Butyrate ameliorate skeletal muscle atrophy in Diabetic Nephropathy via enhancing gut barrier function and GPR43 mediated PI3K/AKT/mTOR signals

Gang Tang[1], Yi Du[1], Haochen Guan[1], Jieshuang Jia[1], Nan Zhu[1], Yuping Shi[1], Shu Rong[1], and Weijie Yuan[1]

[1]Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine

December 2, 2020

Abstract

Muscle protein catabolism in patients with diabetic nephropathy (DN) results in striking losses of muscle proteins, which increases morbidity and mortality risks. Emerging evidence shows that short-chain fatty acids (SCFAs) play an important role in the maintenance of health and disease development. Recently, the connection between butyrate (a SCFA) and DN has been revealed, although the relationship between butyrate and muscle atrophy is still not clear. In our study, we found a significant decrease in butyrate in DN using metabolomics analyses. The addition of butyrate remarkably intestinal barrier function. Concurrently, butyrate could alleviate muscle atrophy and promote PI3K/AKT/mTOR signals, and suppress oxidative stress and autophagy in the skeletal muscle of db/db mice as well as high glucose/lipopolysaccharide (HG/LPS)-induced C2C12 cells. To further explore the mechanism, we found that GPR43, the key SCFAs signaling molecule, was significantly decreased in the skeletal muscle of db/db mice and HG/LPS-induced C2C12 cells. Overexpression of GPR43 could activate PI3K/AKT/mTOR signals and inhibit oxidative stress and autophagy in HG/LPS-induced C2C12 cells. Silencing of GPR43 blocked PI3K/AKT/mTOR signals improved by butyrate, as well as suppression of oxidative stress and reduction of autophagy. Ultimately, butyrate alleviated muscle atrophy in DN via GPR43-mediated PI3K/AKT/mTOR pathway

Title Page

Butyrateameliorate skeletal muscle atrophy inDiabetic Nephropathy via enhancing gut barrier function and GPR43mediatedPI3K/AKT/mTOR signals

Gang Tang[1]*, Yi Du[1]*, Haochen Guan[1]*, Jieshuang Jia[1], Nan Zhu[1],Yuping Shi[1], Shu Rong[1#], Weijie Yuan[1#]

1. Department ofNephrology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine,100 Haining Road, Shanghai 200080, P.R. China

 # Correspondence Weijie Yuan and Shu Rong, Department of Nephrology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, 100 Haining Road, Shanghai 200080, China. E-mail: ywj4168@163.com; *sophiars@126.com*

*** These authors contributed equally to this work**

Acknowledgements

This study was supported by the National Natural Science Foundation of China (grant no. 81700621) and by the National Natural Science Foundation of China (grant no. 8197062).

Conflicts of interest

The authors declare there is no conflicts of interest regarding the publication of this paper.

Data Availability Statment

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

Abstract

Muscle protein catabolism in patients with diabetic nephropathy (DN) results in striking losses of muscle proteins, which increases morbidity and mortality risks. Emerging evidence shows that short-chain fatty acids (SCFAs) play an important role in the maintenance of health and disease development. Recently, the connection between butyrate (a SCFA) and DN has been revealed, although the relationship between butyrate and muscle atrophy is still not clear. In our study, we found a significant decrease in butyrate in DN using metabolomics analyses. The addition of butyrate remarkably intestinal barrier function. Concurrently, butyrate could alleviate muscle atrophy and promote PI3K/AKT/mTOR signals, and suppress oxidative stress and autophagy in the skeletal muscle of db/db mice as well as high glucose/lipopolysaccharide (HG/LPS)-induced C2C12 cells. To further explore the mechanism, we found that GPR43, the key SCFAs signaling molecule, was significantly decreased in the skeletal muscle of db/db mice and HG/LPS-induced C2C12 cells. Overexpression of GPR43 could activate PI3K/AKT/mTOR signals and inhibit oxidative stress and autophagy in HG/LPS-induced C2C12 cells. Silencing of GPR43 blocked PI3K/AKT/mTOR signals improved by butyrate, as well as suppression of oxidative stress and reduction of autophagy. Ultimately, butyrate alleviated muscle atrophy in DN via GPR43-mediated PI3K/AKT/mTOR pathway.

Keywords: muscle atrophy; butyrate; GPR43; diabetic kidney disease; db/db mice

1. Introduction

Protein-energy wasting (PEW) is a state of disordered catabolism resulting from metabolic and nutritional derangements in chronic disease states. Patients with DN and end-stage renal disease (ESRD) exhibit muscle atrophy that contributes to frailty and morbidity(Hanna *et al.* , 2020; Obi *et al.* , 2015). Preventing PEW is the most pressing clinical concern in DN. Catabolic pathways that cause protein wasting include activation of the ubiquitin-proteasome system (UPS), caspase-3, lysosomes, and myostatin, a negative regulator of skeletal muscle growth(Wang XH and Mitch, 2014). Muscle atrophy is a manifestation of PEW. Recently, oxidative stress and autophagy were found to be remarkably associated with muscle atrophy (Abrigo *et al.* , 2018; Wang XH and Mitch, 2014). Mounting evidence has shown that the PI3K/AKT/mTOR pathway is a major factor mediateing the regulation of muscle protein metabolism(Franke *et al.* , 2003; Katso *et al.* , 2001; Wang XH *et al.* , 2009; Wang XH and Mitch, 2014).

Research has shown that SCFAs play important roles in health maintenance and disease development. SCFAs are a subset of fatty acids produced by the gut microbiota during the fermentation of partially and nondigestible polysaccharides(Tan *et al.* , 2014). Butyrate is a class of SCFAs, and reportedly plays a protective role against diabetes and kidney disease(Andrade-Oliveira *et al.* , 2015; Xu *et al.* , 2018). Our previous study found that butyrate alleviated DN. Furthermore, we conducted both *in vivo* and *in vitro*experiments to prove that butyrate ameliorates renal fibrosis(Du*et al.* , 2020).

Intestinal dysbiosis is a common phenomenon in DN. Uraemia significantly contributes to dysbiosis and alters the biochemical environment of the gut(Anders *et al.* , 2013; Nallu *et al.* , 2017; Vaziri*et al.* , 2013). This can lead to alteration of TJ proteins in intestinal epithelial cells, which results in impairment of intestinal barrier function. Research has suggested that the disruption of colonic epithelial tight-junction apparatus in uraemic rats(Vaziri *et al.* , 2012). These events enable leakage of noxious contents, including phenols, cresols, and LPS, into the intestinal wall and systemic circulation(Nallu *et al.* , 2017; Vaziri *et al.* , 2016). The presence of various toxic biomolecules can also aggravate muscle atrophy, along with associated diseases, such as diabetes and DN. Energy metabolism disorder is a specific characteristic of DN. Mechanisms that may cause muscle atrophy in a rat model of CKD and db/db mice were investigated(Kim *et al.* , 2019; Wang M *et al.* , 2019). In the muscles of all models of diabetes or CKD, levels of protein synthesis serine/threonine-protein

kinase mTOR markers appeared decreased. Akt signaling and its downstream signaling are unique in that it controls both protein synthesis and protein degradation(Sandri, 2013). mTOR is at the centre of several cellular processes, such as downstream of Akt signaling. Researchers have suggested that the development of a new molecule specifically activating the Akt pathway may be extremely useful for counteracting muscle atrophy.

Another major mechanism resulting in muscle atrophy in DN is autophagy and lysosomal proteolysis. Importantly, the formation of autophagosomes is stimulated by decreased levels of phosphatidylinositol 3-kinase (PI3K). An increase in the levels of the PI3K/Akt signaling pathway is critical for autophagy suppression(Sandri, 2013; Wang XH and Mitch, 2014). Recent studies have demonstrated that oxidative stress can initiate autophagosome formation and autophagic degradation by acting as cellular signaling molecules(Glick *et al.* , 2010). However, the effect of butyrate on these mechanisms and muscle atrophy remains unclear.

To explore the role of butyrate in muscle atrophy caused by DN, we investigated the level of butyrate in the blood of DN patients and healthy people. Meanwhile, db/db mice received supplementary butyrate and normal saline for comparison. Urinary albumin, serum creatinine (SCr), blood urea nitrogen (BUN), and muscle weight were used as indicators of renal function and skeletal muscle atrophy, respectively. The levels of ZO-1, occludin, mucin, and Muc2 were determined to show changes in intestinal barrier function in db/db mice with or without butyrate supplementation. Malondialdehyde (MDA) and the activity of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) were used to observe the oxidative stress state, while p62 and LC3-II were studied to explore the effect of butyrate on autophagy in skeletal muscle tissue in db/db mice and C2C12 myoblasts. The activation of the PI3K/AKT/mTOR pathway was valuated to reflect the state of protein synthesis. To study the underlying mechanism by which butyrate ameliorated muscle atrophy, we focused on GPR43, the key molecule in SCFAs singling, mediating butyrate's effect on intracellular signaling.

2. Materials and Methods

2.1 Study population

Blood samples were obtained from 15 inpatients and 42 healthy people at the Department of Nephrology and Physical Center, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine. Clinical variable were showed in Supplementary T1. Serum samples metabolites were diagnoses with DN with PEW according to the criteria proposed by the Joint Committee of Diabetic Nephropathy and International Society of Renal Nutrition and Metabolism (ISRNM)(Fouque*et al.* , 2008; Haneda *et al.* , 2015). The study was approved by the ethics committee of Shanghai General Hospital.

2.2 Sample Preparation

Samples were thawed in an ice-bath to diminish sample degradation. Plasma(25μL) was added to a 96-well plate. The the plate was then transferred to the Eppendorf epMotion Workstation (Eppendorf Inc., Humburg, Germany). Ice cold methanol(120μL) with partial internal standards was automatically added to each sample and vortexed vigorously for 5 (min). The plate was centrifuged at 4000 $\times g$ for 30 min (Allegra X-15R, Beckman Coulter, Inc., Indianapolis, IN, USA). Then, the plate was returned to the workstation. Supernatant(30μL) was transferred to a clean 96-well plate, and freshly prepared derivative reagents (20μL) were added to each well. The plate was sealed and the derivatization was carried out at 30°C for 60 min. After derivatization, 330μL ice-cold 50%methanol solution was added to dilute the sample. The plate was then stored at -20°C for 20 min and centrifugated at 4000 $\times g$ at 4°C for 30 min. 135μL supernatant was transferred to a new 96-well plate with 10μL internal standards in each well. Serial dilutions of the derivatized stock standards were added to the remaining wells. Finally the plate was sealed for LC-MS analysis.

2.3 Metabolomics analysis

Metabonomics analysis was performed using the Q300 Kit (Metabo-Profile, Shanghai, China). An ultraperformance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) system (ACQUITY UPLC-Xevo TQ-S, Waters Corp., Milford, MA, USA) was used to quantitate all targeted metabolites in this

study (Metabo-Profile Biotechnology (Shanghai) Co., Ltd). The optimized instrument settings are briefly described as follows. For HPLC, column: ACQUITY HPLC BEH C18 1.7 × 10-6 m VanGuard precolumn (2.1 x 5 mm) and ACQUITY HPLC BEH C18 1.7 x 10-6 m analytical column (2.1 x 100 mm), column temp.: 40 degC, sample manager temp.: 10 degC, mobile phases: A = water with 0.1% formic acid; and B = acetonitrile/IPA (70:30), gradient conditions: 0–1 min (5% B), 1–11 min (5%–78% B), 11–13.5 min (78%–95% B), 13.5–14 min (95%–100% B), 14–16 min (100% B), 16–16.1 min (100-5%B), 16.1–18 min (5% B), flow rate: 0.40 mL min-1, and injection volume: 5.0 μL. For the mass spectrometer, capillary: 1.5 (ESI+), 2.0 (ESI-) Kv, source temperature: 150 °C, desolvation temperature: 550 °C, and desolvation gas flow: 1000 L h-1.

For data processing, the raw data files generated by UPLC-MS/MS were processed using the iMAP platform (v1.0; Metabo-Profile, Shanghai, China) to calculate the concentration of each analyte in the samples. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were also performed with iMAP (v1.0). VIP Variable importance in projection (VIP) was obtained based on the OPLS-DA model. Metabolites with VIP [?]1 and p-value <0.05 (univariate analyses were based on whether the data were normally distributed) were regarded as statistically significant (differentially expressed metabolites: DEMs). The Z-score indicates how many standard deviations an observation is above or below the mean of the control group. The Vplot that integrates the fold change and P -values, was used to depict the significantly different metabolites.

2.4 Animals

Male db/db and db/m mice, 4weeks old, were purchased from the Nanjing Institute of Model Animals. All mice were kept at the animal centre of the Shanghai General Hospital at 22degC under a 12 h light/12 h dark cycle with free access to water. When the mice were 8 weeks old, their blood glucose levels were randomly measured and those with?16.7[?]mM of blood glucose were considered to be diabetic. The diabetic mice were divided into the DN group (n=5) and DN+Bu group (n=5). A standard diet was administered to the mice belonging to the NC and DN groups while those of the DN+Bu group were fed with a Bu diet, which was prepared by blending the powdered form of Bu (Sigma-Aldrich, MO, USA) with the standard diet using a food processor, followed by centrifugation at 18 xg , such that, the final proportion of Bu in the diet was 1% (Medicine, Nanjing, China). The diet containing Bu was pelleted and stored in a freezer at?-20degC until use. Mice administered the Bu diet received 1g/kg/day of butyrate at the normal rate of caloric intake(Dong*et al.* , 2017). When the mice were sacrificed after oral administration of Bu for 12 weeks, the thigh, gastrocnemius, soleus and tibial anterior (TA) muscles, and serum were collected. Their blood glucose and LPS levels were recorded on Mondays 4, 8, 12, 16, and 20 weeks after birth, while urine was collected on the Monday morning of week 20. Urinary albumin, SCr and BUN were detected using a fully automated biochemical analyser (Rayto, Shenzhen, China). Butyrate levels were also determined.

All experiments involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai General Hospital in compliance with the Guide for the Care and Use of Laboratory Animals and the US National Institute of Health, project number 2019DW001.

2.5 Cell Culture

C2C12 murine myoblast cells (C2C12) were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Gibco, New York, USA) supplemented with 10% foetal bovine serum (FBS, Gibco, New York, USA), 2mmol L-glutamine, and 1% penicillin-streptomycin (PS) solution (NCM biotech, Suzhou, China) in a humidified atmosphere containing 5% CO2/95% air at 37degC. Cells were grown to confluence (approximately six days), at which point the medium was changed to differentiation media (DM) composed of DMEM plus 2% horse serum (HS) (HyClone, New York, USA), and 1% penicillin-streptomycin (PS) solution to initiate differentiation. LPS (Sigma-Aldrich) was used to induce oxidative stress and atrophy models in C2C12 myoblasts(Baker *et al.* , 2018). After differentiation for six day, myotubes were exposed to one of three conditions: I, vehicle Control (NC): DM; ii, high glucose/LPS (HG/LPS): DM+ 30mM/L of glucose+ 100ng/mL LPS ; iii, butyrate (HG/LPS+Bu): DM+ 30mM/L of glucose+ 100ng/mL LPS + 0.5mM Bu.

2.6 Tissue processing

The colon and ileum were isolated at necropsy and stored at -80degC. The first 1/3 of the ascending colon was cut for histology and the next 2/3 was cut and stored at -80degC for other studies. The gastrocnemius(1/4), isolated from the mice post-mortem, weas harvested, and fixed in 4% of paraformaldehyde, embedded in paraffin, and cut into 5μm thick sections. The remaining gastrocnemius was stored at -80°C for follow-up studies.

2.7 RT-qPCR

TRIzol reagent (Invitrogen, CA, USA) was used to isolate total RNA, and RT-qPCR was performed using the TB Green Premix Ex Taq kits (Takara, Shiga, Japan), according to the manufacturer's instructions. Primer sequences were checked for specificity and assay efficiency by performing standard curve analysis, and the sequences were as follows: IL-6, F: 5'-CTCCCAACAGACCTGTCTATAC-3', R: 5'-CCATTGCACAACTCTTTTCTCA-3'; TNF-α, F: 5'-ATGTCTCAGCCTCTTCTCATTC-3', R: 5'-GCTTGTCACTCGAATTTTGAGA-3'; IL-1β, F: 5'-TGATGAAAGACGGCACACCC-3', R: 5'-TGTCCCGACCATTGCTGTTT-3'; CRP, F: 5'-CCTTCGTATTTCCCGGAGTGTC-3', R: 5'-CTCACATCAGCGTGGGCATAG-3'; GAPDH, F: 5'-CTGGAGAAACCTGCCAAGTATG-3', R: 5'-GGTGGAAGAATGGGAGTTGCT-3').

2.8 Enzyme-linked immunosorbent assay (ELISA)

Serum levels of IL-6, TNFα, IL-1β, and CRP were determined using IL-6 Quantikine ELISA Kit (M6000B, R&D, Minneapolis, MN, USA), TNF-alpha Quantikine ELISA Kit (MTA00B, R&D, Minneapolis, MN, USA), IL-1 beta/IL-1F2 Quantikine ELISA Kit (MLB00C, R&D, Minneapolis, MN, USA), C-Reactive Protein/CRP Quantikine ELISA Kit (MCRP00, R&D, Minneapolis, MN, USA), respectively.

2.9 Haematoxylin-eosin (HE) staining

After tissue processing, paraffin-embedded gastrocnemius sections were deparaffinized with xylene, washed with ethanol and then washed with water, and stained with haematoxylin and eosin. Then, six images were taken randomly for each sample, and the myofiber cross-sectional area of these images was analyzed using ImageJ.

2.10 Transmission Electron Microscopy (TEM)

The fresh gastrocnemius muscle was sectioned in 40mm3 blocks, immersed for 1 h in fixative solution (0.5% glutaraldehyde, 2% paraformaldehyde, 7% saccharose, and 4% polyvinylpyrrolidone in 0.1M cacodylate buffer), and rinsed with 1X PBS buffer. These muscle blocks were recut into smaller samples of about 10mm3 and fixed with another fixative solution (2% osmic acid in 0.1M cacodylate buffer) for 1 h. Subsequently, the samples were dehydrated, infiltrated, and embedded in Epon 812 at 60° for 48 h and cut into longitudinal or transversal sections 0.1 μm thick. These sections were stained with 1% uranyl acetate and Reynolds's lead citrate. They were observed on a Leica UC7 HT7700 (HITACHI) microscope to analyze the ultrastructure of mitochondria.

2.11 Immunohistochemistry (IHC)

The colon, ileum and muscle tissue sections were subjected to heat treatment in a citrate solution for antigen clearance and incubated with 3% bovine serum albumin (BSA) blocking solution at room temperature for 30?min. Thereafter, the sections were incubated at 4°C overnight with corresponding primary antibodies (for colon and ileum: ZO-1, 1:200, Abcam, USA; Occludin, 1:200 Cell Signaling Technology, USA. For muscle: GPR43, 1:300, bs-13536R, Bioss, USA). After incubation with goat anti-rabbit secondary antibody (1:200, Servicebio, Wuhan, China) at room temperature for 1h, the slides were treated with DAB using Dako REAL Envision (K5007; Dako; Agilent Technologies, Inc.), according to the manufacturer's instructions.

2.12 Immunofluorescence (IF)

Immunofluorescence staining was conducted according to the manufacturer's instructions. Tissue sections were incubated with corresponding primary antibodies (for colon and ileum: Muc2, 1:100, Santa Cruz Biotechnology, Inc.) at 4°C overnight, followed by incubation with corresponding anti-mouse secondary antibodies for 1 h at room temperature.

Regarding cells, C2C12 myoblasts grown on coverslips were fixed with 4% formaldehyde solution for 20min. After washing with PBS twice, the cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min, washed with PBS twice, and incubated with phalloidin (1:200 in PBS) to visualise the F-actin filaments in myotubes.

For tissue sections and cells, nuclei were stained with Antifade Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) for 5min.

2.13 Alcian blue stain

Alcian blue staining is widely used for mucins(Kameyama *et al.* , 2015). After dewaxing and hydration, the colon sections were exposed to Alcian blue stain for 30 min at room temperature. The sections were then dehydrated and mounted.

2.14 Oxidative Stress Markers

Gastrocnemius (100_mg) preserved at -80degC was treated via ultrasonic grinding and centrifuged at 12,000 rpm for 15 min at 4degC to obtain supernatants. Blood was centrifuged at 3,000 rpm for 20 min to obtain serum. The activities of SOD, CAT, GSH-Px, and MDA were determined using detection kits (SOD: A001-3-2; CAT: A007-1; GSH-Px: A005-1-2; MDA: A003-1, Nanjing Jiancheng Bio-engineering Institute Co., Ltd.) according to the manufacturer's protocols.

2.15 ROS Measurements

The intracellular ROS levels were measured using a Reactive Oxygen Species Assay Kit (Beyotime Biotechnology, China); 2', 7'-dichlorofluorescein-diacetate (DCFH-DA), which is easily oxidized to fluorescent dichlorofluorescein (DCF) by intracellular ROS, is its principal component, and therefore, the ROS levels were quantified. Briefly, the cells were seeded in 6-well plates as described above and following 6 days of differentiation, myotubes were exposed to corresponding conditions. Following the treatment, the cells were incubated with DCFH-DA for 20min at 37degC and then observed using fluorescence microscopy (Leica) and measured at 488 nm excitation and 525 nm emission by a fluorescence spectrophotometer (BioTek).

2.16 Western blot (WB)

The tissues and cell samples were mixed with radioimmunoprecipitation assay (RIPA) lysis buffer containing phenyl methane sulfonyl fluoride (PMSF) and phosphatase inhibitors to isolated proteins. The samples were then centrifuged at 12,000 rpm for 15 min, and the supernatant was collected for protein quantitation using the a bicinchoninic acid (BCA) assay. Processed proteins were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After blocking with 5% skim milk for 1 h, the membranes were incubated overnight at 4degC with the following primary antibodies: ZO-1 (1:500, Abcam, USA), occludin, PI3K, p-PI3K, AKT, p-AKT, mTOR, p-mTOR, p62, LC3B (all 1:500, Cell Signaling Technology, USA), GPR43 (1:500, bs-13536R, Bioss, USA), GAPDH (1:1000, Cell Signaling Technology, USA), and ∝-tubulin (1:1000, Proteintech, China). The membranes were then incubated with goat anti-rabbit (1:10000; abs20002, Absin, China) or goat anti-mouse secondary antibodies (1:10000; abs20001, Absin, China). Band visualisation was performed using an electrochemiluminescence (ECL) reagent (NCM biotech, China). The blots were quantified using ImageJ software.

2.17 Construction of stabilized GPR43 Overexpression C2C12 myoblasts

GPR43 cDNA was amplified using PCR and cloned into the pGMLV-EF1 lentiviral vector (Genomeditech Inc., China). Stable cell lines that expressed GPR43 were selected by two rounds of treatment with 1.5μg/ml

puromycin for two days. We constructed stable GPR43-overexpressing cell lines C2C12-GPR43 and their corresponding stable control cell lines C2C12-pGMLV-Vector.

2.18 siRNA-mediated gene silencing of GPR43

To silence the GPR43 gene, we used a small interfering RNA (siRNA) that targets GPR43 (Genomeditech Inc., China) and the siRNA negative control, which does not target any known sequence. Transfection with 20 nM siRNA duplex was performed using Lipofectamine 2000 reagent (Invitrogen, Life technologies, USA) according to the manufacturer's protocols. Cells were transfected for 48 h and then stimulated with 30mM of glucose + 100 ng/mL LPS in the presence or absence of 0.5mM Bu for 24 h.

2.19 Statistical analysis

All statistical analyses were carried out using the statistical software package SPSS, version 22.0 (IBM SPSS, USA). The data are expressed as the mean?±?standard error of mean (SEM). Comparisons between groups were performed using the two-tailed paired Student's t -test. One-way ANOVA followed by the Student-Newman-Keuls post-hoc test was used to analyse the differences between multiple groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

3.1 Serum butyrate levels are significantly correlated with DN and DN-induced muscle atrophy

To investigate the butyrate levels of patients with DN, targeted metabolomics analyses based on UPLC/MS were applied to investigate serum samples of 15 inpatients paired with 42 healthy people. A total of 146 metabolites were identified in serum, 44 metabolites were significantly upregulated, 32 metabolites were down-regulated, and 70 metabolites exhibited no change in DN serum (filtered by |FC (fold change)| [?] 2 and P < 0.05) (Fig 1 A, B). We found that serum butyrate levels decreased significantly in DN compared with that in healthy people (P <0.05) (Fig1 C). The ROC curve was plotted to evaluate the diagnostic value of muscle atrophy. The area under the ROC curve (AUC) was 0.749, suggesting a modest accuracy for butyrate in the diagnosis of muscle atrophy (Fig 1 D). Furthermore, serum butyrate levels in db/db mice had declined compared to those of the NC group, while the diet containing butyrate contributed to a significant improvement in serum butyrate levels (P <0.01, Fig1 E).

To show the effect of butyrate on renal function, we investigated several biochemical criteria and found that the levels of SCr, BUN and urinary albumin/creatinine were within normal ranges in the NC group, while they were increased in the db/db mice (P <0.01). After supplementation with butyrate, significant reductions in the levels of SCr, BUN, and urinary albumin/Creatinine were observed in the butyrate group (P <0.05, Fig1 G-I). No obvious difference was observed in blood glucose levels when db/db mice was received butyrate supplementation (Fig 1 F).

3.2 Butyrate alleviated inflammation and reduced LPS levels in db/db mice

Circulating inflammation levels generally increased in patients with DN, and high levels of CRP predicted all-cause mortality, especially in patients with hypoalbuminemia. Levels of muscle and circulating proinflammatory cytokines, including IL-6, TNFα, IL-1β, and CRP, were detected in this study. In db/db mice, all proinflammatory cytokines increased significantly compared with those in the NC group (P <0.001). However, the levels of IL-6, TNFα, IL-1β, and CRP were remarkably decreased after administration of the butyrate diet (Fig 2 A-H). Conversely, serum LPS levels were significantly higher in db/db mice than in the NC group (P <0.001), and supplementation with butyrate relieved this effedt (Fig 2 I, J).

3.3 Butyrate enhanced gut barrier function

To explore the alteration in DN, we stained the ileum and colon with Alcian blue (Fig 3 A, B), known as a dye to stain mucin. Mucin expression in the db/db mice was significantly lower than that in the NC group (P <0.001). After butyrate treatment, mucin synthesis increased and mucin expression was remarkably elevated in all ileum and colon samples(P <0.05,P <0.001).

Alcian blue stained all mucins, which were secreted by goblet cells. Muc2 was the most distinctive and prominent mucin secreted in the intestinal tract. Thus, we used IF and observed that the expression of Muc2 in the ileum and colon was significantly reduced in the db/db mice (P <0.001). And butyrate treatment significantly improved Muc2 levels (P <0.01, P <0.001) (Fig3 C, D).

These results indicate that mucins and Muc2 all exhibited significant changes in the ileum and colon. However, the overall expression of mucins and Muc2 in the colon was higher than that in the ileum. Further, we used colon tissues to research the intestinal TJ proteins. Our immunohistochemistry data showed that TJ proteins, ZO-1 and occludin were significantly lower in db/db mice and butyrate treatment improved them (P <0.001, Fig 3 E, F). Meanwhile, immunoblot analysis also suggested that the levels of ZO-1 and occludin of colon in db/db mice were remarkably decreased compared with those in the NC group, and butyrate treatment abrogated the reduction (Fig 3 G).

3.4 Butyrate ameliorated skeletal muscle atrophy

To observe the muscle alteration in different groups, we measured the tight and gastrocnemius muscle mass of mice for comparison. The results showed that tight and gastrocnemius muscle mass were significantly reduced in db/db mice compared with the NC group (P <0.001), while butyrate treatment blocked this decrease (P <0.01, Fig4 A-D). In the micro measurement, HE staining also showed that the cross-sectional area of muscle fibres in db/db mice was significantly smaller than that in the NC group (P <0.001), while butyrate treatment alleviated the atrophy (P <0.001, Fig 4 E, F). In addition, myotubes incubated with HG/LPS for 48 h displayed an obvious level of morphological atrophy, with a nearly 50% reduction in myotube diameter (P <0.001), but no change in myotube number (Fig 4 G-I). Butyrate treatment, along with HG/LPS, remarkably resolved HG/LPS-induced atrophy (P <0.001) with no difference in myotube number.

3.5 Butyrate suppressed autophagy and oxidative stress, and activated the PI3K/AKT/mTOR pathway in db/db mice and HG/LPS-induced C2C12 myoblasts

Autophagy-lysosomal proteolysis plays an important role in DN-induced muscle atrophy. During autophagy, double membranes form around defective organelles or cytoplasmic proteins that are disrupted or damaged. To evaluate the effect of butyrate on autophagy in muscle atrophy, we observed the formation of autophagosomes and autolysosomes via TEM in mouse skeletal muscle tissue (Fig5 A). The results showed that the number of autophagosomes and autolysosomes in db/db mice was distinctly higher than that in the NC group (P <0.01). After butyrate treatment, the number of autophagosomes and autolysosomes was remarkably reduced (P <0.05). To further verify the inhibitory effect of butyrate on autophagy, we used western blotting to measure the expression of LC3II and p62 in skeletal muscle and C2C12 myoblasts. We found that levels of LC3II was significantly increased (P <0.01), while p62 expression was decreased both in skeletal muscle of db/db mice and C2C12 myoblasts incubated with HG/LPS (P <0.05). However, butyrate treatment reversed the upregulation of LC3II and the downregulation of p62 (P <0.05, Fig 5 B, C).

Previous studies have reported that ROS could induce the autophagy. Thus, we examined the influence of butyrate on oxidative stress. Catalase (CAT), glutathione peroxidase (GSH-Px), malondialdehyde (MDA), and superoxide dismutase (SOD) are known biomarkers of the antioxidant defence system and lipid peroxidation. In our studies, we found that the levels of CAT, GSH-Px, and SOD were distinctly decreased in the skeletal muscle of the DN group (P <0.001), indicating that the antioxidant defence system was impaired in DN (Fig 5 D). The damage also occurred in C2C12 myoblasts incubated with HG/LPS, while the ROS activity was increased (P <0.01, Fig 5 E and F). However, the impaired antioxidant defence system was reversed by butyrate treatment, which also alleviated the ROS activity. Correspondingly, both in the skeletal muscle of db/db and C2C12 myoblasts incubated with HG/LPS, the levels of MDA were significantly improved (P <0.05), while they appeared to be reduced with butyrate treatment (Fig5 D, F).

To further investigate the molecular mechanism underlying butyrate's effect on muscle atrophy, we focused on the oxidative stress-mediated PI3K/AKT/mTOR pathway, the key signaling pathway of protein synthesis. The western blotting results showed that the expression of p-PI3K, p-AKT, and p-mTOR was all significantly

decreased in skeletal muscle in the DN group and C2C12 myoblasts induced by HG/LPS. A remarkably upward trend of p-PI3K, p-AKT, and p-mTOR appeared with butyrate treatment both in tissues and cells (Fig 5 G, H).

3.6 Butyrate inhibited the reduction of GPR43 in db/db mice and HG/LPS-induced C2C12 myoblasts

Many studies have proven that GPR43 is a key molecule involved in butyrate- mediated metabolism. However, there is a paucity of information regarding GPR43 in muscle atrophy. Using IHC and IF, we found that the levels of GPR43 in the skeletal muscle of db/db mice were distinctly decreased compared with those in the NC group (P <0.01, Fig 6 A, B). To obtain further evidence, GPR43 expression was measured using western blotting. Compared with the NC group, we observed a remarkable downregulation of GPR43 ($P<$ 0.01, Fig 6 C, D) in the db/db mice and HG/LPS-induced C2C12 myoblasts. After butyrate treatment, significant upregulation of GPR43 appeared in all experiments ($P < 0.01$).

To further verify the mechanisms by which GPR43 affects muscle atrophy, we successfully constructed the stabilized GPR43 overexpression (pGMLV-GPR43) C2C12 myoblasts and siRNA-mediated gene silencing of GPR43 (Fig 6 E, F). We used siRNA 1 (siRNA-GPR43) with the best silencing effect in subsequent experiments.

3.7 GPR43 attenuated atrophy by suppressing oxidative stress and autophagy, and activating the PI3K/AKT/mTOR pathway in HG/LPS-induced C2C12 myoblasts

The TRITC-phalloidin staining results showed that overexpression of GPR43 prominently reversed the reduction of myotube diameters in HG/LPS-induced C2C12 myoblasts ($P < 0.001$), while silencing of GPR43 did not improve this decreasing trend (Fig7 A, B). Meanwhile, regardless of whether GPR43 expression changed, the number of myotubes remained unchanged.

To observe the influence of GPR43 on autophagy, we used western blotting to evaluate the levels of LC3II and p62. Compared with the HG/LPS group, we found that the LC3II expression was distinctly decreased ($P<$ 0.05), while p62 expression increased in the pGMLV-GPR43 group ($P < 0.01$, Fig 7 C). However, silencing GPR43 prevented its role in reversing LC3II upregulation and p62 downregulation (Fig 7 D).

We analysed ROS levels by examining fluorescence and oxidative stress-related factors, including CAT, GSH-Px, SOD, and MDA in C2C12 myoblasts. Our studies found that overexpression of GPR43 remarkably reduced HG/LPS-induced strong ROS fluorescence ($P < 0.001$), whereas the staining intensity did had not significantly decrease upon silencing of GPR43, compared with that induced by HG/LPS (Fig7 E, G). HG/LPS also resulted in decreased CAT, GSH-Px, and SOD activity, and increased MDA, which were reversed by overexpression of GPR43, but not GPR43 silencing (Fig 7 F, H).

To evaluate whether the beneficial effect of GPR43 on muscle atrophy was related to the PI3K/AKT/mTOR pathway in C2C12 myoblasts, we determined the levels of p-PI3K, p-AKT, and p-mTOR using western blotting. Results indicated that overexpression of GPR43 significantly improved p-PI3K, p-AKT, and p-mTOR expression ($P < 0.05$), which were reduced by HG/LPS treatment. However, the effect disappeared with GPR43 silencing (Fig 7 I, J). This indicated that GPR43 promoted activation of the PI3K/AKT/mTOR pathway to alleviate muscle atrophy.

3.8 Butyrate alleviated muscle atrophy via GPR43 against ROS and further activate the PI3K/AKT/mTOR pathway

According to the above experiments, overexpression of GPR43 and butyrate treatment both attenuated HG/LPS-induced C2C12 myoblast atrophy. To examine whether butyrate attenuated the atrophy by GPR43, HG/LPS was used to treat pGMLV-GPR43 and siRNA-GPR43 C2C12 myoblasts, respectively. After TRITC-phalloidin staining, we observed that the beneficial effect of butyrate against C2C12 myoblast morphological atrophy disappeared in siRNA-GPR43 C2C12 myoblast, which suggested that butyrate could not alleviate muscle atrophy without GPR43 (Fig 7 A, B). Similarly, butyrate significantly suppressed the upregulation of LC3II and downregulation of p62 in HG/LPS-induced pGMLV-GPR43 C2C12 myoblasts (P

< 0.05), whereas the trend was not reversed in siRNA-GPR43 C2C12 myoblasts (Fig 7 C, D). In addition, increased ROS fluorescence, MDA levels, and decreased CAT, GSH-Px, and SOD activity in HG/LPS-induced siRNA-GPR43 C2C12 myoblasts did not change with or without butyrate treatment (Fig 7 E-H). Further studies showed that the promotion of butyrate to activate the PI3K/AKT/mTOR pathway also disappeared in HG/LPS-induced siRNA-GPR43 C2C12 myoblasts. These results indicated that butyrate inhibited the oxidative stress-mediated PI3K/AKT/mTOR pathway, possibly by upregulating GPR43 expression.

Discussion

Butyrate is the least abundant SCFA. Previous studies have demonstrated that it has antioxidant, anti-inflammatory, and immunomodulatory effects, regulates intestinal barrier function, and modulates diabetic-endotoxemia (Chang *et al.* , 2014; Gao *et al.* , 2009; Gonzalez *et al.* , 2019; Xu*et al.* , 2018). However, there are few reports on the protective effect of butyrate against oxidative damage and fibrosis in DN kidneys (Krokowicz *et al.* , 2014), while no reports regarding the correlation between butyrate and muscle atrophy in DN. In this study, we quantitated serum sample metabolites and found a significant butyrate reduction in patients with DN and modest accuracy in the diagnosis of muscle atrophy. Furthermore, we demonstrated that DN-induced loss of renal function and skeletal muscle atrophy can be reversed by butyrate. Further, inflammatory factors in muscles and serum were also remarkably reduced with butyrate treatment in db/db mice. In addition, butyrate significantly slowed down the rising trend of serum LPS, which was consistent with enhanced intestinal barrier function after butyrate treatment in db/db mice. Increased ROS production and decreased antioxidant enzymes commonly result in oxidative stress, which contributes to DN-induced muscle atrophy. Our present study also confirmed that mitochondrial dysfunction and subsequent production of ROS decreased antioxidant enzymes activities, including CAT, GSH-Px, and SOD, which were reversed after butyrate treatment in db/db mice. Furthermore, we verified that the above process involved the PI3K/AKT/mTOR pathway (the key pathway related to protein synthesis), which was activated by butyrate in db/db mice. In addition, we demonstrated that GPR43 is the central molecule regulating oxidant stress, the PI3K/AKT/mTOR pathway, and autophagy in muscle atrophy of DN through overexpression or silencing of GPR43.

'Diabetic-endotoxemia' is a new concept according to the characteristic of low-grade chronic inflammation (elevated inflammatory cytokines and LPS) in Type 2 diabetes mellitus (T2DM)(Gomes *et al.* , 2017). As one of the long-term complications of diabetes, diabetic nephropathy (DN) is a primary cause of end-stage renal disease(Dronavalli *et al.* , 2008). Diabetic-endotoxemia has also been recognized as a risk factor that is closely associated with both the onset and the progression of T2DM and subsequent DN (Dronavalli *et al.* , 2008). Recently, it is believed that gut dysbacteriosis may contribute to gut-leak, which plays a pivotal role in the source of this endotoxemia (Denis *et al.* , 2015). Numerous studies have confirmed that increased LPS levels contribute to CKD-induced muscle atrophy via *in vivo* or*in vitro* experiments(Baker *et al.* , 2018; Wang XH and Mitch, 2014). Thus, we hypothesized and demonstrated that butyrate ameliorated DN-induced muscle atrophy by preserving gut integrity, which suppressed the transposition of LPS from the gut to circulation. Austin et *al* . found that butyrate ameliorated renal failure in CKD rats by improving mucin expression (Gonzalez *et al.* , 2019), which was similar to our results in db/db mice. Reduction in serum LPS levels inhibited muscle atrophy. Therefore, our studies proved that butyrate mitigated muscle atrophy by enhancing intestinal barrier function.

Mitochondria are a key source of ROS, which are produced by normal metabolism in the body(Murphy, 2009). Mitochondrial dysfunction and decreased antioxidant enzyme activities result in elevated ROS production, which leads to oxidative stress. Furthermore, oxidative stress contributes to the pathogenesis and development of muscle injury(Ryan *et al.* , 2011). And Wang et *al* . proved that ROS production and oxidative stress also contributed to CKD-induced muscle atrophy(Wang M *et al.* , 2019). Some researchers have observed mitochondrial dysfunction in the skeletal muscle of CKD rats(Enoki *et al.* , 2017). In fact, we also observed morphological swelling in db/db mice. Mitochondrial dysfunction leads to loss of mitochondrial mass, and reduces the energy production. Oxidative stress, in turn, damages the mitochondria. Together, these constitute a vicious cycle, producing more ROS-aggravating muscle atrophy. In fact, ROS

play a positive role at low, regulated concentrations under physiological conditions(Di Meo *et al.* , 2016). They participate in the regulation of many cellular processes, including differentiation, proliferation, growth, apoptosis, cytoskeletal regulation, migration, and contraction (Krause, 2007). However, the delicate balance between appropriate redox states and oxidative stress is disrupted under pathological conditions. Oxidative stress is manifested by increased ROS generation and destruction of many cellular organelles and proteins, which involves muscle atrophy. In this study, we observed the harmful influence of oxidative stress on muscle atrophy in db/db mice.

We also observed increased autophagy and activation of the PI3K/AKT/mTOR pathway in the muscle of db/db mice and cell models. Although the influence of CKD on the activation of autophagy has not been rigorously evaluated, recent studies have suggested that autophagy plays a role in exacerbating muscle atrophy in CKD (Abrigo *et al.* , 2018; Su*et al.* , 2017). In one study, the rate of proteolysis in isolated muscles decreased when lysosomal functions were inhibited with weak bases (Wang XH and Mitch, 2014). Studies have shown that autophagy is activated by oxidative stress. However, the mechanisms by which ROS affect DN-induced muscle atrophy by regulating autophagy remain unclear. Importantly, it has been proven that the formation of autophagosomes is stimulated by decreased levels of p-PI3K, and CKD suppresses PI3K/Akt signaling, which also stimulates the autophagy–lysosome system (Sandri, 2013). ROS have been reported to suppress or promote autophagy via activation of Src/PI3K/AKT or inhibition of the AKT/mTOR pathway in different models of muscle atrophy (Pal *et al.* , 2014; Talbert*et al.* , 2013). Therefore, the antioxidant effect of butyrate suggests that it can suppress oxidative stress and consequently inhibit PI3K/AKT/mTOR pathway-mediated autophagy to protect DN-induced muscle atrophy.

Butyrate acts as an extracellular molecule in the above-mentioned intracellular activities, which raises the question about a component that mediates this signal transduction. Interestingly, GPR43, also known as free fatty acid receptor 2 (FFA2/FFAR2), is the primary receptor for SCFAs(Tan*et al.* , 2014). GPR43 expression has been identified along the entire gastrointestinal tract, including cells of both the immune and nervous systems (Tan *et al.* , 2014). Recent studies have shown that SCFAs may regulate adipocyte development in adiposity, modulate the development or differentiation of immune cells, and play an anti-inflammatory role (Dewulf *et al.* , 2013; Maslowski *et al.* , 2009; Senga *et al.* , 2003; Voltolini *et al.* , 2012). However, there are no reports regarding GPR43 expression and its regulatory effect on muscle atrophy. We hypothesised that butyrate reverses DN-induced muscle atrophy by regulating GPR43. First, we demonstrated that GPR43 expression in muscle in db/db mice were significantly lower than those in the NC group. Furthermore, our study showed that butyrate treatment improved tight and gastrocnemius mass in db/db mice, which was confirmed by GPR43-mediated activation of the PI3K/AKT/mTOR pathway, which was a central process that reduced of ROS production, resulting in suppressing of oxidative stress and inhibition of autophagy. Therefore, GPR43, as the specific receptor of SCFAs, could be more useful in promoting butyrate to treat DN-induced muscle atrophy. however, the strict molecular mechanism needs further research to be done.

Conclusions

In this study [Fig 9], we performed the metabonomics analysis and found butyrate, the key metabolite. We demonstrated the beneficial effects of butyrate on intestinal barrier function and muscle atrophy in db/db mice and HG/LPS-induced C2C12 myoblasts. We demonstrated, for the first time, that GPR43 expression was present in skeletal muscle and regulated the protection of butyrate in DN-induced muscle atrophy. This effect was mainly achieved by the PI3K/AKT/mTOR pathway, which regulates the oxidative stress-mediated autophagy in DN-induced muscle atrophy. Our studies suggest that butyrate may be clinically useful as a novel drug for the protection against muscle atrophy via promotion of the GPR43-mediated PI3K/AKT/m-TOR pathway in DN.

Author contributions

G.T., S.R. and W.J.Y. conceived and designed the study. G.T., Y.D. and H.C.G. carried out the acquisition, analysis and interpretation of data. G.T., Y.D., H.C.G., J.S.J., N.Z. and Y.P.S wrote the manuscript. S.R. and W.J.Y. supervised the study.

Declaration of transparency and scientific rigour

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research recommended by funding agencies, publishers and other organisations engaged with supporting research.

Reference

Abrigo J, Elorza AA, Riedel CA, Vilos C, Simon F, Cabrera D et al. (2018). Role of oxidative stress as key regulator of muscle wasting during cachexia. Oxid Med Cell Longev, 2018: 2063179.

Anders HJ, Andersen K, Stecher B (2013). The intestinal microbiota, a leaky gut, and abnormal immunity in kidney disease. Kidney Int, 83: 1010-1016.

Andrade-Oliveira V, Amano MT, Correa-Costa M, Castoldi A, Felizardo RJ, de Almeida DC et al. (2015). Gut bacteria products prevent aki induced by ischemia-reperfusion. J Am Soc Nephrol, 26: 1877-1888.

Baker LA, Martin NRW, Kimber MC, Pritchard GJ, Lindley MR, Lewis MP (2018). Resolvin e1 (rv e1) attenuates lps induced inflammation and subsequent atrophy in c2c12 myotubes. J Cell Biochem, 119: 6094-6103.

Chang PV, Hao L, Offermanns S, Medzhitov R (2014). The microbial metabolite butyrate regulates intestinal macrophage function via histone deacetylase inhibition. Proc Natl Acad Sci U S A, 111: 2247-2252.

Denis MC, Desjardins Y, Furtos A, Marcil V, Dudonne S, Montoudis A et al. (2015). Prevention of oxidative stress, inflammation and mitochondrial dysfunction in the intestine by different cranberry phenolic fractions. Clin Sci (Lond), 128: 197-212.

Dewulf EM, Ge Q, Bindels LB, Sohet FM, Cani PD, Brichard SM et al. (2013). Evaluation of the relationship between gpr43 and adiposity in human. Nutr Metab (Lond), 10: 11.

Di Meo S, Reed TT, Venditti P, Victor VM (2016). Role of ros and rns sources in physiological and pathological conditions. Oxid Med Cell Longev, 2016: 1245049.

Dong W, Jia Y, Liu X, Zhang H, Li T, Huang W et al. (2017). Sodium butyrate activates nrf2 to ameliorate diabetic nephropathy possibly via inhibition of hdac. J Endocrinol, 232: 71-83.

Dronavalli S, Duka I, Bakris GL (2008). The pathogenesis of diabetic nephropathy. Nat Clin Pract Endocrinol Metab, 4: 444-452.

Du Y, Yang YT, Tang G, Jia JS, Zhu N, Yuan WJ (2020). Butyrate alleviates diabetic kidney disease by mediating the mir-7a-5p/p311/tgf-beta1 pathway. FASEB J

Enoki Y, Watanabe H, Arake R, Fujimura R, Ishiodori K, Imafuku T et al. (2017). Potential therapeutic interventions for chronic kidney disease-associated sarcopenia via indoxyl sulfate-induced mitochondrial dysfunction. J Cachexia Sarcopenia Muscle, 8: 735-747.

Fouque D, Kalantar-Zadeh K, Kopple J, Cano N, Chauveau P, Cuppari L et al. (2008). A proposed nomenclature and diagnostic criteria for protein-energy wasting in acute and chronic kidney disease. Kidney Int, 73: 391-398.

Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C (2003). Pi3k/akt and apoptosis: Size matters. Oncogene, 22: 8983-8998.

Gao Z, Yin J, Zhang J, Ward RE, Martin RJ, Lefevre M et al. (2009). Butyrate improves insulin sensitivity and increases energy expenditure in mice. Diabetes, 58: 1509-1517.

Glick D, Barth S, Macleod KF (2010). Autophagy: Cellular and molecular mechanisms. J Pathol, 221: 3-12.

Gomes JMG, Costa JA, Alfenas RCG (2017). Metabolic endotoxemia and diabetes mellitus: A systematic review. Metabolism, 68: 133-144.

Gonzalez A, Krieg R, Massey HD, Carl D, Ghosh S, Gehr TWB et al. (2019). Sodium butyrate ameliorates insulin resistance and renal failure in ckd rats by modulating intestinal permeability and mucin expression. Nephrol Dial Transplant, 34: 783-794.

Haneda M, Utsunomiya K, Koya D, Babazono T, Moriya T, Makino H et al. (2015). A new classification of diabetic nephropathy 2014: A report from joint committee on diabetic nephropathy. Clin Exp Nephrol, 19: 1-5.

Hanna RM, Ghobry L, Wassef O, Rhee CM, Kalantar-Zadeh K (2020). A practical approach to nutrition, protein-energy wasting, sarcopenia, and cachexia in patients with chronic kidney disease. Blood Purif, 49: 202-211.

Kameyama A, Dong W, Matsuno YK (2015). Succinylation-alcian blue staining of mucins on polyvinylidene difluoride membranes. Methods Mol Biol, 1314: 325-331.

Katso R, Okkenhaug K, Ahmadi K, White S, Timms J, Waterfield MD (2001). Cellular function of phosphoinositide 3-kinases: Implications for development, homeostasis, and cancer. Annu Rev Cell Dev Biol, 17: 615-675.

Kim KW, Baek MO, Choi JY, Son KH, Yoon MS (2019). Analysis of the molecular signaling signatures of muscle protein wasting between the intercostal muscles and the gastrocnemius muscles in db/db mice. Int J Mol Sci, 20

Krause KH (2007). Aging: A revisited theory based on free radicals generated by nox family nadph oxidases. Exp Gerontol, 42: 256-262.

Krokowicz L, Stojcev Z, Kaczmarek BF, Kociemba W, Kaczmarek E, Walkowiak J et al. (2014). Microencapsulated sodium butyrate administered to patients with diverticulosis decreases incidence of diverticulitis–a prospective randomized study. Int J Colorectal Dis, 29: 387-393.

Maslowski KM, Vieira AT, Ng A, Kranich J, Sierro F, Yu D et al. (2009). Regulation of inflammatory responses by gut microbiota and chemoattractant receptor gpr43. Nature, 461: 1282-1286.

Murphy MP (2009). How mitochondria produce reactive oxygen species. Biochem J, 417: 1-13.

Nallu A, Sharma S, Ramezani A, Muralidharan J, Raj D (2017). Gut microbiome in chronic kidney disease: Challenges and opportunities. Transl Res, 179: 24-37.

Obi Y, Qader H, Kovesdy CP, Kalantar-Zadeh K (2015). Latest consensus and update on protein-energy wasting in chronic kidney disease. Curr Opin Clin Nutr Metab Care, 18: 254-262.

Pal R, Palmieri M, Loehr JA, Li S, Abo-Zahrah R, Monroe TO et al. (2014). Src-dependent impairment of autophagy by oxidative stress in a mouse model of duchenne muscular dystrophy. Nat Commun, 5: 4425.

Ryan MJ, Jackson JR, Hao Y, Leonard SS, Alway SE (2011). Inhibition of xanthine oxidase reduces oxidative stress and improves skeletal muscle function in response to electrically stimulated isometric contractions in aged mice. Free Radic Biol Med, 51: 38-52.

Sandri M (2013). Protein breakdown in muscle wasting: Role of autophagy-lysosome and ubiquitin-proteasome. Int J Biochem Cell Biol, 45: 2121-2129.

Senga T, Iwamoto S, Yoshida T, Yokota T, Adachi K, Azuma E et al. (2003). Lssig is a novel murine leukocyte-specific gpcr that is induced by the activation of stat3. Blood, 101: 1185-1187.

Su Z, Klein JD, Du J, Franch HA, Zhang L, Hassounah F et al. (2017). Chronic kidney disease induces autophagy leading to dysfunction of mitochondria in skeletal muscle. Am J Physiol Renal Physiol, 312: F1128-F1140.

Talbert EE, Smuder AJ, Min K, Kwon OS, Szeto HH, Powers SK (2013). Immobilization-induced activation of key proteolytic systems in skeletal muscles is prevented by a mitochondria-targeted antioxidant. J Appl Physiol (1985), 115: 529-538.

Tan J, McKenzie C, Potamitis M, Thorburn AN, Mackay CR, Macia L (2014). The role of short-chain fatty acids in health and disease. Adv Immunol, 121: 91-119.

Vaziri ND, Wong J, Pahl M, Piceno YM, Yuan J, DeSantis TZ et al. (2013). Chronic kidney disease alters intestinal microbial flora. Kidney Int, 83: 308-315.

Vaziri ND, Yuan J, Rahimi A, Ni Z, Said H, Subramanian VS (2012). Disintegration of colonic epithelial tight junction in uremia: A likely cause of ckd-associated inflammation. Nephrol Dial Transplant, 27: 2686-2693.

Vaziri ND, Zhao YY, Pahl MV (2016). Altered intestinal microbial flora and impaired epithelial barrier structure and function in ckd: The nature, mechanisms, consequences and potential treatment. Nephrol Dial Transplant, 31: 737-746.

Voltolini C, Battersby S, Etherington SL, Petraglia F, Norman JE, Jabbour HN (2012). A novel antiinflammatory role for the short-chain fatty acids in human labor. Endocrinology, 153: 395-403.

Wang M, Hu R, Wang Y, Liu L, You H, Zhang J et al. (2019). Atractylenolide iii attenuates muscle wasting in chronic kidney disease via the oxidative stress-mediated pi3k/akt/mtor pathway. Oxid Med Cell Longev, 2019: 1875471.

Wang XH, Du J, Klein JD, Bailey JL, Mitch WE (2009). Exercise ameliorates chronic kidney disease-induced defects in muscle protein metabolism and progenitor cell function. Kidney Int, 76: 751-759.

Wang XH, Mitch WE (2014). Mechanisms of muscle wasting in chronic kidney disease. Nat Rev Nephrol, 10: 504-516.

Xu YH, Gao CL, Guo HL, Zhang WQ, Huang W, Tang SS et al. (2018). Sodium butyrate supplementation ameliorates diabetic inflammation in db/db mice. J Endocrinol, 238: 231-244.