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Original article

Glycine supplementation during calorie restriction accelerates fat loss and protects against further muscle loss in obese mice

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SUMMARY

Background & aim: Calorie restriction (CR) reduces co-morbidities associated with obesity, but also reduces lean mass thereby predisposing people to weight regain. Since we demonstrated that glycine supplementation can reduce inflammation and muscle wasting, we hypothesized that glycine supplementation during CR would preserve muscle mass in mice.

Methods: High-fat fed male C57BL/6 mice underwent 20 days CR (40% reduced calories) supplemented with glycine (1 g/kg/day; n = 15, GLY) or L-alanine (n = 15, ALA). Body composition and glucose tolerance were assessed and hindlimb skeletal muscles and epididymal fat were collected.

Results: Eight weeks of a high-fat diet (HFD) induced obesity and glucose intolerance. CR caused rapid weight loss (ALA: 20%, GLY: 21%, $P < 0.01$), reduced whole-body fat mass (ALA: 41%, GLY: 49% $P < 0.01$), and restored glucose tolerance to control values in ALA and GLY groups. GLY treated mice lost more whole-body fat mass (14%, $p < 0.05$) and epididymal fat mass (26%, $P < 0.05$), less lean mass (27%, $P < 0.05$), and had better preserved quadriceps muscle mass (4%, $P < 0.01$) than ALA treated mice after 20 d CR. Compared to the HFD group, pro-inflammatory genes were lower ($P < 0.05$), metabolic genes higher ($P < 0.05$) and S6 protein phosphorylation lower after CR, but not different between ALA and GLY groups. There were significant correlations between %initial fat mass (pre CR) and the mRNA expression of genes involved in inflammation ($r = 0.51$ to 0.68, $P < 0.05$), protein breakdown ($r = -0.66$ to -0.37, $P < 0.05$) and metabolism ($r = -0.59$ to -0.47, $P < 0.05$) after CR.

Conclusion: Taken together, these findings suggest that glycine supplementation during CR may be beneficial for preserving muscle mass and stimulating loss of adipose tissue.

1. Introduction

Obesity increases the risk of developing multiple pathological conditions and is commonly associated with metabolic syndrome (MetS), type 2 diabetes mellitus (T2DM), and cardiovascular disease [1]. Weight loss is key to combat the comorbidities from obesity and can have profound health benefits by improving metabolic control [2]. Dietary modulation of energy balance through a reduction in energy intake [calorie restriction (CR), defined as a 30–60% decrease in food intake without malnutrition] appears the most effective strategy for managing weight [for review see [3]].

Weight loss during CR is not due to loss of adipose tissue alone. An undesirable consequence of CR is the associated loss of fat-free mass [4,5]. Up to 50% of the body weight lost through dieting (i.e. 3 month period of reduced daily caloric intake by 400–800 kcal) is attributed to the loss of fat free mass [6], particularly skeletal

Abbreviations: ALA, alanine; Atf4, activating transcription factor 4; AUC, area under the curve; Bnip3, BCL2/adenovirus E1B interacting protein 3; Ccl2, chemokine(C–C motif) ligand 2; Ccl5, chemokine(C–C motif) ligand 5; CON, control; CR, calorie restriction; eIF2a, eukaryotic translation initiation factor 2A; eIF3F, eukaryotic translation initiation factor 3; F4/80, EGF-like module containing, mucin-like, hormone receptor-like sequence 1; Foxo1, forkhead box O1; Foxo4, forkhead box O4; GAST, gastrocnemius muscle; GTT, glucose tolerance test; GLY, glycine; HFD, high fat diet; Il-6, interleukin-6; LC3B, microtubule-associated protein 1 light chain 3 beta; LSM, least-squares method; mTOR, mechanistic target of rapamycin; Murf1, muscle ring finger-1; NF, normalization factor; QUAD, quadriceps muscle; PLAN, plantaris muscle; Ppara, peroxisome proliferator activated receptor alpha; Ppard, peroxisome proliferator activated receptor delta; Pparg, peroxisome proliferator activated receptor gamma; SOL, soleus muscle; TA, tibialis anterior muscle; Tbp, tata-box binding protein; Tnfa, tumor necrosis factor alpha.

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muscle. An excessive loss of skeletal muscle mass reduces the capacity for glucose uptake, thereby increasing the risk of developing metabolic diseases and weight regain [7]. Exercise in conjunction with CR attenuates, but does not completely prevent, the loss of fat free mass [5]. Therefore, strategies that can maintain skeletal muscle mass during states of negative energy balance are required, particularly for those who require rapid weight loss, but are unable to exercise.

Ingestion of a high-protein (1.2–1.5 g/kg/day), low-fat diet can modulate skeletal muscle mass and attenuate muscle loss during CR [8–12]. Additional protein intake stimulates protein synthesis especially during conditions where daily protein intake is low [13]. Small elevations in plasma and/or muscle amino acid concentrations, particularly leucine, increases muscle protein synthesis, and stimulates the secretion of anabolic hormones. Therefore it has been suggested that supplementation with this specific amino acid could be effective in attenuating the loss of muscle mass. Interestingly, leucine administration during CR in rats did not attenuate muscle wasting [14]. Our recently published observations demonstrate that a specific non-essential amino acid, glycine, can preserve skeletal muscle mass and function during wasting conditions [15].

Glycine, is often considered biologically neutral, but recent studies from our laboratory show that glycine administration attenuates cancer-induced skeletal muscle wasting in mice by 50% [15]. In addition, glycine reduces muscle inflammation, macrophage infiltration and the production of reactive oxygen species in a mouse model of cancer cachexia. In this study, we investigated the effect of glycine supplementation on muscle mass of high-fat fed obese mice subjected to a period of CR (40% reduced calories). We tested the hypothesis that glycine supplementation during CR would preserve muscle mass.

2. Methods

2.1. Animals

Sixteen week old male C57BL/6 mice (n = 75) were used in this study. Mice were obtained from the Animal Resources Centre (Canning Vale, WA, Australia), and housed in the Biological Research Facility at The University of Melbourne under a 12-h light/dark cycle with drinking water available *ad libitum*. All experiments were approved by the Animal Ethics Committee of The University of Melbourne and conducted in accordance with the Australian code of practice for the care and use of animals for scientific purposes as described by the National Health and Medical Research Council of Australia.

2.2. Experimental protocol

To induce obesity, 16 week old mice received a high-fat diet (HFD, n = 45). Mice were fed these respective diets for 8 weeks and weighed weekly to monitor weight gain. After the first 8 weeks, 15 (HFD) mice were assessed for body composition, and glucose tolerance to verify that the high-fat diet induced obesity. After these tests the mice were killed and tissues collected for further analysis.

HFD mice (n = 30) were allocated randomly into two groups of equal body mass (n = 15/group; alanine [ALA] and glycine [GLY]) for 20 days of CR (40% reduced calories). Mice were housed individually during this time to ensure that each individual calorie restricted mouse (ALA and GLY) received their entire allocation of food. ALA and GLY groups were fed a 40% CR AIN93G diet supplemented with additional trace minerals and vitamins (Specialty Feeds, WA, Australia) supplemented with either L-alanine or glycine, respectively (1 g/kg/day). Mice were monitored closely every day for adverse signs or symptoms (such as excessive weight loss or abnormal appearance). Control mice (CON, n = 15) were fed standard-diet AIN93G and food intake was measured weekly to determine the amount of food to be given to the calorie restricted mice.

2.3. Diets

To induce obesity during the first 8 weeks of the experiment, mice fed the high-fat diet were provided with *ad libitum* access to SF03-002 fat modified AIN93G (% by weight: 19.4% protein; 36% total fat; Specialty Feeds, WA, Australia). During the CR period, 15 mice were individually housed and provided with *ad libitum* access to standard AIN93G rodent diet (% by weight: 19.4% protein; 7% fat; Specialty Feeds) and served as controls (CON) for ALA and GLY groups. The diets for ALA and GLY groups during the 20 day CR period were based on the AIN93G diet and supplemented with additional trace minerals and vitamins and either additional L-alanine (Sigma–Aldrich Co, Castle Hill, NSW, Australia) or glycine (Sigma–Aldrich), respectively. Glycine and L-alanine were supplemented within the AIN93G feed at 1.9% by weight, so that the mice on the calorie restricted diets received 1 g/kg/day of the respective amino acid. Alanine and glycine groups were limited to 60% of the food intake of the CON group during the CR period. Extra vitamins and minerals were added to AIN93G diet during CR so that the CR mice still received normal amounts of these important nutrients. This was based on the assumption that calorie restricted mice received 60% of the expected 4 g/day of feed consumed by CON mice, as previously demonstrated [16]. The dose of glycine was based on a previous study that had shown it effective in attenuating muscle loss [15].

2.4. Body composition and mass

Body mass was monitored throughout the study by weighing mice in an open container on an electronic balance (Ohaus, Port Melbourne, VIC, Australia). This was performed weekly for the first 8 weeks (HFD) and daily during the CR period. Body composition (fat and lean mass, free water, and total water) was assessed by an MRI scan (EchoMRI™-100, EchoMRI, Houston, Texas) during week 8 (day 0), at the midpoint of CR (day 10) and at the end of CR (day 20). Three scans were performed and the average of these scans used for analysis.

2.5. Glucose tolerance test

Glucose tolerance tests were performed on mice after an overnight fast (16 h). Briefly, basal glucose levels were measured via blood collection from the tail vein by fine needle puncture using a glucometer (Accu-Chek Performa, Roche Diagnostics, Castle Hill, VIC, Australia). Mice were given an intraperitoneal injection (*i.p.*) of 1 g/kg body mass of 0.1 g/mL glucose (Sigma–Aldrich) dissolved in sterilized saline, and blood glucose was measured at 15, 30, 60, 90 and 120 min post glucose injection [17].

2.6. Tissue collection

At the end of the intervention periods (8 weeks of HFD or 20 days of CR), mice were anesthetized with an *i.p.* injection of sodium pentobarbitone (Nembutal, 60 mg/kg, Sigma–Aldrich) such that they were unresponsive. Epididymal fat and the tibialis anterior (TA), plantaris (PLAN), gastrocnemius (GAST), soleus (SOL) and quadriceps (QUAD) muscles were carefully excised, blotted on filter paper and weighed on an analytical balance (Ohaus, Port Melbourne, VIC, Australia). All frozen tissues were stored at −80 °C for subsequent analyses.

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