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Glycine administration attenuates skeletal muscle wasting in a mouse model of cancer cachexia

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SUMMARY

Background and aims: The non-essential amino acid, glycine, is often considered biologically neutral, but some studies indicate that it could be an effective anti-inflammatory agent. Since inflammation is central to the development of cancer cachexia, glycine supplementation represents a simple, safe and promising treatment. We tested the hypothesis that glycine supplementation reduces skeletal muscle inflammation and preserves muscle mass in tumor-bearing mice.

Methods: To induce cachexia, CD2F1 mice received a subcutaneous injection of PBS (control, $n = 12$) or C26 tumor cells ($n = 32$) in accordance with the protocols developed by Murphy et al. [Murphy KT, Chee A, Trieu J, Naim T, Lynch GS. Importance of functional and metabolic impairments in the characterization of the C-26 murine model of cancer cachexia. Dis Models Mech 2012;5(4):533–545.]. Subcutaneous injections of glycine ($n = 16$) or PBS ($n = 16$) were administered daily for 21 days and at the conclusion of treatment, selected muscles, tumor and adipose tissue were collected and prepared for Real-Time RT-PCR or western blot analysis.

Results: Glycine attenuated the loss of fat and muscle mass, blunted increases in markers of inflammation (F4/80, $P = 0.01$ & IL-6 mRNA, $P = 0.01$) and atrophic signaling (MuRF, $P = 0.047$; atrogin-1, $P = 0.04$; LC3B, $P = 0.06$ and; BNIP3, $P = 0.10$) and tended to attenuate the loss of body mass ($P = 0.07$), muscle function ($P = 0.06$), and oxidative stress (GSSG/GSH, $P = 0.06$ and DHE, $P = 0.07$) seen in tumor-bearing mice. Preliminary studies that compared the effect of glycine administration with isonitrogenous doses of alanine or citrulline showed that the observed protective effect was specific to glycine.

Conclusions: Glycine protects skeletal muscle from cancer-induced wasting and loss of function, reduces the oxidative and inflammatory burden, and reduces the expression of genes associated with muscle protein breakdown in cancer cachexia. Importantly, these effects were glycine specific.

1. Introduction

Cancer cachexia is a multifactorial syndrome characterized by an ongoing loss of skeletal muscle mass with or without loss of fat mass that leads to progressive functional impairment.[2] Cachexia is associated with reduced mobility, functional independence and response to anti-neoplastic treatments as well as an increased risk of post-surgical complications, and accounts for more than 20% of all cancer-related deaths.[1] Disease progression in cachectic patients is reliant on the complex interplay between the tumor and the host inflammatory response. The increase in circulating inflammatory cytokines (e.g. TNFα, IL-6, IFNγ and IL-1β) reduces skeletal muscle protein synthesis and increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), triggering muscle degradative pathways.[3] Consequently, mice with cancer cachexia have smaller muscles and higher muscle concentrations of reactive oxygen species (ROS) and inflammatory cytokines.[1] Tumor-factors can also modulate food intake, affecting the gut and altering neuronal regulation of food intake, i.e., anorexia. Although anorexia alone cannot account for cancer-associated cachexia, its presence is associated with a poorer prognosis and is prevalent in late stage cancer patients.[4] Conventional nutritional support (i.e. increased CHO, FAT, PRO intake) is therefore ineffective in maintaining muscle mass. Therefore, novel nutritional therapeutic strategies should focus on modulating skeletal muscle protein metabolism or blocking the synthesis or action of inflammatory cytokines.[5]

Small elevations in plasma and/or muscle amino acid concentration, particularly leucine and arginine, increases muscle protein synthesis, and stimulates the secretion of anabolic hormones. In addition, the branched chain amino acid (BCAA) leucine can

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directly modulate the initiation of mRNA translation and hence protein synthesis in skeletal muscle.[6] Consequently, the potential of specific amino acids to stimulate protein synthesis, reduce protein breakdown, and attenuate muscle loss in cachexia, has received considerable attention. Indeed, administration of BCAA's preserves muscle mass in cancer cachexia.[7] Interestingly, recent studies indicate that essential amino acids, arginine and/or BCAA are not the only amino acids that can manipulate muscle protein synthesis and breakdown.

The non-essential amino acid, glycine, is often considered biologically neutral, but studies have indicated that it could be an effective anti-inflammatory agent that preserves muscle function during wasting conditions.[8] Glycine administration activates glycine-gated chloride (Cl^-) channels in inflammatory cells, mediating an influx of Cl^- into the cell, thereby making voltage gated Ca^{2+} channels more difficult to open and attenuating increases in $[Ca^{2+}]_i$, cytokine production, and whole-body (systemic) inflammation in several models.[9] Since increased inflammation plays a key role in the loss of skeletal muscle and adipose tissue with cancer cachexia, glycine supplementation could represent a simple, safe and promising treatment option. In this study, we tested the hypothesis that glycine treatment reduces systemic inflammation and attenuates the loss of skeletal muscle and function in a C26 mouse model of cancer cachexia that exhibits losses of skeletal muscle mass and function that parallel those seen in human patients.[1]

2. Materials and methods

2.1. Animals

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 stipulated by the National Health and Medical Research Council (Australia). Fourteen-week-old male CD2F1 mice, with a mean body mass of 30.7 ± 0.5 g (range: 25.8–40.4 g) were allocated into one of three experimental groups: a severely cachectic C26 tumor-bearing group treated with PBS ($n = 16$), a severely cachectic C26 tumor-bearing group treated with glycine ($n = 16$), or a control group treated with PBS ($n = 12$). All mice remained in their original box of four and groups were matched for body mass. Animal numbers were based on previous work in our lab using the same model of cancer cachexia.[1] The experiment was performed on two cohorts of mice due to animal availability. The first cohort of mice ($n = 24$, 8 per group) was used to determine changes in body, tumor, muscle and fat mass as well as muscle fiber cross-sectional area, but underwent end-point analyses, not presented herein, that meant tissue was not suitable for further analyses. The second cohort of mice ($n = 20$, four control mice, eight PBS-treated and eight glycine treated C26 tumor-bearing mice) underwent all analyses. All mice were obtained from the Animal Resources Centre (Canning Vale, Western Australia) and housed in the Biological Research Facility at The University of Melbourne under a 12:12-h light–dark cycle. Animals were monitored daily for adverse signs and symptoms. Water was available *ad libitum*, and standard laboratory chow was provided, changed and monitored daily. The amount of food consumed per cage of four mice per day was determined and expressed as cumulative food intake per mouse.

2.2. C26 cell line and inoculation

Frozen C26 cells inducing severe cachexia were kindly donated by Prof. Martha Belury (The Ohio State University, Columbus, OH). Frozen cells were thawed rapidly in a 37 °C water bath and transferred to a 100-mm culture plate (Corning, Corning, NY) containing growth media consisting of RPMI Medium 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% (vol/vol) FBS (Invitrogen), 1% L-glutamine (Invitrogen) and 1% penicillin/streptomycin (Invitrogen), and incubated at 37 °C with 5% CO2. Cells were maintained in growth media and passaged when 70–80% confluent. Before injection of C26 cells into mice (day 0), cells were counted using a hemocytometer (Bright Line, Hausser Scientific, Horsham, PA), pelleted via centrifugation (1600 g for 5 min at 25 °C) and resuspended at 5×10^6 cells/ml of sterilized PBS. Between 10 am and 12 pm in a laboratory within the animal facility, all mice were anesthetized via an intraperitoneal (i.p.) injection of a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg; VM Supplies, Chelsea Heights, VIC, Australia), such that they were unresponsive to tactile stimuli. Mice were shaved on the right flank and given a subcutaneous (*s.c.*) injection of either 5×10^5 C26 cells suspended in 100 μl of sterilized PBS, or 100 μl of sterilized PBS only (control). Mice recovered from anesthesia on a heat pad and were given an *s.c.* injection of atipamezole (Antisedan; 1 mg/kg body weight; VM Supplies) to partially reverse the effects of xylazine and promote more rapid recovery from sedation. Body mass and tumor size were monitored daily after inoculation.

2.3. Glycine administration

C26 tumor-bearing mice were allocated into either control ($n = 16$) or glycine treated groups ($n = 16$). Treated animals received 1 g/kg of glycine (Sigma-Aldrich Co., Castle Hill, NSW; Australia) in PBS once daily via *s.c.* injections for 21 days. As dietary protein intake is ~27.2 g kg^{-1} day^{-1} this dose represents an increased amino acid intake of only ~3.7%. Control mice received an identical volume of saline only. Injections were performed between 8 am and 11 am each day and the order of treatment was alternated between groups, with one full box receiving treatment at a time. Glycine is orally available and has proved effective as a nutritional supplement in a number of settings.[9] However, since tumor-bearing mice exhibit a progressive reduction in food intake starting from 7 to 10 days[1] and we have also observed both reduced and variable water intake, we decided as a proof-of-principle exercise to administer glycine subcutaneously to ensure the animals received a standardized dose of 1 g/kg/day across the treatment period. Animals were fed *ad libitum* to allow the study of any effect of glycine on food intake to modulate the severity of cachexia. Saline was used as the control treatment instead of an isonitrogenous control due to the complexity of amino acid metabolism and the uncertainty of the *in vivo* effects of individual amino acids in cancer cachexia. For example, glycine is commonly considered biologically inert and is often used as an isonitrogenous control, either in isolation or as a component of a non-essential amino acid preparation, when investigating amino acid based dietary interventions.[10] However, to rule out the influence of increased nitrogen intake, we conducted two separate preliminary studies to compare the effect of glycine administration with isonitrogenous doses of alanine (assumed negative control, preliminary study 1) or citrulline (assumed positive control, preliminary study 2) using tumor size, loss of tumor free body mass, and tibialis anterior (TA) muscle mass as the dependent variables. Preliminary study two used the same methods (i.e. injection of C26 cells, end-point measures after 21 days) as described for the primary study. Food intake was not measured in this experiment. In preliminary study one, there were two exceptions. First, glycine and alanine were delivered via drinking water, which resulted in a progressively lower glycine dose due to reduced water consumption across the experiment. The dose started at 1.3 g kg day^{-1}, but dropped to around 0.65 g kg day^{-1} over the last