Original Article Oxidative stress contributes to abnormal glucose metabolism and insulin sensitivity in two hyperlipidemia models

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Abstract: Objective: Lipid metabolism disturbance can result in insulin resistance and glucose intolerance; however, the features of glucose metabolism are still elusive in different dyslipidemia. Our study intended to explore the characteristics and molecular mechanisms of glucose metabolism abnormal in hypercholesterolemia and hypertriglyceridemia models. Methods: Two mouse models were used in this study, one was lipoprotein lipase gene-deleted (LPL+/-) mice, and the other was high fat dietary (HFD) mice. Levels of total cholesterol (TC), triglyceride (TG), highdensity lipoprotein-cholesterin (HDL-c) and low-density lipoprotein-cholesterin (LDL-c) in serum were measured by full-automatic biochemical analyzer. Intraperitoneal glucose tolerance test (IPGTT) was performed to evaluate insulin sensitivity and β-cell function. Malondialdehyde (MDA) and total superoxide dismutase (T-SOD) levels in serum were measured by colorimetric determination. mRNA expression of superoxide dismutase 1 (SOD1), catalase (CAT), glutathione peroxidase 1 (Gpx1), nuclear factor erythroid 2-related factor 2 (Nrf2a) and peroxisome proliferatoractivated receptor-gamma coactivator 1 alpha (PGC-1a) in liver, skeletal muscle, visceral fat and subcutaneous fat were measured by Real-Time PCR. Results: Compared with HFD mice, the levels of serum TG were significantly higher in LPL^{+/-} mice, whereas the levels of TC, HDL-c, LDL-c were significantly lower. The plasma glucose levels were increased at each time point of intra-peritoneal glucose tolerance test (IPGTT) in both groups. Furthermore, the level of serum fasting insulin and homeostasis model assessment index-insulin resistance (HOMA-IR) increased with a decreased ISI in both groups. In addition, the plasma MDA of HFD group was higher than that of lipoprotein lipasedeficiency (LPL^{+/-}) group, while the activity of T-SOD in HFD group was lower than that in LPL^{+/-} group. Real-Time PCR revealed that the expressions of SOD1, CAT and Gpx1 in liver and subcutaneous fat were lower in HFD group than those in LPL^{+/-} group, but higher in skeletal muscle and visceral fat. Conclusions: There are different in glucose metabolism between high TG mice and high TC mice. Impaired insulin sensitivity is more serious in HFD mice than that in LPL^{+/-} mice. Oxidative stress could contribute to insulin resistance in hyperlipidemia mice.

Keywords: Lipotoxicity, hypertriglyceridemia, hypercholesterolemia, oxidative stress

Introduction

Hyperlipidemia is one of the most common diseases, which is a risk factor for cardiovascular diseases and type 2 diabetes mellitus [1-3]. T2DM is characterized by two different mechanisms: insulin resistance and β -cell dysfunction, both of which are associated with hyperlipidemia [4, 5]. Hyperlipidemia can be divided into three types, hypercholesterolemia, hypertriglyceridemia and their combination. Hypercholesterolemia is the presence of high levels of cholesterol in the blood [6]. Recent evidences suggested that alterations of plasma cholesterol levels may contribute to impaired insulin secretion and insulin sensitivity [7, 8]. Some studies have indicated that high triglyceride level is a risk factor for the development of T2DM and individuals with hypertriglyceridemia exhibited more serious insulin resistance than individuals with normal triglyceride level [9, 10]. Although mounting evidence showed hyperlipid-

Gene name	Forward Primer (5'-3')	Reverse Primer (5'-3')
Nrf2	CACTGACAGAAATGGACAGCA	CACTGGGCTCTGCTATGAAAG
GPx1	GGACTACACCGAGATGAACGA	GAGCCTTCTCACCATTCACTTC
SOD1	GTACCAGTGCAGGACCTCATTT	TTTCTCATGGACCACCATTGTA
CAT	AGTCTTCGTCCCGAGTCTCTC	CTGGTCGGTCTTGTAATGGAA
PGC1a	ATGAAGCCTATGAGCACGAAA	CAAAGCGGTCTCTCAATTCTG
GAPDH	TCTCCTGCGACTTCAACA	TGGTCCAGGGTTTCTTACT

Table 1. Primers of real time PCR

Note: Nrf2, nuclear factor erythroid 2-related factor 2; Gpx1, glutathione peroxidase 1; SOD1, superoxide dismutase 1; CAT, Catalase; PGC-1α, Peroxisome proliferatoractivated receptor-gamma coactivator 1 alpha; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase.

emia is closely related to glucose metabolism and insulin sensitivity; however, the precise molecular mechanisms are still elusive.

Reactive oxygen molecules have been typically viewed as the toxic by-products of metabolism. Chronic and increased production of these reactive molecules or a reduced capacity for their elimination, termed as oxidative stress, could lead to abnormal changes in intracellular signaling and result in insulin resistance [11, 12]; but few studies focused on the levels of reactive oxygen molecules in dyslipidemia models.

Our study intended to explore characteristics of glucose metabolism and tissue-targeted insulin sensitivity in two different hyperlipidemia models and to probe the possible mechanisms of oxidative stress in this process.

Materials and methods

Animal model

8-weeks-old lipoprotein lipase (LPL)+/- female mice (n = 8) and age-matched wild-type littermate mice (n = 16) were purchased from Peking university institute of cardiovascular sciences. All of them were housed at laboratory animal center of the secondary military medical university with the controlled temperature (about 23°C), a 12:12 hours lighting (dark photoperiod) and free access to water and food for 8 weeks. The wild-type mice were randomly assigned to two groups, one was fed with standard normal diet (control group, n = 8), and the other was fed with high-fat diet (HFD group, n = 8). Meanwhile, LPL^{+/-} mice were fed with standard normal diet (LPL^{+/-} group, n = 8). The standard normal diet included carbohydrate (63%), fat (12.5%), and protein (24.6%); while high fat diet included carbohydrate (33.8%), fat (45%), protein (19.4%), and cholesterol (1%). The research was conducted in conformity with PHS policy and the studies were approved by the laboratory animal center of the secondary military medical university.

Analysis of plasma lipid profile

Blood was collected from the jugular vein and the plasma

was stored at -80°C. Plasma lipid profiles, which included total cholesterol (TC), triglyceride (TG), high-density lipoprotein-cholesterol (HDL-c) and low-density lipoprotein-cholesterol (LDL-c), were measured by full-automatic biochemistry analyzer (Hitachi 7020, Japan).

Glucose tolerance tests and insulin assay

Glucose tolerance tests were performed after 12 h fasting periods. Mice were injected intraperitoneally with glucose (1 g/kg body weight), and blood glucose levels were monitored at the time of 0 (baseline), 5, 15, 30, 60, 120 min after glucose injection. The concentration of Insulin was measured with a Mouse Insulin Enzyme-linked immunoassay (ELISA) kits (Shibayagi, Japan). Homeostasis model assessment (HOMA) index was calculated with the formula: HOMA-insulin resistance (IR) = fastinginsulin concentration × fasting glucose concentration/22.5, while the Insulin sensitivity index (ISI) was calculated with the formula: ISI = In[1/(fasting insulin concentration × fasting glucose concentration)]. Serum malondialdehyde (MDA) and total superoxide dismutase (T-SOD) level were measured with an enzymatic colorimetric assay.

Quantitative real-time PCR

Tissues (liver, skeletal muscle, visceral adipose tissue, subcutaneous adipose tissue) were excised from three kinds of mice and were homogenized in Trizol reagent (Life Technologies, USA). Total RNA was isolated and then converted into cDNA using cDNA Reverse Transcription Kits (TaKaRa, Japan). Gene expression was tested by real-time PCR (**Table 1**).

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Figure 1. Establishment of two mouse models with hyperlipidemia and different metabolic characteristics. A. Body weight; B. The levels of TG; C. The levels of TC; D. The levels of HDL-c; E. The levels of LDL-c; F. The plasma glucose levels at each point time of IPGTT; G. The levels of serum fasting insulin; H. The levels of HOMA-IR. Note: TG, triglyceride; TC, total cholesterol; HDL-c, high-density lipoprotein-cholesterol; LDL-c, low-density lipoprotein-cholesterol; HOMA, homeostasis model assessment index; IR, insulin resistance; LPL^{+/-}, lipoprotein lipase-deficiency; HFD, high fat dietary; **P*<0.05.

Statistics

Results of calculations were presented as means \pm SEM. Differences in means between two groups were evaluated with unpaired twotailed Student *t* tests, and those among multiple groups with one-way ANOVA followed by Bonferroni post-hoc tests. All statistics was performed with Statistical Package for Social Sciences (SPSS) software (version 16, SPSS, Inc, Chicago, IL, USA). *P* values of < 0.05 were considered statistically significant.

Results

Mouse models

After two months of feeding, both the body weights of LPL^{+/-} group and HFD group were increased significantly compared with the control group (**Figure 1A**). The level of serum TG in LPL^{+/-} group was significantly higher than that in the HFD group and in control group (**Figure 1B**), while in HFD group, the levels of TC, HDL-c, and LDL-c were significantly higher than those

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Figure 2. Expression of oxidative stress indicators. A. The serum levels of MDA; B. The activity of T-SOD; C. In liver tissue, relative mRNA expressions of SOD1, CAT, Gpx1; D. In muscle skeletal tissue, relative mRNA expressions of SOD1, CAT, Gpx1; E. In visceral fat, relative mRNA expressions of SOD1, CAT, Gpx1; F. In subcutaneous fat, relative mRNA expressions of SOD1, CAT, Gpx1; F. In subcutaneous fat, relative mRNA expressions of SOD1, CAT, Gpx1; G, H. The expression of Nrf2 and PGC-1 α in liver, skeletal, visceral fat and subcutaneous fat. Note: MDA, malondialdehyde; T-SOD, total superoxide dismutase; SOD1, superoxide dismutase 1; CAT, Catalase; Gpx1, glutathione peroxidase 1; Nrf2, nuclear factor erythroid 2-related factor 2; PGC-1 α , Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; LPL^{+/-}, lipoprotein lipase-deficiency; HFD, high fat dietary; **P*<0.05.

in LPL^{+/-} and control group, which suggested that we successfully established hypertriglyceridemia and hypercholesterolemia models (**Figure 1C-E**).

Levels of glucose metabolism in different groups

Compared with control group, the plasma glucose levels were rising at each time point of Intraperitoneal glucose tolerance test (IPGTT) in LPL^{+/-} group, but no significant difference was showed; in HFD group, the plasma glucose levels had significant increase when compared with the control. Accordingly, it could be concluded that there was more obvious increase in the plasma glucose level of the HFD group than that of LPL-deletion-group (**Figure 1F**).

Serum fasting insulin and HOMA-IR among different groups

The levels of serum fasting insulin and HOMA-IR were increased and the level of ISI was decreased in LPL^{+/-} group compared with the control group. However, there was no significant difference between them (**Figure 1G**). In HFD group, the parameters above showed the same trend and significant differences compared with LPL^{+/-} group and control group (**Figure 1H**).

Concentration of serum MDA and the activity of total SOD

Compared with the control group, the serum level of MDA in LPL^{+/-} group was significantly higher, while with no significant difference of the activity of T-SOD (**Figure 2A**). In HFD group, levels of serum MDA were significant increased, whereas the activities of T-SOD were significant decreased (**Figure 2B**).

Reactive oxygen molecules expression in different tissues in dyslipidemia models

Oxidative stress was assessed with three major anti-oxidases-superoxide dismutase 1 (SOD1), catalase (CAT) and glutathione peroxidase 1 (Gpx1). In liver tissue, the relative mRNA expression of SOD1, CAT, Gpx1 in LPL+/- group were 0.63, 0.64, 0.77 times lower than those in control group but with no significant difference; in HFD group, above parameters were 0.23, 0.33, 0.06 times lower than those in control group and with significant difference (Figure 2C). In muscle skeletal tissue, the expression of CAT in LPL^{+/-} group was 1.05 times higher, while Gpx1 and SOD1 were 0.65, 0.59 times lower than those in control group; in HFD group, the expression of SOD1 and CAT were 0.81 and 1.07 times of those in control group, while Gpx1 was 0.68 times of that in control group with significant difference (Figure 2D). In visceral fat, the expression of CAT, Gpx1 in LPL^{+/-} group were 1.46 and 0.51 times of those in control group, and SOD1 was 0.31 times lower than that in control group; in HFD group, the expression of Gpx1 was 1.45 times of that in the control group, while SOD1 and CAT were 0.43 and 0.53 times lower with significant difference (Figure 2E). In subcutaneous fat, the expressions of SOD1, CAT and Gpx1 were 0.87, 1.61 and 1.00 times of those in control group in LPL^{+/-} group; in addition, above parameters were 0.77, 0.92 and 0.43 times lower in HFD group than those in the control group. However, no significant difference of the three antioxidant enzymes in subcutaneous fat tissue was showed in two mouse models of hyperlipidemia (Figure 2F).

Relative mRNA (Nrf2, PGC1a) expression in different tissues

In liver tissue, relative mRNA expressions of nuclear factor erythroid 2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 α) in LPL^{+/-} group were 0.93 and 0.74 times lower than those in control group; in HFD group, the expression were 1.67, 0.78 times of those in control group. In skeletal muscle, the expressions Nrf2 of and PGC-1 α in LPL^{+/-} group were 1.60, 0.63 times of those in control group, while in HFD group, the two parameters were 1.16, 0.54 times. In visceral fat, the expression of Nrf2 and PGC1a in LPL^{+/-} group were 1.46 and 0.31 times of those in control group; while in HFD group, the two parameters were 0.96 and 0.95 times. In subcutaneous fat, the expressions of Nrf2 and PGC-1a in LPL^{+/-} group were 1.48 and 1.03 times higher than those of control group, while in HFD group, the two parameters were 0.96, 1.18 times (Figure 2G, 2H). Although there was no significant difference of mRNA expression of Nrf2, PGC1a among different groups, it showed that Nrf2 had a rising trend in two hyperlipidemia groups when compared with the control one. At the same time, a decreasing trend of PGC-1 α was exhibited in two hyperlipidemia groups.

Discussion

Dyslipidemia plays a vital role in the pathogenesis of T2DM [7, 8, 13]. It was reported that the incidences of type 2 diabetes in patients with hypercholesterolemia group, hypertriglyceridemia and combined hyperlipidemia were 5.8, 3.9, 7.1 times higher than that in control group respectively after 8-year-follow-up [14], which indicated that different types of lipid profiles have different effects on glucose metabolism.

Hypertriglyceridemia denotes high blood levels of triglycerides, the most abundant fatty molecule in most organisms [15]. A big national study which included 46239 Chinese adult participants had shown that an elevated serum triglyceride level was significantly associated with an increased risk of diabetes [9]. Lipoprotein lipase (LPL) is an important enzyme in hydrolyzing triglyceride. In our study, we used the LPL^{+/-} mice to create the hypertriglyceridemia animal model because LPL-/- mice would die in two days from the birth [16]. According to our experimental data, we found the 16-monthsold LPL+/- mice presented dyslipidemia and insulin resistance, but normal glucose metabolism. As time went on, 28-months-old LPL+/mice had come up with abnormal glucose

metabolism [17]. Therefore, high TG causes insulin resistance and then lead to impaired glucose metabolism consequently.

It is well known that hypercholesterolemia could lead to atherosclerosis [18]. In recent years, the relationship between hypercholesterolemia and type 2 diabetes has been more clearly. Basciano's study in hamsters demonstrated that dietary rich in cholesterol led to severe hepatic steatosis and glucose intolerance, and progressed to diabetes mellitus in the end [19]. In our study, we observed that high-fat diet mice have come up with insulin resistance and obviously impaired glucose metabolism at 16 months when LPL+/- mice had not presented impaired glucose metabolism. Accordingly, high-fat diet-induced hypercholesterolemia could impair glucose metabolism earlier than LPL gene defect-induced inherited hypertriglyceridemia did.

In this study we also measured insulin sensitivity of different tissues in two different hyperlipidemia mice and came up with the results that in LPL^{+/-} mice, the insulin sensitivity in liver, skeletal-muscle and visceral fat were impaired, among which the damage in skeletal-muscle was more serious, but the sensitivity was enhanced in subcutaneous fat; in HFD group, the sensitivity in liver, skeletal-muscle and visceral fat were impaired, but the damage was more serious in liver and visceral fat, no damage was shown in subcutaneous fat. Recent studies showed that the decrease of visceral fat mass and the increase of subcutaneous fat mass could enhance insulin sensitivity [20, 21]. Wang's study [22] showed in 16-months-old LPL^{+/-} mice, the lipid deposition in liver, skeletalmuscle, visceral fat and subcutaneous fat were increased, but only in subcutaneous fat had a significant difference. Since LPL+/- mice had more subcutaneous fat and enhanced insulin sensitivity in subcutaneous fat, it was easier for us to understand why insulin sensitivity in hypertriglyceridemia group was higher than that in hypercholesterolemia group.

The mechanisms of hyperlipidemia resulting in insulin resistance and glucose intolerance are still unclear. In the present study, we found that oxidative stress may contribute to the phenotypes [23, 24]. Reactive oxygen species (ROS), the representatives of oxidative stress, were present in cells and tissues at low yet measurable concentrations. The concentrations of ROS were depending on the balance between the rate of production and clearance, which were associated with various cellular enzymatic and nonenzymatic antioxidant compounds, such as SOD, CAT, and Gpx [12, 25]. MDA, as the production of lipid peroxides, was also the most commonly used index for evaluation of damaged oxidative stress [26, 27]. In our study we also measured the mRNA expression of Nrf2 and PGC-1 α . Generally, the elevation of Nrf2 could promote the gene transcription of anti-oxidases, which might protect cells from oxidative stress damage. PGC-1a could combine with many nuclear receptors and then regulate gene transcription; however, the function of PGC-1 α was weaker than Nrf2.

In this study, we found that oxidative stress was more serious in hypercholesterolemia mice than in hypertriglyceridemia ones. In hypercholesterolemia group, oxidative stress was showed in liver, skeletal-muscle and visceral fat, but it was more serious in liver and visceral fat than that in skeletal-muscle. In hypertriglyceridemia group, oxidative stress was existed in skeletal-muscle and visceral fat, while in skeletal-muscle it was more severe. It is well know that increased concentration of reactive molecules triggered the activation of serine/threonine kinase cascades, such as inhibitor kappa B kinase (IKK), c-Jun amino-terminal kinases (JNK), mammalian target of rapamycine (mTOR) and protein kinase C isoforms (PKC), that in turn, phosphorylate multiple targets, including the insulin receptor and the insulin receptor substrate (IRS) proteins. Increased serine phosphorylation of IRS reduced its ability to undergo tyrosine and might accelerate the degradation of insulin receptor substrate-1 (IRS-1), followed by a reduced protein kinase-B phosphorylation and glucose transporter 4 (GLUT4) translocation to the plasma membrane [28]. In addition, prolonged exposure to ROS affected the transcription of glucose transporters: whereas the level of glucose transporter 1 (GLUT1) was increased and GLUT4 level was reduced [29]. This mechanism might explain the link between oxidative stress and insulin resistance, but further study need to be done. In a word, it could be concluded that oxidative stress occurred in both hypertriglyceridemia and hypercholesterolemia models. Moreover, anti-oxidases may play tissue specific roles in different hyperlipidemia models.

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Disclosure of conflict of interest

None.

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