Pyridostigmine Protects Against Diabetic Cardiomyopathy by Regulating Gut Microbiota and Branched-Chain Amino Acid Catabolism to Attenuate Mitochondria Dysfunction

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Abstract

Background: Recent studies have reported that disruption of gut microbes and their metabolites is associated with diabetic cardiomyopathy, but the mechanism by which gut microbes improve diabetic cardiomyopathy remains unclear.

Method: Male C57BL/6J mice with high-fat diet and streptozotocin-induced diabetic cardiomyopathy were studied in comparison with control littermates. Diabetic mice were either untreated or subjected to daily intragastric of pyridostigmine. After 10 weeks of hyperglycaemia, vagus activity, cardiac function and cardiac structure were measured by heart rate variability assessment, echocardiography, and immunohistochemistry. The intestinal barrier and gut microbiota were evaluated by fluorescence in situ hybridization and high-throughput sequencing. Additionally, plasma and cardiac branched-chain amino acid (BCAA) distribution and cardiac BCAA catabolism were determined. The structure and respiratory function of mitochondria were measured to assess cardiac mitochondria performance.

Results: Intestinal permeability and tight junctions were impaired, bacterial translocation was increased, vagal activity was decreased in mice with diabetic cardiomyopathy mice. Additionally, gut microbes in mice with diabetic cardiomyopathy were disrupted, especially key microbes related to diabetes and BCAA production. Pyridostigmine, which reversibly inhibits cholinesterase to improve autonomic imbalance, enhanced vagus nerve activity, improved insulin resistance and cardiac damage, and alleviated intestinal barrier injury and gut microbiota disruption. Specifically, pyridostigmine decreased the abundance of diabetes-non-protective microbes and increased that of diabetes-protective microbes and BCAA-producing microbes. Pyridostigmine decreased cardiac BCAA concentrations by impairing gut microbe-mediated BCAA production. Furthermore, pyridostigmine upregulated BCAT2 and PP2Cm expression and decreased P-BCKDHA/BCKDHA and BCKDK expression, thus improving cardiac BCAA catabolism. Interestingly, the mitochondrial structural and functional disruption in mice with diabetic cardiomyopathy was attenuated after pyridostigmine administration, which may indicate one of the mechanisms by which BCAAs reduce cardiac damage.

Conclusions: In conclusion, intestinal barrier, gut microbiota and vagal activity were impaired in mice with diabetic cardiomyopathy. Pyridostigmine ameliorated insulin resistance and cardiomyopathy, with an effect related to regulated gut microbes and its metabolite BCAA catabolism to attenuate mitochondria dysfunction of heart. These results provide novel insights for the development of a therapeutic strategy for diabetes-induced cardiac damage that targets gut microbes and BCAA catabolism.

Introduction

An estimated 451 million people (age 18-99 years) worldwide had diabetes in 2017. This number is expected to increase to 693 million by 2045 [1]. Diabetic cardiomyopathy (DCM), one of the main complications of diabetes, is the leading cause of heart failure and death in diabetic patients [2]. Recent studies have revealed that the disruption of gut microbiota homeostasis is closely associated with

diabetes and metabolic syndrome. Diabetic patients and animals show significantly different gut microbiota compositions than their non-diabetic counterparts [3]. Disruption of the gut microbiota has also been linked to cardiovascular conditions, such as coronary heart disease, hypertension, heart failure, ventricular fibrillation, and vascular dysfunction [4, 5]. A series of bacterial metabolites, such as indoles, secondary bile acids, trimethylamine-N-oxide, short-chain fatty acids, and branched-chain amino acids (BCAAs), have been demonstrated to affect host physiologic homeostasis [6, 7]. BCAA levels tend to be increased in the circulatory system when the gut microbiota is enriched with genes involved in BCAA biosynthesis [8].

BCAA supplementation is often beneficial to energy expenditure; however, increased circulating BCAA levels are linked to diabetes [7, 9]. The serum metabolomes of insulin-resistant individuals are characterized by increased levels of BCAAs, which are correlated with a gut microbiome that has strong potential for BCAA biosynthesis [8, 10]. Furthermore, high circulating BCAA levels have been found to be accompanied by tissue-specific inactivation of BCAA-catabolizing enzymes in human and animal studies [11]. In addition, BCAAs augment the production of mitochondria-derived reactive oxygen species (ROS) with subsequent increase in oxidative damage and mitochondrial dysfunction. This mitochondrial dysfunction has been identified as a relevant mechanism in cardio-metabolic diseases, underlying cardiovascular risk factors such as diabetes, hypertension and atherosclerosis [12]. From this perspective, targeting the gut microbiota to improve circulating BCAA dysfunction could be a pivotal strategy for improving cardiac function. However, the mechanism by which the gut microbiota and its BCAA metabolites ameliorate cardiac damage in diabetes is still unclear.

Recent studies have revealed that autonomic imbalance and diminished vagus nerve activity occur frequently in humans with diabetes and in animal models of diabetes [13, 14]. Autonomic imbalance participates in the pathological processes of many cardiovascular diseases [15, 16]. Pyridostigmine, a cholinesterase inhibitor, improves vagal activity and regulates glucose metabolism to protect mitochondrial structure and function and decreases oxidative stress to reduce myocardial vulnerability to injury in diabetic mice [14]. However, thus far, no studies have analysed the protective effects of pyridostigmine on the intestinal barrier and gut microbiota homeostasis. In the present study, we used the cholinesterase inhibitor pyridostigmine to stimulate vagal activation in mice with diabetic cardiomyopathy and studied the effects on insulin resistance and cardiac damage, with a focus on the intestinal barrier and gut microbiota, as well as on BCAA catabolism.

Materials And Methods

Animals and experimental Models

Male C57BL/6J mice (4 weeks old) were supplied by the Experimental Animal Center of Xi'an Jiaotong University. The animals were maintained under standard laboratory conditions and housed in a temperature-controlled room with ad libitum access to water and food unless otherwise indicated. All experimental procedures were performed in accordance with the Guidelines for the Care and Use of

Laboratory Animals (National Institutes of Health Publication, eighth edition, 2011). This study was approved by the ethics committee of Xi'an Jiaotong University.

After acclimatization for 2 weeks, the mice were initially administered either a normal chow diet (ND; D12450, Research Diets, USA) or a 60% high-fat diet (HFD; D12492, Research Diets) for 12 weeks. The HFD-fed mice were then intraperitoneally injected with 35 mg/kg body weight streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA) for 3 d. The ND mice received equivalent volumes of 0.1 M citrate buffer for 3 d. Serum glucose levels were measured by tail blood glucometry (Roche, Basel, Switzerland) 2 weeks after the injection. Mice with random blood glucose levels ≥ 7.9 mmol/L were considered diabetic mice and were recruited for subsequent experiments. Then, both diabetic and control mice were administrated or not administered pyridostigmine (3 mg/kg/d, i.g.) for 10 weeks and continually fed with either the HFD or ND. Accordingly, three groups were defined: the control + vehicle group (CON), the diabetic cardiomyopathy mice + vehicle group (DCM), and the diabetic cardiomyopathy mice + pyridostigmine group (DCM + PYR).

Echocardiographic measurement

Cardiac morphology and function were assessed by transthoracic echocardiography (VisualSonics Vevo 2100, VisualSonics, Inc., Toronto, Canada) in mice anaesthetized with 1.5–2% isoflurane. The left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular internal dimensions in systole and diastole (LVIDs and LVIDd, respectively) were evaluated.

Heart rate variability (HRV) analysis

HRV was calculated as the mean difference between sequential RRs for the complete set of ECG waveforms. ECG was performed using a PowerLab system. For each 5-min stream of ECG waveforms, the mean time between successive QRS complex peaks, mean heart rate, and mean HRV analysis-generated time measures were acquired. The time-domain measures included the standard deviation of the normal-to-normal beat interval (SDNN) and the root mean square of successive differences (RMSSD).

Insulin tolerance test (ITT) and glucose tolerance test (GTT)

Mice were fasted overnight, and GTTs and ITTs were performed after intraperitoneal injection of 2 g/kg glucose (Sigma-Aldrich) and 0.75 U/kg insulin (Sigma-Aldrich), respectively. Glucose levels were measured at 0, 30, 60, 90 and 120 min with an AccuChek glucometer (Roche).

In vivo intestinal paracellular permeability assay

Intestinal paracellular permeability was assessed using fluorescein isothiocyanate-dextran 4 kDa (FITC-D4; Sigma-Aldrich) as a paracellular tracer. Before the assay, mice were fasted for 6 h. The mice were then orally gavaged with FITC-D4 (500 mg/kg of body weight). 2 hours after gavage, blood was collected from the facial vein, and the serum was prepared for fluorescence measurements (excitation, 490 nm; emission, 520 nm).

Blood, faeces and tissue collection and biochemical analysis

After the end of the experiments, faecal samples were collected and stored at −80 °C. After the mice were anesthetized, blood samples were obtained from the abdominal aorta, intestinal and cardiac tissues were removed, and lipopolysaccharide (LPS), acetylcholine (ACh), and diamine oxidase (DAO) were detected with a biochemical detection system (AU2700; Olympus, Melville, NY, USA). Serum insulin and BCAA levels, cardiac BCAA levels were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (Abcam, Cambridge, UK) with the manufacturers' standards and protocols.

Haematoxylin and eosin (H&E) and Masson’s trichrome staining

Mouse intestinal and cardiac tissues were fixed in formalin and embedded in paraffin for sectioning into 5-μm-thick sections. The sections were stained with H&E and Masson's trichrome (Heart Biological Technology Co., Ltd., Xi’an, China) and analysed for morphological changes.

Transmission electron microscopy (TEM)

Mouse intestinal and cardiac tissues were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4 °C. The samples were postfixed with 1% osmium tetroxide, dehydrated in a graded ethanol series, embedded in epoxy resin, and then cut into ultrathin sections. After counterstaining with uranyl acetate and lead citrate, the sections were examined by TEM (H-7650; Hitachi, Tokyo, Japan).

Immunohistochemistry

For immunohistochemical analysis, sections were deparaffinized through xylene and ethanol series. All sections were boiled in 10 mmol/L sodium citrate antigen retrieval buffer at 95 °C for 20 min, and the slides were washed 3 times with PBS. Sections were exposed to 3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity and then washed 3 times with PBS. Next, the sections were blocked with 10% goat serum for 1 h and then incubated overnight at 4 °C with anti-bax (1:200 dilution; Bioworld, Minnesota, USA), anti-bcl-2 (1:200 dilution; Bioworld), anti-cleaved caspase 3 (1:200 dilution; Bioworld), anti-zonula occludens -1 (ZO-1; 1:150 dilution; Bioworld), anti-occludin (1:200 dilution; Abcam), and anti-claudin 1 (1:200 dilution; Abcam), and anti-AChE (1:200 dilution; Proteintech, Wuhan, China) antibodies. After 3 washes with PBS, the sections were incubated with secondary antibodies for 30 min at 37 °C and then washed 3 times with PBS. Diaminobenzidine was used to develop the antibody staining, and haematoxylin counterstaining was used to visualize the nuclei. Images were obtained under a light microscope (BX53, Olympus, Japan).

Immunofluorescence

Paraffin-embedded samples were prepared according to standard histological procedures. Nitrotyrosine (1:200 dilution; Santa Cruz, CA, USA) was used for immunostaining. The sections were then incubated with a goat anti-mouse IgG superclonal secondary antibody for 1 h. Fluorescence images were analysed with Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA).

Fluorescence in situ hybridization (FISH)

Intestinal segments were fixed in a methanol-Carnoy mixture and embedded in paraffin. The paraffin sections were de-waxed and washed in 100% ethanol. FISH was performed as previously described [17]. Universal EUB338 oligonucleotide probes were used for 16S rRNA. The tissues were counterstained with 4’6-diamidino-2-phenylindole, and fluorescent images were acquired using a fluorescent microscope (Eclipse Ci, Nikon, Tokyo, Japan) equipped with CCD camera.

Western blotting

Intestinal and cardiac tissues proteins were extracted with protease inhibitor-containing lysis buffer. The sample proteins were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes. Nonspecific binding to the membranes was blocked by incubating the membranes in Tris-buffered saline containing 5% nonfat milk and 0.1% Tween 20 at room temperature for 1 h. The immunoblots were probed with antibodies against p-Akt (Ser473), Akt, branched-chain amino transferase 2 (BCAT2), branched-chain α-keto acid dehydrogenase kinase (BCKDK), phosphatase 2C in mitochondria (PP2Cm; Proteintech), branched-chain α-keto acid dehydrogenase (BCKDHA; Bioworld), mitochondrial complexes 1-5 (Invitrogen, Carlsbad, CA, USA), p-BCKDHA (Ser293; Thermo Fisher, Massachusetts, USA), Cytochrome C (Cell Signaling Technology, Danvers, MA, USA), and GAPDH (CMCTAG, Milwaukee, WI, USA) overnight at 4 °C and then incubated with the corresponding secondary antibodies at room temperature for 1 h. The bands were visualized with ECL-Plus reagent (Millipore), and quantified using Gel-Pro Analyzer (Media Cybernetics, Bethesda, MD, USA).

High-throughput sequencing

Genomic DNA was extracted using an E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer’s protocols. The concentrations and purity of the resultant DNA were determined using a NanoDrop ND-2000 (NanoDrop, USA), and the quality was checked by running aliquots on gels. The sample were stored at –80 °C for further analysis.

The 16S rRNA gene was amplified by polymerase chain reaction (PCR) with the primers 341F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') targeting the hypervariable V3-V4 region of the bacterial 16S rRNA gene. PCR were performed in triplicate with Phusion High-Fidelity PCR Master Mix (New England Biolabs) using 30 ng of template DNA. The PCR products were purified with AMPureXP beads and quantified/qualified with an Agilent 2100 Bioanalyzer (Agilent, California, USA). The PCR products of different samples were mixed equally and used to construct an Illumina pair-end library using a Next Ultra™ DNA Library Prep Kit for Illumina (NE, USA). Then, the amplicon library was sequenced in paired-end mode (2 × 300 bp) on an Illumina MiSeq platform (Illumina, San Diego, USA) according to standard protocols.

RNA extraction and real-time PCR

Total RNA was isolated and extracted from cardiac and intestinal tissues using TRNzol Universal (BioTeke, Beijing, China) according to the manufacturer's protocol. The extracted RNA was quantified and assessed for integrity using a NanoDrop ND-2000 (Thermo Fisher). A kit (BioTeke) was used to perform first-strand cDNA synthesis according to the procedure recommended by the manufacturer. Real-time PCR was performed on an Exicycler 96 PCR detection system (Bioneer, Daejeon, Korea). β-Actin was used as the invariant control. The sequences of the real-time PCR primers are shown in Table 1. The relative mRNA expression levels of individual genes were calculated after normalization to the corresponding β-actin mRNA levels.

Statistical analysis

The data are expressed as the means ± SEM. The data were statistically analysed using one-way ANOVA followed by Tukey's multiple comparison test (three groups). Student's t-test was applied for comparison of two groups. All figures were prepared using GraphPad Prism 7.04 (GraphPad Software Inc., La Jolla, CA).

Results

Pyridostigmine improved vagal activity and insulin resistance in mice with diabetic cardiomyopathy

In the present study, a diabetic cardiomyopathy mouse model was established using HFD and STZ, and the mice were administered pyridostigmine (Fig. 1a). As expected, compared with the CON group, the DCM group exhibited lower SDNN and RMSSD values in the time domain, but the change was partially prevented in the DCM + PYR group (Fig. 1b). The results revealed that AChE activity was not notably augmented and that ACh levels were lower in the DCM group than in the CON group, but the change was prevented by pyridostigmine administration (Fig. 1c-d). In addition, the activity of cardiac and intestinal AChE was higher in the DCM group but lower in the DCM + PYR group than in the CON group (Fig. 1e). Intestinal and cardiac ACh concentrations were lower in the DCM group but higher in the DCM + PYR group than in the CON group (Fig. 1f). Together, these data suggest that pyridostigmine improved vagal tone by suppressing AChE activity and increasing ACh concentration.

Fasting serum glucose levels were elevated in DCM group, but pyridostigmine treatment significantly decreased serum glucose levels in mice of the DCM + PYR group (Fig. 1g). Serum insulin concentrations were lower in the DCM group than in the CON group, but pyridostigmine had no significant effect on insulin concentrations (Fig. 1h). Additionally, while diabetes altered Akt phosphorylation, pyridostigmine increased Akt phosphorylation in cardiac tissue, indicating that the drug had a positive effect on insulin sensitivity in cardiac tissue(Fig. 1i). Moreover, the GTT and ITT results revealed diminished glucose and insulin tolerance under diabetic conditions, indicating that insulin sensitivity was impaired in mice with diabetic cardiomyopathy. However, in the DCM + PYR group, glucose and insulin tolerance were restored (Fig. 1j-k). Together, these results demonstrated that pyridostigmine improved insulin resistance in mice with diabetic cardiomyopathy.

Pyridostigmine attenuated intestinal barrier injury in mice with diabetic cardiomyopathy

Tight junctions (TJs) are important components of the intestinal barrier, and ZO-1 occludin and claudin-1 are the key factors. The expression of ZO-1, occludin and claudin-1 was examined by Immunohistochemistry. The results demonstrated that ZO-1, occludin and claudin-1 expression was reduced in intestinal tissues in mice with diabetic cardiomyopathy. These abnormalities were reversed by pyridostigmine administration (Fig. 2a). In addition, the TEM results revealed that the intestinal mucosal epithelium was neatly arranged with tight TJs between epithelial cells in the CON group (Fig. 2b). Conversely, in the DCM group, the intestinal epithelial cells were swollen, and the TJs between epithelial cells appeared damaged with widened intercellular spaces. In the DCM + PYR group, TJs were distributed in an orderly manner, and the widening of the intercellular spaces was mild (Fig. 2b).

Bacterial invasion of the epithelium was observed by FISH with a universal 16S rRNA gene probe. The CON group only found positive areas outside the intestinal mucosa, while positive areas were found on the lamina propria and submucosa in the DCM group, while pyridostigmine reversed it partially (Fig. 2c). Intestinal permeability was measured via determination of the concentration of serum FITC-D4. As shown, the serum FITC-D4 levels in mice with diabetic cardiomyopathy were higher than those in control mice, but pyridostigmine reversed the increase (Fig. 2d). In addition, the concentrations of the serum metabolic endotoxaemia markers LPS and DAO were higher in the DCM group than in the CON group but lower in the DCM + PYR group (Fig. 2 e-f).

Pyridostigmine regulated gut microbiota homeostasis in mice with diabetic cardiomyopathy

Sequencing of the V3-V4 region of the 16S rRNA gene was performed on faecal samples. PLS-DA of bacterial operational taxonomic units (OTUs) of the three groups showed separation of the populations (Fig. 3a). Interestingly, the gut microbial taxonomy was different among CON, DCM and DCM + PYR groups. The α-diversities of the gut microbiota analysed using the ACE, Chao and observed species indexes showed discrepancies in microbial species among the three groups (Fig. 3b). The gut microbiota diversity was lower in mice with diabetic cardiomyopathy than in control mice, and it decreased after treatment with pyridostigmine, indicating that pyridostigmine meaningfully modulated the gut microbiota.

Analysis of faecal samples identified 56 OTUs that were differentially abundant among CON, DCM and DCM + PYR groups. To present the data in aggregate, we counted the potentially protective (more abundant in the CON group than in the DCM group; n = 26) and potentially non-protective (more abundant in the DCM group; n = 30) OTUs. In addition, we calculated a score by weighting each OTU based on its relative abundance in the sample (hereafter called the abundance score) (Fig. 3c). The heat map shows that the abundance of protective OTUs (Lactobacillus and Allobaculum) was lower and that the abundance of non-protective OTUs (Ruminococcus, Parabacteroides, Dorea and Clostridium) was higher in the DCM group than in the CON group. Pyridostigmine increased the abundance of protective OTUs and decreased the abundance of non-protective OTUs (Fig. 3c).

The composition of the gut microbiota was affected after pyridostigmine supplementation in mice with diabetic cardiomyopathy

To identify the specific bacterial taxa associated with diabetes and pyridostigmine, we compared the gut microbiota of control mice, diabetic cardiomyopathy mice and pyridostigmine-supplemented mice using the linear discriminant analysis (LDA) effect size (LEfSe) method. A cladogram of the structure of the gut microbiota and the predominant bacteria is shown, and the greatest differences in taxa between the control mice, mice with diabetic cardiomyopathy and pyridostigmine-supplemented mice are displayed. In the DCM group, the relative abundance levels of the phylum Firmicutes, the order Lactobacillales, the families Lactobacillaceae and Bifidobacteriaceae, and the genera Lactobacillus, Bifidobacterium and Allobaculum were decreased, while the relative abundance levels of the order Clostridiales, the families Porphyromonadaceae and Lachnospiraceae and the genus Dorea were increased. Pyridostigmine supplementation significantly elevated the relative abundance levels of the family Bacteroidaceae and the genus Bacteroides, while it reduced the relative abundance levels of the order Clostridiales, the families Porphyromonadaceae and Lachnospiraceae and the genus Dorea. These differentially abundant taxa between groups were further supported by LEfSe (Fig. 4a). As shown in Fig. 4b, the orde-level analysis demonstrated that the abundance levels of Lactobacillales and Desulfovibrionales were decreased and that the abundance of Clostridiales was increased in the DCM group, pyridostigmine partially reversed these changes. Compared to control mice, diabetic mice displayed significantly lower relative abundance levels of Lactobacillaceae and Erysipelotrichaceae and high higher relative abundance levels of Porphyromonadaceae, Lachnospiraceae, and Ruminococcaceae, while pyridostigmine treatment protected against these changes. At the genus level, diabetic mice displayed significantly lower relative abundance levels of Allobaculum and Lactobacillus and higher relative abundance levels of Ruminococcus and Parabacteroides, while pyridostigmine treatment reserved these effects (Fig. 4b).

The relative abundance levels of the bacterial taxa analysed above not only are related to the pathological state of diabetes but also may be related to the synthesis of BCAAs. Analysis of the microbes related-BCAA producing showed that the relative abundance levels of Clostridiales and Lachnospiraceae were increased in mice with diabetic cardiomyopathy compared with control mice, and the relative abundance levels of Clostridiales and Lachnospiraceae decreased after administration of pyridostigmine (Fig. 4c).

Pyridostigmine improved BCAA catabolism in cardiac in mice with diabetic cardiomyopathy

To further verify how pyridostigmine plays a protective role with regard to BCAA concentrations in serum and BCAA catabolism in cardiac tissue in diabetic mice, the alterations in these variables were examined in all groups. Serum BCAA concentrations were significantly higher in mice with diabetic cardiomyopathy than in control mice, but the abnormal increase was reversed by pyridostigmine administration (Fig. 5a). Additionally, Spearman's correlation analysis showed that the relative abundance levels of Clostridiales and Lachnospiraceae were positively correlated with the BCAA concentration in mouse serum (Fig. 5b).

To investigate the effetcs of pyridostigmine on BCAA catabolism, the levels of catabolic enzymes BCAT2, p-BCKDHA, BCKDHA, BCKDK and PP2Cm were investigated by Western blot analysis and real-time PCR, respectively. The real-time PCR results showed that the mRNA levels of BCAT2 and PP2Cm were lower, while those of BCKDK were higher in mice with diabetic cardiomyopathy than in the control mice. These changes were partially normalized by pyridostigmine administration. There was no significant change in the mRNA levels of BCKDHA in mice with diabetic cardiomyopathy, but was decreased by pyridostigmine (Fig. 5c). In addition, the phosphorylation of BCKDHA and the protein expression of BCKDK were increased, while the protein expression of BCAT2 and PP2Cm were decreased. However, all of the alterations in these parameters observed in mice with diabetic cardiomyopathy were partially relieved by pyridostigmine treatment (Fig. 5d). Taken together, these results showed that cardiac BCAA catabolism was reduced in cardiac tissue in the context of diabetic cardiomyopathy but that pyridostigmine improved BCAA catabolism(Fig. 5e).

Pyridostigmine decreased cardiac BCAA concentrations to attenuate cardiac dysfunction in diabetic mice

Pharmacological promotion of systemic BCAA catabolism lowers circulating and cardiac BCAA concentrations, and improves cardiac function in both hemodynamic and ischemic challenges [18]. Our results showed that the BCAA concentrations in cardiac tissue were higher in mice with diabetic cardiomyopathy than in control mice. The abnormality was greatly attenuated by pyridostigmine administration (Fig. 6a).

As shown in Fig. 6b, the mice in the DCM group exhibited higher LVEF and LVFS and lower LVIDs and LVIDd than those in the CON group. Pyridostigmine administration led to improvements in the LVEF, LVFS, LVIDs and LVIDd. The cardiomyocyte and fibrotic areas were greater in the DCM group than in the CON group and were reduced by pyridostigmine administration (Fig. 6c-d). In addition, compared with control mice, mice with diabetic cardiomyopathy showed lower cardiac Bcl-2 expression and higher Bax and cleaved caspase 3 expression, while pyridostigmine treatment ameliorated these alterations and reversed mitochondria-related apoptosis (Fig. 6e).

Pyridostigmine alleviated cardiac mitochondrial dysfunction in mice with diabetic cardiomyopathy

To further verify how BCAAs helped improve cardiac function in mice with diabetic cardiomyopathy with pyridostigmine administration, we examined mitochondrial function in cardiac tissue. Compared with that in the CON group, the arrangement of myocardial mitochondria was disordered in the DCM group, as indicated by increased proliferation and swelling, increased numbers of vacuoles, a loosened and broken mitochondrial ridge structure, and decreased matrix density, and these changes were reversed by pyridostigmine (Fig. 7a). The ratio of mitochondrial to cytosolic cytochrome C in diabetic mice was markedly lower than that in control mice, and pyridostigmine treatment restored the normal ratio, indicating that pyridostigmine suppressed the release of mitochondrial cytochrome C (Fig. 7b).

We next sought to determine whether mitochondrial function was improved with pyridostigmine treatment. We found that lower expression of the complex subunits I, II, III and V and lower ATP content in

cardiac tissue in diabetic mice than in control mice (Fig. 7c-d). The abnormalities were greatly alleviated by pyridostigmine administration, demonstrating that pyridostigmine improved mitochondrial function in mice with diabetic cardiomyopathy. Additionally, immunofluorescence staining revealed nitrotyrosine expression in the cardiac tissue of mice with diabetic cardiomyopathy. However, the expression of nitrotyrosine was reduced significantly in the DCM + PYR group (Fig. 7e). This study showed that high levels of BCAAs may led to mitochondrial dysfunction and oxidative stress (Fig. 7f).

Discussion

The gut microbiota and its metabolites are closely associated with metabolic syndrome and cardiovascular health conditions, including obesity, diabetes, atherosclerosis, hypertension and heart failure [19, 20]. Therefore, novel pharmacological agents to treat impairment of gut microbiota and BCAA metabolism are urgently needed. The present study demonstrated the following: (1) The intestinal barrier function (related to TJs, intestinal permeability and bacterial translocation) and gut microbial homeostasis (of diabetes-related bacteria and BCAA-producing bacteria) were impaired in diabetic mice and that this impairment was accompanied by reduced vagal activity, which eventually led to cardiac damage. (2) Pyridostigmine enhanced vagal activity and alleviated intestinal barrier injury and gut microbiota disruption (disruption of the key driving flora related to diabetes as well as BCAA-producing microbes), thereby reducing BCAA synthesis, ameliorating insulin resistance and cardiac damage. (3) More importantly, mitochondrial BCAA catabolism was decreased in cardiac tissue in the context of diabetes, while pyridostigmine regulated the mRNA and protein expression of BCAA catabolism enzymes (BCAT2, p-BCKDHA/BCKDHA, PP2Cm, BCKDK) to decrease BCAA concentrations and reduce myocardial damage by alleviating mitochondrial damage. Taken together, these findings showed that pyridostigmine enhanced vagal activity, attenuated intestinal barrier injury and gut microbial disruption, and improved BCAA catabolism to reduce BCAA concentrations in cardiac tissue. The reductions in BCAA concentrations prevented cardiac damage in mice with diabetic cardiomyopathy by regulating mitochondrial function and structure. This study provides novel insights for the development of a therapeutic strategy for diabetes-induced cardiac damage that targets the gut microbiota and BCAA catabolism.

Patients with diabetes have chronic hyperglycaemia, which easily leads to tissue damage and organ dysfunction in the heart, blood vessels, and other organs [21]. Diabetic cardiomyopathy, a major cardiovascular complication in diabetic patients, is defined as structural and functional myocardial impairment without coronary artery disease or hypertension that is characterized mainly by myocardial hypertrophy and fibrosis, metabolic dysregulation, and defects in myocardial contractile properties [22]. In the present study, a diabetic cardiomyopathy mouse model was established via HFD feeding and STZ administration. The diabetic cardiomyopathy model mice showed elevated fasting serum glucose levels, decreased LVEF and LVFS, increased LVIDs and LVIDd, cardiomyocyte fibrosis and cardiomyocyte hypertrophy. Previous studies have shown that autonomic imbalance, as indicated by attenuated parasympathetic nerve tone and increased sympathetic nerve activity, is involved in the pathological processes of diabetes [23, 24]. Some studies in this laboratory have shown that pyridostigmine increased

vagal activity, improved cardiac damage in the diabetes and obesity models [14, 25]. Consistent with these results, our study showed that pyridostigmine increased parameters of vagal activity, as evidenced by increased ACh, decreased AChE, higher SDNN and RMSSD in mice with diabetic cardiomyopathy. This is the first time to determine the change of intestinal vagal activity in mice with diabetic cardiomyopathy.

Long-term diabetes leads to severe peripheral, autonomous, and central neuropathy in combination with clinical gastrointestinal symptoms. The brain-gut axis thus expresses a neurophysiological profile, and HRV can be correlated with clinical gastrointestinal symptoms [26]. Interestingly, vagal nerve electrical stimulation potently reduces intestinal inflammation by restoring intestinal homeostasis, whereas vagotomy has the reverse effect [27]. Imbalances in intestinal barrier dysfunction and the gut microbiota have been linked to various diseases, including atherosclerosis, hypertension, heart failure, obesity, and diabetes [19, 20]. The research showed that diabetic cardiomyopathy was associated with modifications to the gut microbiota, some of which appeared to affect on cardiac function and structure [28]. However, whether improving vagus nerve activity can regulate the intestinal barrier and gut microbiota in mice with diabetic cardiomyopathy has not been reported.

Intestinal dysbiosis associated with intestinal barrier disruption may participate in diabetes mellitus development by increasing intestinal permeability, which would trigger an inflammatory response leading to peripheral insulin resistance and ultimately to diabetes mellitus [29]. Consistent with these above results, the TEM result in the current study showed that TJs were distributed in an orderly manner and that widening of intercellular spaces was mild after pyridostigmine treatment. In addition, pyridostigmine increased the expression of the TJ proteins in the intestinal epithelium and decreased intestinal permeability in mice with diabetic cardiomyopathy. Intestinal barrier dysfunction has been found to lead to bacterial translocation, metabolic endotoxaemia caused by LPS release into the blood and insulin resistance [30], which is consistent with our observations. The results of our study showed that pyridostigmine decreased the bacterial translocation of the intestinal mucosa and reduced the serum concentrations of the LPS and DAO, thus alleviating metabolic endotoxaemia in mice with diabetic cardiomyopathy.

Gut microbes are critical for intestinal epithelial barrier function and for the maintenance of physiological homeostasis; the gut microbiota is a promising therapeutic target for diabetes and its complications, as it plays a significant role in the related pathogenic processes [31]. Previous studies have shown that the α-diversity of the gut microbiota is connected with obesity and diabetes [32]. According to previous research, the α-diversity in mice with diabetic cardiomyopathy was decreased, but this decrease can be reversed by pyridostigmine. Accumulating studies have reported that diabetic patients and animals show significantly different gut microbiota compositions than their non-diabetic counterparts [3, 33]. Li et al. demonstrated that Clostridiales was associated with increased albuminuria, an early hallmark of diabetic nephropathy, and that Clostridiales species were most abundant in zero-fibre diet-fed diabetic mice [34]. However, Larsen et al. reported that proportion of bacteria in the phylum Firmicutes was significantly lower in the diabetic adult males group than in the healthy control group [28]. As the abundance of the family Erysipelotrichaceae is reduced in pre-diabetic mice, Hu et al. proposed that mice with reduced

Erysipelotrichaceae abundance may later develop diabetes [35]. Qin et al. reported that the abundance of Lachnospiraceae bacteria in the gut was positively correlated with type 2 diabetes, implying that Lachnospiraceae might be associated with the occurrence of the disease [36]. Gu et al. demonstrated that Porphyromonadaceae was positively correlated with fasting insulin and fasting blood glucose [37]. Zhang et al. reported that the family Bacteroidaceae was dysregulated in a diabetic mouse model [38]. Previous studies have shown that the abundance levels of Lactobacillus and Allobaculum, beneficial bacteria that can directly protect intestinal barrier function, are reduced in diabetic animal models [39, 40]. Furthermore, Bifidobacterium, Lactobacillus and Bacteroides have been shown to be beneficial bacteria that can directly affect the immune system of the host, inducing intestinal immunity and enhancing immune function [41]. Additionally, accumulating studies have shown that the abundance levels of Ruminococcus, Parabacteroides, Dorea, and Clostridium are increased, while those of Lactobacillales, Lactobacillaceae, Bacteroides, Bifidobacterium, and Bifidobacteriaceae are decreased, in the gut microbiota in diabetic animal models and patients [41-44]. Consistented with the above findings, pyridostigmine enhanced the abundance of Lactobacillales, Lactobacillaceae, Erysipelotrichaceaem, Lactobacillus and Allobaculum. In contrast, it reduced the abundance of Clostridiales, Porphyromonadaceae, Lachnospiraceae, Ruminococcaceae, Ruminococcus, Parabacteroides, Dorea and Clostridium. Notably, Firmicutes, Lactobacillales, Bifidobacteriaceae, Bacteroidaceae, Allobaculum, Lactobacillus, Bifidobacterium and Bacteroides were the predominant bacterial groups in the control and pyridostigmine treatment groups. The predominant bacterial groups in the diabetic cardiomyopathy model group were Clostridiales, Porphyromonadaceae, Lachnospiraceae and Dorea.

Disturbance of the intestinal flora affects functional metabolites of the intestinal microbiota, such as BCAAs, short-chain fatty acids and trimethylamine-n-oxide [9, 45]. Recent research has shown that elevated BCAA levels are associated with a gut microbiome pattern characterized by enriched BCAA biosynthetic potential. In addition, the relative abundance of BCAA-producing Clostridiales bacteria has been found to be increased in the HFD-fed mice [46]. Bile acids produced in response to a HFD may promote the growth of Clostridium; thus, elevated production of BCAAs via proteolysis is related to the increased Clostridium abundance [47]. However, the family Lachnospiraceae has been reported to be positively associated with circulating BCAAs in different European populations [7, 48]. The results of this study showed that BCAA-biosynthesizing microbes, including those in the order Clostridiales and the family Lachnospiraceae, exhibited reduced abundance after pyridostigmine administration in mice with diabetic cardiomyopathy. Epidemiological research has shown that serum BCAA levels are elevated in insulin-resistant individuals and that these elevations are correlated with elevated abundance of BCAA-producing microbes [8]. A growing array of proof-of-concept experiments have demonstrated that BCAA metabolic dysfunction is tightly related to diabetes phenotypes [11], and high concentrations of BCAAs in the circulatory system have been recommended as biomarkers for the early diagnosis of obesity, diabetes or non-alcoholic fatty liver disease [7]. Consistent with the above results, circulating BCAA concentrations were higher in mice with diabetic cardiomyopathy than in control mice in the current study, and pyridostigmine reversed the increase. Further Pearson correlation analysis indicated that the abundance

levels of Clostridiales and Lachnospiraceae were positively correlated with the serum concentrations of BCAAs in mice.

Normally, surplus BCAAs in circulatory system can be catabolized via abundant BCAA-catabolizing enzymes in various tissues [11, 49]. BCKD is a multienzyme complex that exists in mitochondria and is regulated by kinase (BCKD phosphorylation inactivation) and phosphatases (BCKD dephosphorylation activation). The activity of the BCKD complex is decreased in the livers of diabetic patients and animals, which causes BCAA and BCKA to accumulate in plasma [50]. In the context of diabetes, BCAA catabolism in skeletal muscle tissue is impaired, leading to accumulation of high BCAA levels [51]. Indeed, the heart, muscles, and kidneys are known to metabolize BCAAs and may be particularly affected by deficiencies in this cofactor [52]. The study showed that cardiac BCAA catabolism and insulin signaling was impaired in human heart failure, while enhancing BCAA oxidation could improve cardiac function in the failing mouse heart [53]. A previous study has suggested that miR-22 overexpression is coupled with PP2Cm downregulation, BCAA accumulation, and mTOR hyperactivation and is possibly linked to alterations in glucose utilization and suppression of autophagy in dilated cardiomyopathy [54]. Suppression of whole-body BCAA catabolism via deletion of PP2Cm elevates circulating and cardiac BCAA levels and worsens the cardiac response to ischaemia/reperfusion injury [55]. Conversely, pharmacological promotion of systemic BCAA catabolism lowers circulating and cardiac BCAA levels and improves cardiac function during both hemodynamic and ischaemic challenges [56]. Consistent with these findings, the present study showed that the BCAA-catabolizing enzymes were downregulated in the cardiac tissues of mice with diabetic cardiomyopathy. However, pyridostigmine improved the catabolism of BCAAs in the cardaic tissues of mice with diabetic cardiomyopathy. Overall, pyridostigmine inhibited the biosynthesis of BCAAs by the gut microbiota, thereby potentially reducing the supply of BCAAs. On the other hand, pyridostigmine effectively triggered tissue-specific BCAA catabolism to accelerate catabolism of BCAAs in the cardiac tissue.

The mechanisms by which BCAAs affect cardiac function remain poorly understood. Transient exposure of isolated rodent hearts to high concentrations of BCAAs impairs contractility. The impairment may occur in part via inhibition of mitochondrial ATP production, as high levels of BCAAs inhibit both pyruvate and αKG dehydrogenases. Newgard et al. reported that high levels of BCAAs led to insulin resistance of skeletal muscle, possibly because incomplete oxidation of large amounts of BCAAs leads to the accumulation of large numbers of lipid metabolism intermediates in skeletal muscle [9]. The excessive mitochondrial oxidation leads to skeletal muscle dysfunction, which eventually develops into skeletal muscle resistance [13, 51, 55]. BCAAs and their metabolites affect mitochondrial respiratory chain function. Large amounts of BCAAs may overload the mitochondria and affect the activity of mitochondrial enzymes, leading to aggravation of ischemia-induced mitochondrial dysfunction and increased ROS production [57]. Studies have shown that downregulation of PP2Cm causes liver damage, increases hepatocyte apoptosis, increases sensitivity to calcium-induced mitochondrial permeability transition pore opening, increases myocardial oxidative stress and causes cardiomyocyte apoptosis [58]. In addition, the occurrence of diabetic cardiomyopathy is accompanied by disruption of normal mitochondrial function and structure and myocardial antioxidant capacity and by excessive oxidative

stress [59]. This study showed that mitochondrial structure and function were disrupted in the cardiac tissues of diabetic mice. However, pyridostigmine ameliorated mitochondrial dysfunction, reduced cardiac hypertrophy and fibrosis and improved cardiac function in mice with diabetic cardiomyopathy.

Limitations

Although we primarily achieved the goal of our study, showing that in vivo pyridostigmine succeeds in preventing cardiac dysfunction in diabetic mice. Limitations of the present study are also worth noting. The assay kit used in our experiments is a simple and rapid assay for quantifying ACh by a colorimetric method. The better way is to use enzyme-based biosensors to achieve higher sensitivity or microdialysis technique allowing an ‘in vivo’ determination of ACh.

Conclusions

In conclusion, our study indicated that diabetes resulted in autonomic imbalance and cardiac damage that was associated with gut barrier disorder and BCAA catabolism. Importantly, pyridostigmine enhanced vagal activation and exerted positive effects on insulin resistance and cardiac injury in the context of diabetes. Moreover, the primary mechanisms responsible for these findings involved regulation of intestinal barrier injury, gut microbiota disruption, and BCAA catabolism and consequent attenuation of mitochondrial dysfunction and normalization of cardiac remodeling. Overall, our study provides evidence for the roles of gut microbiota disruption and BCAA catabolism in diabetes-induced cardiac damage and novel insights for the development of therapeutic strategies related to vagal activation.

Abbreviations

BCAAs: branched-chain amino acids; ROS: reactive oxygen species; BCAT2: branched chain aminotransferase 2; PP2Cm: phosphatase 2C in mitochondria; BCKDHA: branched-chain α-keto acid dehydrogenase; BCKDK: branched-chain α-keto acid dehydrogenase kinase; ND: normal chow diet; HFD: high-fat diet; STZ: streptozotocin; LVEF: left ventricle ejection fraction; LVFS: left ventricle fractional shortening; LVIDs: left ventricle internal dimension in systole; LVIDd: left ventricle internal dimension in diastole; HRV: heart rate variability; SDNN: standard deviation of the time between normal-to-normal beats; RMSSD: root mean square of successive differences; ITT: insulin tolerance tests; GTT: glucose tolerance tests; LPS: lipopolysaccharide; ACh: acetylcholine; DAO: diamine oxidase; ELISA: enzyme-linked immunosorbent assay; H&E: hematoxylin and eosin; TEM: transmission electron microscopy; ZO-1: zonula occludens-1; FISH: fluorescence in situ hybridization; FITC-D4: fluorescein isothiocyanate-Dextran 4000; LDA: linear discriminant analysis; LEfSe: linear discriminant analysis effect size; PCR: polymerase chain reaction.

Declarations

Authors’ contributions

WZ, XH and YY conceived and designed the experiments; YY, MZ and QW performed the experiments; YY and MZ analyzed the data; All authors drafted and revised the paper. All authors read and approved the final manuscript.

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Data availability

Please contact author for data requests.

Ethics approval and consent to participate

All animal protocols were approved by the Ethics Committee of Xi'an Jiaotong University.

Consent for publication

All authors have declared their consent for this publication.

Competing interests

The authors declare that they have no competing interests.

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subjects. Diabetes. 2012;61:1210-1216.

Tables

Table 1

Primer sequences in real-time PCR

Gene	Forward primer sequence (5’ - 3’)	Reverse primer sequence (5’ – 3’)
BCKD	AGGGCCGGATCTCCTTCTACAT	CCTTGCCTGGGTCATTCACG
BCAT2	GTCATCTTGCCTGGAGTAGTTCG	TTGCTTGCCTTCATACAGGATTT
PPM1K	GCCAGGTGTTCTCGGTTTGA	TGGTTTGCCGTACTTGATGC
BCKDK	CGTAGCCTTCCTTTCATCATTG	CCTCATCTGCCTGGTCCTTG
β-actin	CTGTGCCCATCTACGAGGGCTAT	TTTGATGTCACGCACGATTTCC