focused on extremely protein restrictive diets, in which protein levels were reduced by about 60–100%,[4,12] or on specific essential amino acid (EAA), such as methionine[13] and branched-chain AA,[14,15] which were restricted by 50% or more, far below the estimated average requirement for human and rodents, likely unsustainable and unhealthy due to the severe deficiency of EAA.[16,17] We demonstrated that moderate PR diets, whose protein levels were reduced by about 20%, shrank circulating AA pool size[18] and profoundly altered the pathways concerning immune function in the small intestine even with the supplementation of crystal EAA in diets.[19] However, the impact of moderate PR without malnutrition on insulin resistance remains unclear.

In addition, insulin serves as one of the most important hormones in overall metabolic regulation in bodies. However, few merited attentions were put on the role of gut–liver metabolism in the improvement of insulin sensitivity by PR. Thereby, the present study was conducted to detect whether moderate PR could improve insulin sensitivity and relevant metabolic mechanism using a pig model.

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2. Experimental Section

2.1. Ethics Statement

All experimental protocols concerning animal trials were approved by the Institutional Animal Care and Use Committee of China Agricultural University (ID: SKLAB-B-2010-003) and Sichuan Agricultural University (Sichuan, China), and had therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

2.2. Experimental Diets, Animals, Samples Harvested, and Data Mined

Experimental diets were formulated to be isoenergetic, and crystal EAA were added to meet the National Research Council (NRC, 2012)[20] nutrient recommendations for pigs (Table S1, Supporting Information). The PR was implemented by feeding diets whose crude protein (CP) levels were reduced by 15–25% relative to the control in Experiment 1–3, respectively. The crystal EAA were supplemented to guarantee EAA requirements. For pigs used in hepatic transcriptome analysis, dietary CP level was decreased by 30% while lysine, methionine, threonine, and tryptophan were supplemented to meet the requirements, and other EAA were restricted no more than 25% of the requirements. Therefore, the PR in the present study was moderate restriction relative to extremely protein-restricted diets in previous studies.

Commercially available crossbred pigs of Duroc × Landrace × Yorkshire were used in all Experiments.

In Experiment 1, to avoid the gender effect, 16 castrated male growing pigs (44 days old), with an initial body weight (BW) of 13.5 ± 0.5 kg, were randomly assigned to alternative dietary treatments ($n = 8$), and offered the control diet (CON, 18% CP) or a moderate PR diet (PR, 14% CP). Pigs were kept in individual cages in a national experimental farm (Sichuan Agricultural University, Ya'an, Sichuan, China) for a period of 28 days. Pigs were given free access to feed and water. During the period of the study, the feed intake (kg d^{-1}) remained unchanged, and final BW was decreased upon moderate PR as described previously.[19]

At the end of the trial, blood samples were collected from the precaval vein after overnight starvation for 16 h in the cages of the farm. The plasma was harvested and immediately stored at –20 °C for analysis of plasma lipids, glucose, and insulin. After that, pigs were sacrificed by exsanguination post-anesthesia and the jejunal mucosa, liver, and longissimus dorsi were sampled, frozen in liquid nitrogen immediately and stored at –80 °C for further analysis.

The jejunal mucosal proteomic dataset (PXD004069) representing the proteomic adaption of jejunal mucosa in response to moderate PR was downloaded from the ProteomeXchange Consortium. Differentially expressed proteins (DEPs) were determined based on the ratios of differently labeled proteins with fold change (FC) >1.5 or <0.67. FC was calculated by the abundance of a specific protein upon moderate PR divided by that of CON. The KEGG database was used to classify and group DEPs. Fisher Exact test was used to identify the significantly enriched KEGG pathways.

In Experiment 2, a total of 36, 140 days old gilts with 89.5 ± 0.9 kg BW were subjected to moderate PR by feeding the control diet (14% CP) and a protein-restricted diet (12% CP) for 28 days. The feed intake (kg d^{-1}) and final BW were not altered between two groups as previously described.[21] At the end of the trial, pigs with the average final BW from each replicate were selected ($n = 6$ for each group) and transported to a local abattoir. After at least 4-h rest, the pigs were sacrificed after being electrically stunned and exsanguinated. The liver and longissimus dorsi were sampled as described in Experiment 1.

In Experiment 3, 12, 60 days old, castrated male growing pigs ($n = 6$) with 22.7 ± 1.3 kg BW were housed individually. After 3 days of adaption, pigs were equipped with catheters in portal vein and cared as described previously.[18] Subsequently, pigs were pair-fed the same amount of the control (18% CP) and moderate PR diet (14% CP), respectively. Portal venous blood was sampled at 0.5 h preprandial and 0.5, 1.5, and 7.5 h postprandial through a catheter equipped in the portal vein. The plasma samples were separated and stored at –20 °C for metabolic analysis. Given that the pigs were subjected to moderate PR only for 8 h, the change in BW was ignored.

Hepatic transcriptome data (accession number SUB2170304), representing 12, 28 days old, castrated male growing pigs with an initial BW of 9.57 ± 0.64 kg offered 20% CP of diet (CON) or 14% CP of diet for 30 days (the feed intake and final BW were not reported),[22] were downloaded from the NIH Short Read Archive database. This data represented the liver transcriptomic adaption upon moderate PR. Transcripts with FC ≥ 1.5 or ≤ 0.67 and p-value ≤ 0.01 were considered to be significantly different.

2.3. Measurement of Plasma Lipids, Glucose, and Insulin

The concentrations of plasma VLDL, LDL, HDL, triglyceride (TG), and total cholesterol (TC) were assayed using commercial kits (Zhongsheng Beikong Bio-Technology Inc., Beijing, China). The contents of plasma glucose and insulin were measured by commercial kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, China). Insulin resistance was estimated using the homeostatic model assessment method (HOMA-IR),[23] which was expressed as insulin (mIU L^{-1}) × glucose (mmol L^{-1})/22.5.

2.4. Western Blotting

Frozen liver and muscle tissues obtained from Experiment 1 and 2 were rapidly powdered in liquid nitrogen and lysed in RIPA buffer with protease- and phosphatase-inhibitors, followed by sonication and centrifugation. A sum of 60 μg protein from each sample was loaded, separated by 10% SDS polyacrylamide gels, transferred to a polyvinylidene fluoride membrane (Millipore). The blotted membranes were incubated with corresponding primary antibodies overnight at 4 °C (Table S2, Supporting Information). After three washes, the membranes were incubated with DyLight 800-labeled secondary antibody (Cell Signaling Technology, 5151) for 1 h at room temperature. Band densities were detected with the Odyssey Clx (Gene Company Limited, Hong Kong, China) and quantified using the ImageJ software.

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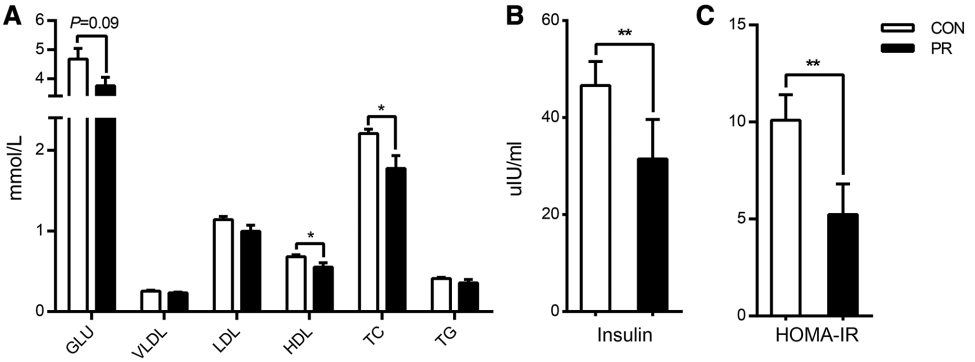

Figure 1. Changes in plasma glucose, lipids, and insulin in response to moderate protein restriction. The plasma was collected from growing pigs in Experiment 1, in which growing pigs were offered to control (18% CP) or a moderate protein-restricted diet (14% CP). Plasma levels of A) glucose, VLDL, LDL, HDL, total cholesterol (TC), triglyceride (TG), and B) insulin in the control (CON) and moderate protein restriction (PR) group. C) HOMA-IR scores were used to reflect insulin resistance in the CON and moderate PR group. Data are expressed as mean ± SEM ($n = 5$–6). *Value significantly different from corresponding value in CON ($p < 0.05$). **Value significantly different from corresponding value in CON ($p < 0.01$).

2.5. Plasma Nontargeted Metabolic Footprint Analysis

Plasma sample analysis was performed with a UPLC-Q-Extractive system after sample preparation as previously described[24] with minor modifications. For details, see the Supporting Information Materials and Methods.

2.6. Statistical Analyses

The data of plasma lipids, glucose, insulin, and western blotting were analyzed using *t*-test procedures of SAS software (Version 9.3, SAS Institute) with each animal as an experimental unit. Values were presented as mean ± SEM and $p < 0.05$ was considered statistical significance.

3. Results

3.1. Plasma Levels of Glucose, Lipids, and Insulin

The plasma was collected from growing pigs in Experiment 1, in which pigs were offered to control (18% CP) or a moderate protein-restricted diet (14% CP). Notably, the concentrations of plasma TC and HDL were significantly reduced upon moderate PR ($p < 0.05$), while no changes were observed in VLDL, LDL, and TG (**Figure 1**A). Meanwhile, plasma glucose content tended to be decreased ($p = 0.09$; Figure 1A), and moreover, plasma insulin and HOMA-IR score were significantly decreased in pigs upon moderate PR ($p < 0.01$; Figure 1B and 1C).

3.2. IRS1/AKT signaling pathway and AMPK signaling pathway

p-IRS1^{Ser307}/IRS1 and p-AKT^{Ser473}/AKT were upregulated in the liver both of growing (Experiment 1) and adult pigs (Experiment 2) upon moderate PR in basal state ($p < 0.05$; **Figure 2**A and 2B). Similarly, p-IRS1^{Ser307}/IRS1 was upregulated in the skeletal muscle of growing pigs ($p < 0.05$), while no change was observed in the skeletal muscle of adult pigs (Figure 2C and 2D). Notably, p-AKT^{Ser473}/AKT was upregulated in the skeletal muscle of both growing and adult pigs upon moderate PR ($p < 0.05$ or $p < 0.01$). Meanwhile, p-AMPKα^{Thr172}/AMPKα was downregulated in the liver of both growing ($p < 0.01$) and adult pigs ($p < 0.05$) (Figure 2E and 2F).

3.3. Alteration of Transcriptomic Profile in the Liver upon Moderate PR

RNA-seq result revealed a total of 23 348 transcripts expressed in the liver of pigs. A total of 1319 differentially expressed transcripts were identified, and among them, 652 transcripts were upregulated and 667 transcripts were downregulated upon moderate PR. Eight KEGG pathways were enriched ($Q < 0.05$) based on 652 upregulated transcripts, among which focal adhesion, PI3K-AKT signaling pathway, thyroid hormone signaling pathway, circadian rhythm, and protein processing in endoplasmic reticulum were closely associated with insulin signaling (**Figure 3**A and Table S3, Supporting Information).

While, 667 downregulated transcripts were clustered into 21 pathways ($Q < 0.05$), involving energy metabolism pathway (oxidative phosphorylation), amino acid metabolism pathways (glycine, serine, and threonine metabolism, glutathione metabolism, valine, leucine and isoleucine biosynthesis, and biosynthesis of amino acids), protein processing in endoplasmic reticulum, glyoxylate, and dicarboxylate metabolism, and lipid metabolism pathway (glyceriolipid metabolism). Interestingly, an endocrine system pathway (PPAR signaling pathway) was also enriched ($Q = 0.051$; Figure 3A and Table S4, Supporting Information).

We delineated the relationship among four insulin signaling related pathways (PI3K-AKT signaling pathway, AMPK signaling pathway, insulin resistance, and insulin signaling pathway) in the liver with differentially expressed genes (DEGs) upon moderate PR, and found that most DEGs involved in the four pathways were upregulated (Figure 3B). Deduced schematic relationships among the pathways were displayed in Figure 3C. Interestingly, key DEGs including *INSR*, *PCK1*, and *PIK3R1* were identified as hubs connecting these four pathways. *INSR* and *PCK1* were

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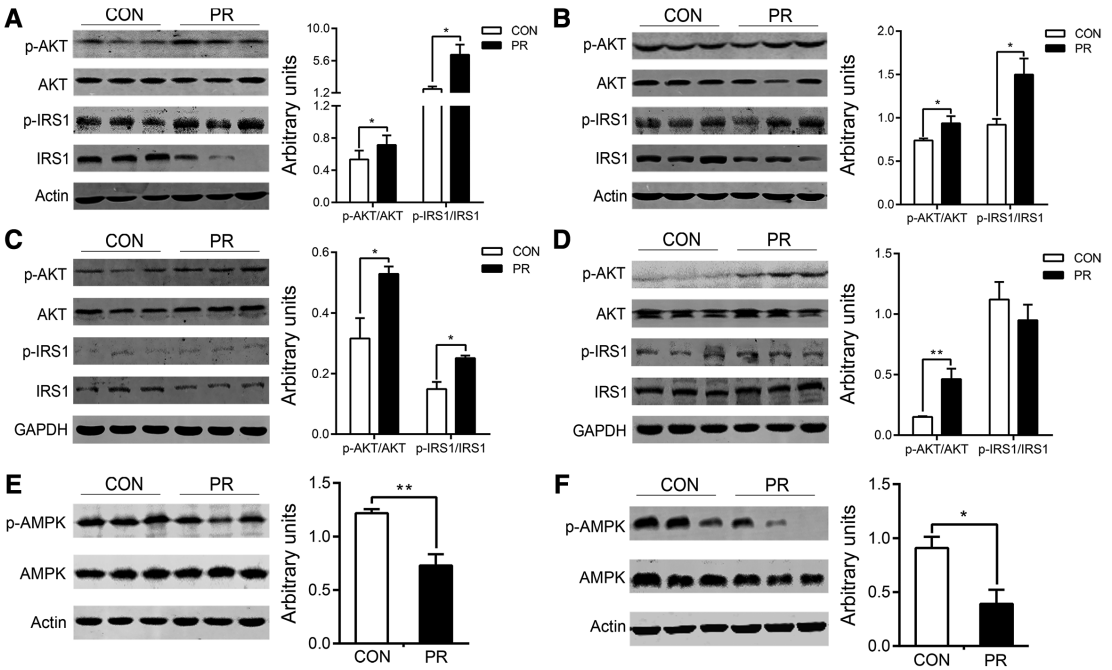

Figure 2. Modified IRS1/AKT signaling in the liver and skeletal muscle and repressed AMPK signaling in the liver in response to moderate protein restriction. Changes in p-AKT^{Ser473} and p-IRS1^{Ser307} in the liver and skeletal muscle of growing pigs (Experiment 1) (A and C) and adult pigs (Experiment 2) (B and D). In addition, changes in p-AMPKα^{Thr172} in the liver of growing pigs (E) and adult pigs (F) were also observed upon moderate PR. Data are expressed as mean ± SEM ($n = 5$–6). *Value significantly different from corresponding value in the control group (CON, $p < 0.05$). **Value significantly different from corresponding value in CON ($p < 0.01$).

increased while *PIK3R1* was decreased in response to moderate PR. Similarly, *CREB3L2* and *TSC1*, as hubs connecting the PI3K-AKT pathway, AMPK signaling pathway, insulin resistance, or insulin signaling pathway, were increased upon moderate PR. We also observed a few DEGs, including *FASN*, *ACACA*, *FBP2*, *SOS1*, *MAP2K2*, *FOXO3*, and *MAPK9*, involved in the crosstalk between two of the insulin signaling related pathways upon moderate PR.

Particularly, both *Prkca* and *ITGAV*, acted as hubs among focal adhesion, PI3K-AKT signaling pathway, and thyroid hormone signaling pathway, were upregulated. Meanwhile, MAPK9 also connected focal adhesion and protein processing in endoplasmic reticulum (Figure S1, Supporting Information). Above observations deciphered the impact of moderate PR on hepatic metabolism in a panoramic view.

3.4. Metabolic Footprint of Portal Venous Plasma upon Moderate PR

A total of 18 different compounds based on the criteria of $p < 0.05$ and FC ≥ 1.5 or ≤ 0.67 were identified in portal venous plasma at serial pre- and postprandial span, which could be clustered into protein metabolism and lipid metabolism (**Table 1**).

At 0.5 h preprandial, the concentration of oligopeptide Arg-Val-Ile-Lys was decreased (FC = 0.50), while Gly-Gly-Asp-His was increased (FC = 1.74) upon moderate PR. Subsequently, the concentration of L-methionine (FC = 2.07), leucy-leucyl-norleucine (FC = 1.58), and pantothenic acid (FC = 1.53) at 0.5 h postprandial were increased compared with CON. The concentration of D-pipecolic acid at 1.5 h postprandial was increased (FC = 1.53) and Ser-Glu-Phe-Ala was decreased (FC = 0.61) upon moderate PR. Pro-Asp-Ile was decreased (FC = 0.55) as well at 7.5 h postprandial.

Most lipid metabolites involved in fatty acid biosynthesis, sphingolipid metabolism, sphingolipid signaling pathway, and steroid hormone biosynthesis, were negatively mediated by moderate PR. Notably, the concentration of sphingosine-1-phosphate (S1P) and linolenic acid at 0.5 h preprandial, 12-oxo-octadecanoic acid at 0.5 h postprandial, 3-ketosphingosine, 12-oxo-octadecanoic acid and (4E,8E10E-d18:3) sphingosine at 1.5 h postprandial, and cortisol at 7.5 h postprandial were reduced upon moderate PR, while stearoylethanolamine was increased at 0.5 h postprandial.

3.5. Alteration of Proteomic Fingerprint concerning Intestinal Metabolism

In the present study, we exploited jejunal mucosal proteomic data (PXD004069, the ProteomeXchange Consortium), and filtered the proteomic adaptions concerning metabolism in small intestine in response to moderate PR. A total of 482 DEPs with FC > 1.5 or < 0.67 were identified, and among them, 235 DEPs were enriched into 25 pathways ($p < 0.05$, **Figure 4**A). The most common pathway cluster was implicated in metabolism (7/25), such as protein digestion and absorption, carbohydrate digestion and absorption, oxidative phosphorylation, citrate cycle, PPAR signaling pathway, ascorbate and aldarate metabolism, and glutathione metabolism, suggesting that the small intestine underwent a

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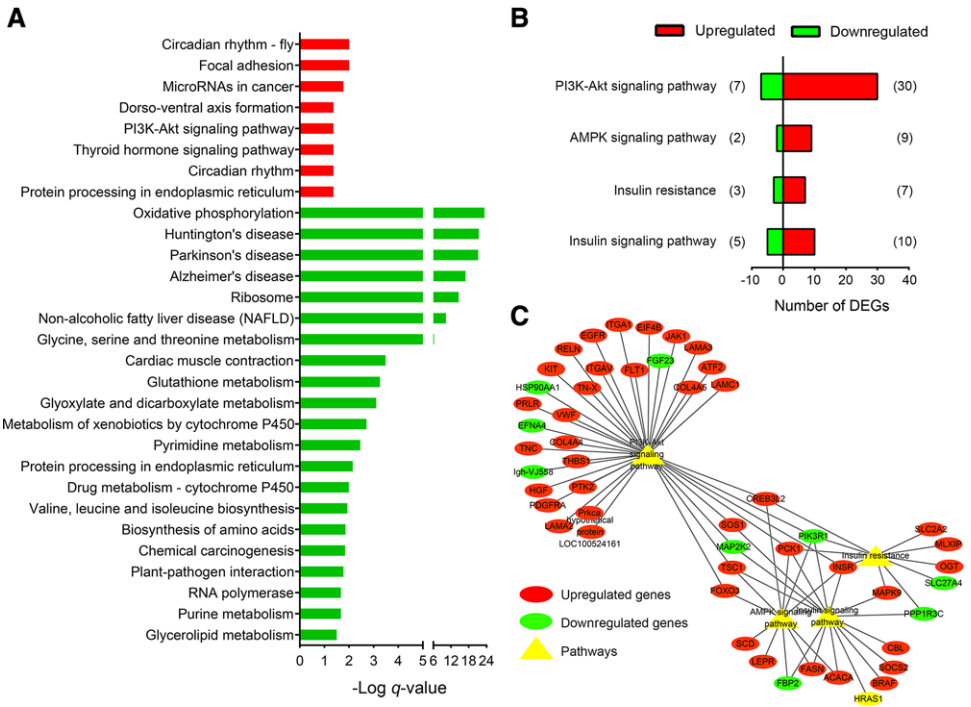

Figure 3. Differentially expressed transcriptomic profile in the liver. A) Upregulated and downregulated KEGG pathways ($Q < 0.05$) respectively based on upregulated and downregulated differentially expressed transcripts. The horizontal line represents –log10 (Q-value) for KEGG pathways. Red represents upregulated pathways, and green represents downregulated pathways upon moderate protein restriction (PR). B) Effects of moderate PR on insulin signaling related pathways based on all differentially expressed genes (DEGs), including insulin resistance, AMPK signaling pathway, insulin signaling pathway and PI3K-AKT signaling pathway. The color of the columns represents gene expression status (red means genes upregulated upon moderate PR, green means downregulated upon moderate PR). C) Transcriptional regulatory network of DEGs in insulin signaling related pathways. Triangles represent KEGG pathways, and ellipses represent genes involved in KEGG pathways. The color of the ellipses represents gene expression status (red means genes upregulated upon moderate PR, green means downregulated in PR). Networks were visualized by Cytoscape (v3.5.1).

profound metabolic remodeling upon moderate PR. In addition, pathways related to diseases (6/25), immune defense (4/25), and cellular functions (4/25) were also observed (Figure 4A).

Most of the DEPs enriched into ribosome and PPAR signaling pathway were decreased, while most of the DEPs involved in citrate cycle, protein digestion and absorption, and focal adhesion, were increased upon moderate PR (Figure 4B). For instance, a total of ten DEPs involved in protein digestion and absorption were increased, while only SLC3A2 was decreased upon moderate PR.

The expression levels of DEPs involved in these pathways were displayed in the heat map generated by hierarchical cluster analysis, and showed a clear difference between CON and PR group (Figure 4C). More details of DEPs were listed in **Table 2**. As for up-regulated proteins, FABP6 (FC = 25.43) and FABP5 (FC = 4.34) were involved in the PPAR signaling pathway, COX1 (FC = 5.35) was enriched in oxidation phosphorylation, and SLC5A1 (FC = 5.11) was involved in carbohydrate digestion and absorption. On the contrary, GK5 (FC = 0.32) and GSTA2 (FC = 0.33) were enriched in the PPAR signaling pathway and glutathione metabolism, respectively. What's more, the succinate dehydrogenase complex (SDHA, SDHC, SDHD), acted as hubs to connect citrate cycle and oxidation phosphorylation, was increased in the jejunal mucosa upon moderate PR. ATP1A1 and ATP1B1, connecting protein/carbohydrate digestion and absorption, were increased as well (Figure 4D).

4. Discussion

PR has been well shown to extend lifespan and ameliorate age-related metabolic diseases, in particular, insulin resistance.[5] Recently, accumulating evidences demonstrated that the restriction of specific individual EAA, such as leucine, methionine, and tryptophan, exerts the beneficial effect of PR to affect the onset of insulin resistance, obesity and other age-related diseases.[7,13,25,26] However, both extreme PR and specific individual EAA restriction could rapidly result in malnutrition.[27] Therefore, our study focused on the mediation of moderate PR on insulin sensitivity and corresponding gut–liver metabolic adaption.

In the present study, we observed that plasma insulin level and HOMA-IR were reduced, and glucose concentration tended to be lowered upon moderate PR, which demonstrated that insulin sensitivity was improved upon moderate PR. Furthermore, diabetic dyslipidemia, characterized by the increased triglycerides and reduced HDL-C, is frequent among patients with T2D.[28] In the present study, plasma concentration of TG, LDL, and VLDL remained unchanged, while the concentration of TC was decreased upon moderate PR. Decreased TC concentration is well believed as a positive sign in improvement of insulin sensitivity.[29,30] However, reduced concentration of HDL observed in the present study was beyond our knowledge, its biological meaning need further study.

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Table 1. Different metabolites in the portal venous plasma from growing pigs[a] in response to moderate protein restriction.

Metabolites	Formula	Theoretical exact *m/z*	Mean measured *m/z*	Retention Time (min)	Fold change[b] (PR/CON[c])	Time (h)[d]	Related pathway
Gly-Gly-Asp-His	$C_{14}H_{20}N_6O_7$	385.1466	385.1461	3.02	1.74	–0.5	Protein metabolism
Indolelactic acid	$C_{11}H_{11}NO_3$	206.0812	206.0809	2.58	0.30	–0.5	Tryptophan metabolism
Arg-Val-Ile-Lys	$C_{23}H_{46}N_8O_5$	515.3664	515.3663	5.64	0.50	–0.5	Protein metabolism
Sphingosine-1-phosphate	$C_{18}H_{38}NO_5P$	380.2560	380.2552	4.72	0.51	–0.5	Sphingolipid signaling pathway
Linolenic Acid	$C_{18}H_{30}O_2$	279.2319	279.2312	8.28	0.56	–0.5	Biosynthesis of unsaturated fatty acids
L-Methionine	$C_5H_{11}NO_2S$	150.0583	150.0582	1.15	2.07	0.5	Protein digestion and absorption
Stearoylethanolamide	$C_{20}H_{41}NO_2$	328.3210	328.3203	9.91	1.80	0.5	Downregulation the expression of SCD-1
Leucyl-leucyl-norleucine	$C_{18}H_{35}N_3O_4$	358.2700	358.2706	9.16	1.58	0.5	Protein digestion or catabolism
Pantothenic Acid	$C_9H_{17}NO_5$	220.1179	220.1175	1.58	1.53	0.5	Metabolism and synthesis of carbohydrate, protein, and fat
12-oxo-octadecanoic acid	$C_{18}H_{34}O_3$	299.2581	299.2575	7.20	0.45	0.5	Fatty acid biosynthesis
Deoxyuridine triphosphate (dUTP)	$C_9H_{15}N_2O_{14}P_3$	468.9809	468.9824	0.77	1.68	1.5	Pyrimidine metabolism
D-Pipecolic acid	$C_6H_{11}NO_2$	130.0863	130.0864	0.87	1.53	1.5	Protein synthesis, amino acid biosynthesis
(4E,8E,10E-d18:3)sphingosine	$C_{18}H_{33}NO_2$	296.2584	296.2576	5.58	0.51	1.5	Sphingolipid signaling pathway
3-Ketosphingosine	$C_{18}H_{35}NO_2$	298.2741	298.2733	6.58	0.54	1.5	Sphingolipid metabolism
12-Oxo-octadecanoic acid	$C_{18}H_{34}O_3$	299.2581	299.2574	7.21	0.55	1.5	Fatty acid biosynthesis
Ser-Glu-Phe-Ala	$C_{20}H_{28}N_4O_8$	453.1980	453.1987	3.38	0.61	1.5	Protein metabolism
Cortisol	$C_{21}H_{30}O_5$	363.2166	363.2157	3.04	0.48	7.5	Steroid hormone biosynthesis
Pro-Asp-Ile	$C_{15}H_{25}N_3O_6$	344.1816	344.1810	2.21	0.55	7.5	Protein metabolism

[a] Pigs from Experiment 3; [b] Fold change, which was based on the normalized data, was calculated by a specific metabolite upon moderate protein restriction divided by that of control; [c] CON, control group; PR, protein restriction; [d] Time was the point that blood samples were obtained from the portal vein before or after feeding. A negative number indicated that blood samples were obtained before feeding, whereas the opposite was indicated by a positive number.

IRS1/PI3K/AKT pathway is a cascade central signaling via which insulin exerts its fundamental role to maintain glucose homeostasis in body.[31,32] In previous study, insulin sensitivity was improved and the phosphorylated IRS1 Ser307 and AKT were also significantly enhanced in the basal state in knockout pigs of $Mstn^{-/-}$,[33] which strongly demonstrated that enhanced phosphorylation of AKT and IRS1 in the basal state was parallel to the increased insulin sensitivity in pigs. In addition, Ser307 of IRS1 in mice is a positive regulatory site that mediates the severity of insulin resistance by maintaining proximal insulin signaling in knock-in mice in which Ser307 was replaced with alanine.[34] In present study, IRS1/PI3K/AKT pathway was significantly stimulated in the liver and skeletal muscle of both growing and adult pigs as the phosphorylation of $IRS1^{Ser307}$ and AKT^{Ser473} was enhanced in the basal state upon moderate PR. What's more, insulin inhibits AMPK activity through the activation of AKT.[35,36] Consistently, the AMPK pathway was repressed in the liver of both growing and adult pigs, which confirmed that insulin sensitivity was improved. To our knowledge, it is the first report that moderate PR could improve insulin sensitivity from childhood to adulthood in the pig model. Considering malnutrition questions resulted from long-term PR or EAA restriction, nonessential AA (NEAA) restriction may be a more feasible dietary intervention for long-term.

In addition, the shift of hepatic transcriptomic profile was also in favor of insulin sensitivity. Besides PI3K-AKT signaling pathway, focal adhesion, circadian rhythm, thyroid hormone signaling pathway, and protein processing in endoplasmic reticulum were enriched in response to moderate PR based on the upregulated transcripts, which had been implicated in insulin signaling mediation.[37–40] Furthermore, we delineated the relationship among four insulin signaling related pathways in the liver using DEGs, and found it shifted to be propitious to insulin activity upon moderate PR. Particularly, the DEGs, acted as hubs of these pathways, should be paid more attention to reveal the mechanism involved in the improved insulin sensitivity by moderate PR. In detail, besides *INSR*, *PIK3R1* had been well demonstrated to mediate insulin signaling and glucose homeostasis.[41] *TSC1* is required for the improvement of hepatic insulin sensitivity upon PR,[12] and exerts its role through the activation of mTORC1.[42] In the present study, *TSC1* was upregulated in the liver upon moderate PR. Additionally, the key DEGs, such as *CREB3L2*, *SOS1*, *MAP2K2*, and *MAPK9*, were identified as hubs among insulin resistance/sensitivity related pathways, and most of their adaptions were consistent with the corresponding roles in mediation of insulin signaling reported in previous studies.[43–46] *PCK1*, however, was upregulated upon moderate PR, which was inconsistent with that observed in db/db mice.[47] This paradox may be due to the different role of *PCK1* in insulin signaling between normal and obese animals. Notably, these DEGs may represent promising candidates for understanding the mechanism underlying the improved insulin sensitivity in response to moderate PR.

Understanding alterations in metabolic footprint of portal vein can provide a better insight into metabolic responses in bodies. Several of different metabolites were identified in portal venous

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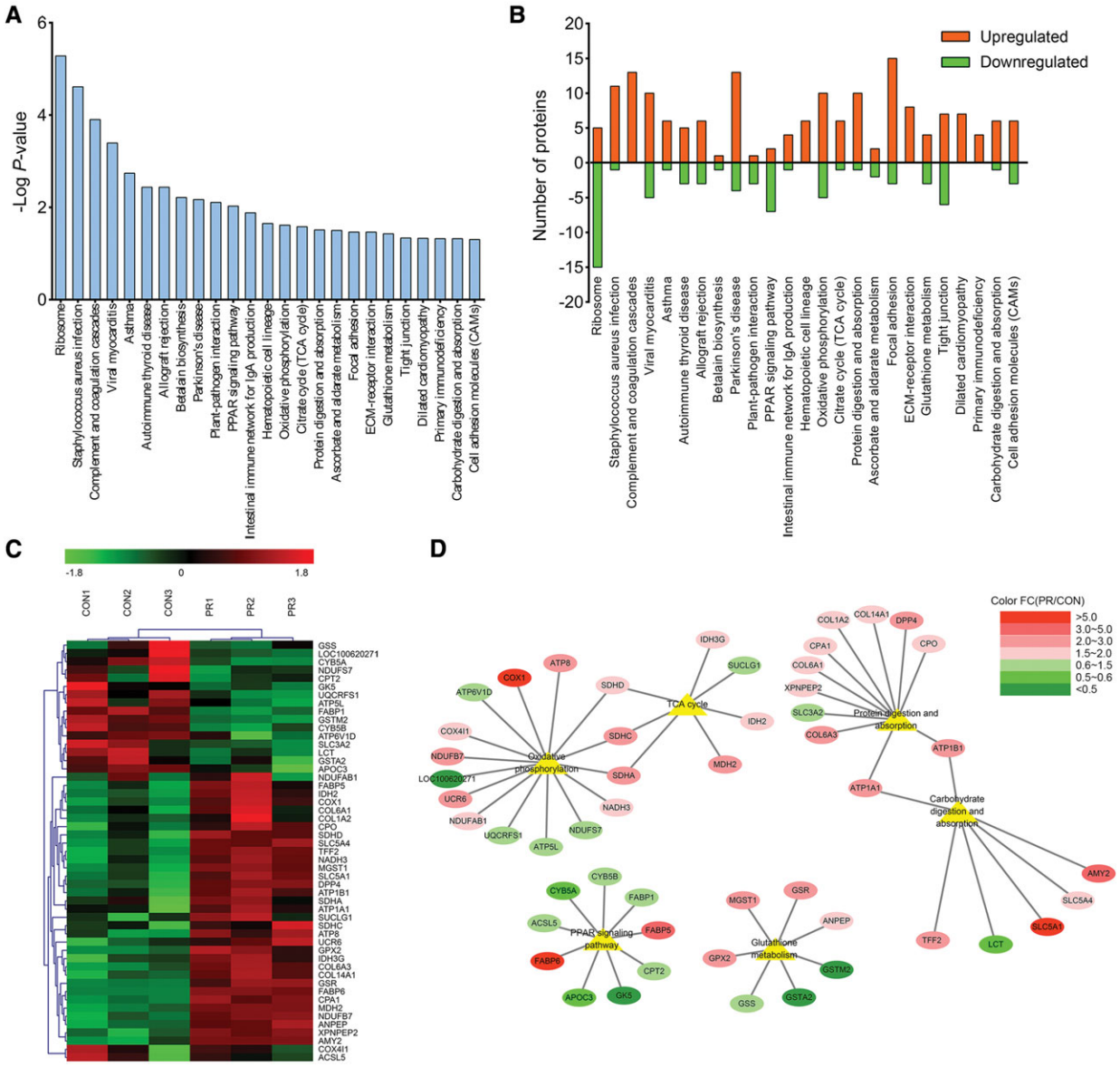

Figure 4. Bioinformatics of differentially expressed proteins identified in the jejunal mucosa (Experiment 1). A) Cluster of KEGG pathways of all differentially expressed proteins (DEPs). The vertical line represents –log10 (*p*-value) for KEGG pathways. B) Effect of moderate protein restriction (PR) on DEPs within each pathway category based on the KEGG analysis. C,D) DEPs related to metabolism were clustered on the heatmap by the normalized expression abundance (C) and networks were visualized by Cytoscape (v3.5.1) (D). FC, fold-change; CON, control group.

plasma upon moderate PR, including numbers of protein/AA metabolites, fatty acid metabolites, and sphingolipid metabolites, as well as pantothenic acid. Among them, elevated plasma S1P has been established as a feature of both human and rodent obesity and correlates with insulin resistance,[48] and it seems to interrupt insulin signaling via promoting beta cell dysfunction.[49] In the present study, S1P was decreased at 0.5 h preprandial upon moderate PR. Moreover, cortisol, which has been shown to contribute to the reduction of insulin sensitivity at early age in Latino children and adolescents,[50] was also observed to decrease at 7.5 h postprandial upon moderate PR. Besides S1P and cortisol, the concentration of oligopeptides, such as Arg-Val-Ile-Lys, Gly-Gly-Asp-His, Ser-Glu-Phe-Ala, and Pro-Asp-Ile, was also varied. Gly-Gly-Leu had been shown to improve glucose homeostasis in both normal and T2D mouse models.[51] Thus, we deduced that the varied concentration of oligopeptides may serve as monitors of insulin sensitivity, although the roles of these oligopeptides need further study. Particularly, pantothenic acid is an important regulator of glucose metabolism and involved in the reduction of insulin resistance in obesity model.[52] Therefore, pantothenic acid, whose concentration was increased in portal venous plasma upon moderate PR, may be served as an adjuvant therapy to diminish the burden of insulin resistance.

Alteration of metabolic footprint in portal venous plasma reflects the shift of both nutrient absorption and resulting

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Table 2. Changed metabolism pathways and relevant differentially expressed proteins in the jejunal mucosa of growing pigs[a)] in response to moderate protein restriction.

UniProtKB accession number	Protein name	Gene name	Fold change (PR/CON[b)])
Protein digestion and absorption			
I3LUR7_PIG	Collagen alpha-3(VI) chain	COL6A3	2.53
AT1B1_PIG	Sodium/potassium-transporting ATPase subunit beta-1	ATP1B1	2.44
I7HD36_PIG	Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	2.37
DPP4_PIG	Dipeptidyl peptidase 4	DPP4	2.25
CBPA1_PIG	Carboxypeptidase A1	CPA1	1.92
I3LS72_PIG	Collagen alpha-1(VI) chain	COL6A1	1.90
XPP2_PIG	Xaa-Pro aminopeptidase 2	XPNPEP2	1.67
F1SHC8_PIG	Carboxypeptidase O	CPO	1.64
F1SFA7_PIG	Collagen alpha-2(I) chain (Alpha-2 type I collagen)	COL1A2	1.61
K7GT00_PIG	COL14A1 protein (Collagen alpha-1(XIV) chain)	COL14A1	1.56
I3LB80_PIG	SLC3A2 protein	SLC3A2	0.65
Carbohydrate digestion and absorption			
F1RLV1_PIG	Sodium/glucose cotransporter 1	SLC5A1	5.11
I3LAV8_PIG	Alpha-amylase	AMY2	3.18
I3LMK2_PIG	Trefoil factor 2	TFF2	2.90
AT1B1_PIG	Sodium/potassium-transporting ATPase subunit beta-1	ATP1B1	2.44
I7HD36_PIG	Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	2.37
SC5A4_PIG	Low affinity sodium-glucose cotransporter	SLC5A4	1.74
I3L7V1_PIG	Lactase	LCT	0.58
Citrate cycle (TCA cycle)			
C560_PIG	Succinate dehydrogenase cytochrome b560 subunit, mitochondrial	SDHC	2.31
SDHA_PIG	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDHA	2.10
I3LP41_PIG	Malate dehydrogenase	MDH2	2.00
IDHP_PIG	Isocitrate dehydrogenase [NADP], mitochondrial (Fragment)	IDH2	1.83
F1S297_PIG	Isocitrate dehydrogenase [NAD] subunit, mitochondrial	IDH3G	1.78
F1SMA9_PIG	Succinate dehydrogenase [ubiquinone] cytochrome b small subunit	SDHD	1.54
SUCA_PIG	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	SUCLG1	0.62
Oxidative phosphorylation			
B5KN74_PIG	Cytochrome c oxidase subunit 1(Complex 4)	COX1	5.35
F1S3W0_PIG	Cytochrome b-c1 complex subunit 6 (Complex 3)	UCR6	2.62
Q2NNM9_PIG	ATP synthase protein 8(Complex 5)	ATP8	2.42
C560_PIG	Succinate dehydrogenase cytochrome b560 subunit, mitochondrial	SDHC	2.31
F1SCH1_PIG	NADH dehydrogenase 1 beta subcomplex subunit 7	NDUFB7	2.28
SDHA_PIG	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	SDHA	2.10
Q9G7R9_PIG	NADH-ubiquinone oxidoreductase chain 3 (Complex 1)	NADH3	1.93
I3LER5_PIG	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	COX4I1	1.70
F1SMA9_PIG	Succinate dehydrogenase [ubiquinone] cytochrome b small subunit,mitochondrial	SDHD	1.54
F1SAB6_PIG	Acyl carrier protein (Complex 1)	NDUFAB1	1.51
F1RPD4_PIG	Uncharacterized protein	LOC100620271	0.46
I3LK43_PIG	NADH: ubiquinone oxidoreductase core subunit S7	NDUFS7	0.60
F1RNZ1_PIG	Cytochrome b-c1 complex subunit Rieske, mitochondrial(Complex 3)	UQCRFS1	0.62
F1SA40_PIG	ATPase H^+ transporting V1 subunit D	ATP6V1D	0.63
F1SAK6_PIG	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit G	ATP5L	0.65
Glutathione metabolism			
MGST1_PIG	Microsomal glutathione S-transferase 1	MGST1	2.81
F1RX66_PIG	Glutathione-disulfide reductase	GSR	2.28
Q09HS4_PIG	Glutathione peroxidase	GPX2	2.16

(*Continued*)

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Table 2. Continued.

UniProtKB accession number	Protein name	Gene name	Fold change (PR/CON[b])
F1SK03_PIG	Aminopeptidase N	ANPEP	1.81
Q29057_PIG	Glutathione S-transferase A2	GSTA2	0.33
Q000H9_PIG	Glutathione S-transferase mu 2	GSTM2	0.42
F1S4 × 9_PIG	Glutathione synthetase	GSS	0.61
PPAR signaling pathway			
F1RR40_PIG	Gastrotropin (Fragment)	FABP6	24.53
Q2EN74_PIG	Epidermal fatty acid-binding protein	FABP5	4.34
F1SKG5_PIG	Glycerol kinase 5	GK5	0.32
APOC3_PIG	Apolipoprotein C-III	APOC3	0.51
CYB5_PIG	Cytochrome b5	CYB5A	0.57
I3LCL6_PIG	Acyl-CoA synthetase 5	ACSL5	0.61
FABPL_PIG	Fatty acid-binding protein, liver	FABP1	0.63
D5LIE7_PIG	Mitochondrial carnitine palmitoyltransferase II	CPT2	0.63
F1S393_PIG	Cytochrome b5 type B	CYB5B	0.64

[a] Growing pigs from Experiment 1; [b] CON, control group; PR, protein restriction.

metabolism occurred in the small intestine. Therefore, we traced back to jejunal mucosal proteomic fingerprint and focused on adaption regarding regulation of metabolism. Enriched pathways protein/carbohydrate digestion and absorption were enhanced in the intestinal mucosa upon moderate PR. Furthermore, energy metabolism pathways including citrate cycle and oxidative phosphorylation were also strengthened. Succinate accumulation acts as a metabolic signaling to link endoplasmic reticulum stress, inflammation, and cAMP/PKA activation, contributing to insulin resistance.[53] In the present study, enriched pathways citrate cycle and oxidative phosphorylation were connected by three increased succinate dehydrogenase components (SDHA, SDHC and SDHD). In addition, FABP5 and FABP6 involved in the repressed PPAR signaling pathway were upregulated upon moderate PR. Both of them had been implicated in the diet-induced insulin resistance.[54,55] Additionally, glutathione metabolism and ascorbate metabolism were also altered upon moderate PR.

Insulin mediates not only glucose utilization but also lipid/protein metabolism in the liver, adipose and muscle tissues.[56] Herein, we characterized that pathways including protein/AA metabolism, energy metabolism, carbohydrate metabolism, and lipid metabolism, along with the involvement of key pathways regulating insulin signaling, were synergistically adapted to moderate PR in the small intestine and liver, and gut–liver metabolism underwent significant modifications upon moderate PR in favor of insulin sensitivity.

Therefore, we provided a panorama of synergetic metabolic adaption from jejunal mucosal proteomic fingerprint to the metabolic footprint in the portal venous plasma and to hepatic transcriptomic fingerprint, which should contribute to the beneficial effect of moderate PR on insulin sensitivity. That is, integrated alterations of protein/AA metabolism and correlative metabolism in the small intestine and liver that triggered the transformation of insulin signaling related pathways via the key metabolites play a vital role in regulation of insulin signaling. What's more, from the comprehensive analysis of multi-omics data together with the results of western blotting and insulin sensitivity indexes, we deduced that the beneficial effect of moderate PR on insulin signaling mainly depended on the quality of absorbable AA even rather so called NEAA. It would help to understand the contribution of AA to the beneficial effect of PR on the improvement of insulin sensitivity, and further facilitate extending of the beneficial effect for long term.

In conclusion, our study has evidenced a novel finding in vivo that moderate PR, or rather NEAA restriction, could potently improve insulin sensitivity from childhood to adulthood in the pig model. It would help to understand the beneficial effect of PR on insulin sensitivity.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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J.Y. obtained financial support, designed and oversaw this study. X.Z. and K.Q. performed the animal experiment and obtained the tissue samples. X.Z. and D.X. conducted the plasma non-targeted metabolic footprint analysis, western blotting, and other analysis. X.Z. and L.W. conducted bioinformatics analyses of transcriptomics and proteomics. J.Y. and X.Z. wrote the manuscript with the contribution from the other authors. All authors read and approved the final manuscript. J.Y. is the guarantor of this work.

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Conflict of Interest

The authors declare no conflict of interest.

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