Dietary delivery of glycomacropeptide within the whey protein matrix is not effective in mitigating tissue ceramide deposition and obesity in mice fed a high-fat diet

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ABSTRACT

Obesity is often accompanied by heightened circulating and tissue inflammation along with an increase in sphingolipids (e.g., ceramides) in metabolically active and insulin-sensitive organs. Whey protein isolate (WPI) has been shown to decrease inflammation and increase insulin sensitivity when given during a high-fat diet (HFD) intervention in rodents. The whey protein bioactive peptide glycomacropeptide (GMP) has also been linked to having anti-inflammatory properties and regulating lipogenesis. Therefore, the purpose of the study was to determine the effect of dietary GMP within the whey protein matrix on tissue inflammation, adiposity, and tissue ceramide accumulation in an obesogenic rodent model. Young adult male mice (10 wk old) underwent a 10-wk 60% HFD intervention. Glycomacropeptide was absent in the control low-fat diet and HFD WPI (−GMP) groups. The HFD WPI (1×GMP) treatment contained a standard amount of GMP, and HFD WPI (2×GMP) had double the amount. We observed no differences in weight gain or reductions in adiposity when comparing the GMP groups to HFD WPI (−GMP). Similarly, insulin resistance and glucose intolerance were not offset with GMP, and skeletal muscle and liver tissue ceramide content was unaltered with the GMP intervention. In contrast, the additional amount of GMP (2×GMP) might adversely affect tissue obesity-related pathologies. Together, dietary GMP given in a whey protein matrix during an HFD intervention does not alter weight gain, insulin resistance, glucose intolerance, and sphingolipid accumulation in the liver and skeletal muscle.

Key words: whey protein, ceramides, inflammation, high-fat diet, obesity

INTRODUCTION

Obesity is driven by many factors, including caloric excess and a decrease in energy expenditure. In most instances, individuals with obesity have increased systemic and tissue inflammation (Kristiansen and Mandrup-Poulsen, 2005; Carlsen et al., 2009) including elevations in interleukin-1β (IL-1β), IL-6, and tumor necrosis factor α (TNF-α; Sinha et al., 2017). Obesity increases the risk of developing insulin resistance and subsequent type 2 diabetes, and in severe circumstances, metabolic dysfunction–associated fatty liver disease. Therefore, it is important to find therapies (e.g., nutraceuticals) to offset the inflammatory and insulin resistant burden that accompanies obesity.

A contributing factor to inflammation and insulin resistance is the formation of sphingolipids, such as ceramides, from dietary and endogenous saturated fatty acids (Holland et al., 2007). In response to a high-fat diet (HFD), ceramides accumulate in insulin-sensitive tissues such as liver and adipose (Shah et al., 2008; Kurek et al., 2014; Kurek et al., 2015). In humans with obesity, ceramides are heightened in circulation as well as in metabolically active organs (Aburasayn et al., 2016), which can lead to many obesity-related pathologies, such as liver steatosis and insulin resistance (Summers et al., 2019). Therefore, approaches that alleviate lipid accumulation may improve obesity-related outcomes (i.e., insulin resistance, tissue-specific ceramide accumulation) caused by an HFD.

To combat obesity-related metabolic complications, we resorted to a dairy-derived compound. Bovine milk is a heavily consumed source of protein in the diet and is used in a variety of products, especially its isolated protein fractions. The whey protein fraction has been...
shown to reduce C-reactive protein levels (CRP) when consumed in individuals with increased basal levels of CRP (e.g., individuals with obesity, hypertension, or COPD; Zhou et al., 2015). Likewise, in rodents, whey protein given along with an HFD, diminished weight gain (Royle et al., 2008; Boscaini et al., 2020), increased insulin sensitivity (Belobrajdic et al., 2004), and reduced liver steatosis (Shertzer et al., 2011). The mechanism behind the effects of whey protein on inflammation and insulin sensitivity is not fully understood.

One of the main bioactive peptides found in whey protein is glycomacropeptide (GMP), constituting ~20% to 25% of the whey protein fraction. Glycomacropeptide is a 64-AA long peptide and ~50% of the GMP is glycosylated to different extends (e.g., mono-, di-, tri-, or tetrasaccharides). To date, 14 different glycosylated forms of GMP have been identified (Neelima et al., 2013). Glycomacropeptide has been shown to reduce inflammation in patients with ulcerative colitis (Hvas et al., 2016). In addition, a daily bolus dose of GMP administration (100–400 mg/kg BW) during a high-fat diet intervention in rats reduced food intake, weight gain, adiposity, and triglycerides (Xu et al., 2013). Also, in mice, GMP has been shown to improve insulin sensitivity, decrease liver triglycerides, and gene expression of cytokines (Song et al., 2018; Yuan et al., 2020; Sauvé et al., 2021). Glycomacropeptide has also been revealed to increase fecal excretion of lipids when given during an HFD (Kim et al., 2005), while also demonstrating an inhibitory role on the proliferation and differentiation of adipocytes in vitro (Xu et al., 2011). These findings suggest that GMP has potential roles in regulating lipid synthesis and modulating adipogenesis. Currently, it is not known how GMP as part of a normal whey protein matrix affects weight gain and how it modulates ceramide accumulation in insulin-sensitive tissues such as liver and skeletal muscle during an HFD.

Thus, the purpose of this study was to investigate the efficacy of the dietary intake of GMP, within the whey protein matrix. We sought to determine the effects on tissue inflammation, adiposity, and tissue ceramide accumulation in a diet-induced obesity (DIO) rodent model, and to determine whether GMP contributes to antidiabetic outcomes. We hypothesized that GMP would offset weight gain, muscle inflammation, insulin resistance, and skeletal muscle and liver ceramide accumulation during an obesogenic diet.

**MATERIALS AND METHODS**

**Animals and General Experimental Design**

Male C57BL/6J mice aged 9 wk were obtained from The Jackson Laboratory (Bar Harbor, ME) and housed with ad libitum food and water access. Mice were maintained in a temperature controlled (22–23°C) facility on a 12:12-h light/dark cycle. After a week of acclimating, mice were randomly assigned (by random number generation) to one of 4 different experimental diet groups (n = 8–11/group, based on previous study; Yuan et al., 2020) for a 10-wk intervention (Figure 1).

**Experimental Diets**

The control low-fat, reduced sucrose diet (TD.210537) consisted of (% kcal) 10.2% fat, 19% protein (rennet casein), and 70.8% carbohydrates (CHO); accumulating to 3.7 kcal/g of food. The HFD WPI (−GMP; TD.210539) was approximately absent of GMP and consisted of (% kcal) 60% fat, 18.8% protein (ion exchange whey protein isolate), and 21.2% CHO; accumulating to 5.1 kcal/g. The HFD WPI (1×GMP; TD.210541)
contained the normal amount of GMP and was made up of a kcal distribution of 60% fat, 18.8% protein (whey protein isolate), and 21.2% CHO; accumulating to 5.1 kcal/g. The HFD WPI (2×GMP; TD.210542) contained twice the normal amount of GMP and consisted of (% kcal) 60.1% fat, 18.7% protein (GMP enriched whey protein isolate), and 21.2% CHO; accumulating to 5.1 kcal/g. Within protein fractions (reported as a percentage of the total amount of kcal), diets contained (% kcal) 0.14%, 0.49%, 4.6%, and 7.7% GMP for control, HFD WPI (−GMP), HFD WPI (1×GMP), and HFD WPI (2×GMP), respectively (Table 1). To maintain a similar protein content in the 2×GMP experimental diet (~19%), some WPI was removed and replaced with GMP. All dietary protein content and GMP were provided by Glanbia Nutritionals (Twin Falls, ID). Complete formulated diets for experimental treatments were assembled and provided by Envigo (Indianapolis, IN). Glycomacropeptide content in manufactured diets was tested by HPLC, and AA content was tested and determined using a Waters Corporation (Milford, MA) kit (system guide 71300129702/Revision B) and ultra-performance liquid chromatography (UPLC), all performed by Glanbia Nutritional (Table 2).

### Table 1. Dietary composition

<table>
<thead>
<tr>
<th>Item</th>
<th>Control HFD WPI (−GMP)</th>
<th>HFD WPI (1×GMP)</th>
<th>HFD WPI (2×GMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>10.2</td>
<td>60.0</td>
<td>60.0</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>70.8</td>
<td>21.2</td>
<td>21.2</td>
</tr>
<tr>
<td>Protein</td>
<td>19.0</td>
<td>18.8</td>
<td>18.8</td>
</tr>
<tr>
<td>GMP</td>
<td>0.1</td>
<td>0.5</td>
<td>4.6</td>
</tr>
<tr>
<td>kcal/g food</td>
<td>3.7</td>
<td>5.1</td>
<td>5.1</td>
</tr>
</tbody>
</table>

1Values for macronutrients are shown as a percentage of the total amount of kilocalories.

2HFD = high-fat diet; WPI = whey protein isolate; GMP = glycomacropeptide. − = absent; 1× = normal amount; 2× = twice the normal amount.

### Table 2. Breakdown of individual AA per experimental diet

<table>
<thead>
<tr>
<th>Item</th>
<th>HFD WPI (−GMP)</th>
<th>HFD WPI (1×GMP)</th>
<th>HFD WPI (2×GMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>5.0</td>
<td>4.6</td>
<td>4.9</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.5</td>
<td>1.9</td>
<td>1.5</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>10.6</td>
<td>10.3</td>
<td>9.8</td>
</tr>
<tr>
<td>Cystine</td>
<td>2.7</td>
<td>2.2</td>
<td>1.8</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>17.1</td>
<td>16.8</td>
<td>17.4</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.6</td>
<td>1.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.8</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.6</td>
<td>6.4</td>
<td>7.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>12.6</td>
<td>10.0</td>
<td>8.3</td>
</tr>
<tr>
<td>Lysine</td>
<td>10.2</td>
<td>8.8</td>
<td>8.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.5</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.4</td>
<td>2.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Proline</td>
<td>4.4</td>
<td>5.5</td>
<td>6.8</td>
</tr>
<tr>
<td>Serine</td>
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<tr>
<td>Threonine</td>
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<td>8.7</td>
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<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Tyrosine</td>
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<tr>
<td>Valine</td>
<td>5.8</td>
<td>6.1</td>
<td>6.7</td>
</tr>
</tbody>
</table>

1Values are shown as a percentage of the total amount of protein in the diet.

2HFD = high-fat diet; WPI = whey protein isolate; GMP = glycomacropeptide. − = absent; 1× = normal amount; 2× = twice the normal amount.

### Body Composition and Physical Function Testing

A nuclear magnetic resonance instrument (Bruker Minispec MQ20 NMR analyzer, Rheinstetten, Germany) was used to assess body composition. In addition, whole-body strength, balance, and coordination were assessed by grip strength and rotarod instrument. Whole-body grip strength was assessed using a grip strength meter with a mesh wire attachment (Columbus Instruments, Columbus, OH). After an acclimation test a week prior, mice were placed on the mesh wire and pulled by the base of their tail, parallel to the mesh wire. Peak force was recorded and an average of 3 trials was recorded. Neuromuscular function was assessed using rotarod testing on a Rotamex-5 (Columbus Instruments, Columbus, OH). The speed began at 0.1 rpm and increased by 0.3 rpm/s increments until it reached 50 rpm. Time was recorded when mice fell off. Each mouse performed the test 4 times, and an average time was recorded. Mice were acclimated on the rotarod a couple of days before testing. All physical function tests were conducted by the same research personnel.

**Glucose Tolerance Test and Fasting Glucose and Insulin**

Glucose tolerance tests were performed by injection of intraperitoneal glucose of 5 μL/g BW of 20% dextrose (Sigma-Aldrich G8769l; 1 mg of glucose per gram of body mass). Mice were fasted for ~4 h before glucose injection. Baseline glucose was measured before the injection and 15, 30, 60, and 120 min after via tail bleed with a glucometer (Contour Next EZ). To determine fasting serum glucose and insulin levels, blood was drawn from the heart after cervical dislocation and placed at room temperature for ~10 min to clot before centrifugation at 12,500 × g for 10 min at 4°C. The top layer (serum) was placed in a separate tube and stored at −80°C until further analysis. To establish the glucose levels in the collected serum, a mouse glucose assay kit (Crystal Chem 81692) was used (according to manufacturer recommendations) and read using an Epoch plate reader (Take3, BioTek, Winooski, VT) at optical density (OD)505. For serum insulin analyses, a low-range ultra-sensitive mouse insulin ELISA kit (Crystal...
described (Bowe et al., 2014).

Metabolic Measurements Using Comprehensive Lab Animal Monitoring System

To measure numerous metabolic parameters, mice were placed in metabolic cages (Columbus Instruments Comprehensive Lab Animal Monitoring System, Columbus, OH, serial no. 180072) for 72 h before the end of the experimental intervention. Mice were single-housed and acclimated for 48 h before taking measurements and collecting data for 24 h (light and dark phase). Respiratory exchange ratio (RER) was calculated from VO₂ production and VCO₂ consumption. The RER is measured by dividing the exhaled CO₂ by the amount of O₂ consumed. An RER value closer to 1 indicates carbohydrates are used as a fuel source, whereas an RER closer to 0.7 indicates fats are used as a fuel source. Additionally, energy expenditure was calculated by dividing heat production (kcal/h) by BW. The ambulatory activity was calculated by summing ambulatory counts in the x and y directions.

Immunofluorescence and Immunohistochemistry

Gastrocnemius. Frozen OCT-embedded gastrocnemius (cut in a longitudinal plane) was sectioned at a thickness of 10 μm using a Leica cryostat (CM1860). Gastrocnemius sections contained equal parts of red and white fibers compared with whole gastrocnemius. To assess myofiber cross-sectional area, sections were blocked in mouse-on-mouse (Vector Laboratories, MKB-2212-1) for 1 h. Sections were then incubated in primary laminin antibody (Sigma-Aldrich, L9393) at a concentration of 1:200 in 2.5% horse serum (Gibco 16050-1-30; in 1× PBS) overnight. Next, slides were incubated in secondary antibody 7-amino-4-methylcoumarin-3-acetic acid (Vector Laboratories; dilution 1:200 in 2.5% horse serum [Gibco] 33342-20; in 1× PBS) overnight. Stained sections were imaged on a Zeiss Slide Scanner Axio Scan.Z1 (Carl Zeiss Inc., Oberkochen, Germany) with the 20× objective lens. Images were analyzed for the total area covered by lipid droplets using Fiji (ImageJ) software (version 2.9.0/1.53t, National Institutes of Health).

Gene Expression

RNA was isolated from liver (~10 mg) and gastrocnemius (~35 mg) tissue utilizing QIAzol lysis reagent (Qiagen 79306), methods as described previously (Petrocelli et al., 2021). Chloroform and isopropanol were used to extract and precipitate the RNA, next the RNA was washed with 75% ethanol and resuspended in nuclease-free water. Using an Epoch (Take3, BioTek, Winooski, VT), the concentration of the isolated RNA was determined. One microgram of RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad 17088-91) and using a Bio-Rad T100 Thermal Cycler (settings: lid 105°C, volume 20 μL, 25°C 5 min, 46°C 20 min, 95°C 1 min, 4°C). Real-time quantitative polymerase chain reaction was performed with 1:8 diluted cDNA (in nuclease-free water) on a CFX Connect real-time PCR detection system (Bio-Rad) in conjunction with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad 17252-70). All data were normalized to ribosomal protein L32 gene: (5′→3′ Forward) GGCTTTTCGGTTCTTAGAGGA, and (Reverse) GGCTTTTGGTTCTCTTAGAGGA. The following primers were purchased from Bio-Rad: tumor necrosis factor (TNF, qMmuCED0004141), interleukin 6 (IL6, qMmuCED00045760), interleukin 1β (IL1B, qMmuCED00045755).

Tissue Triglyceride and Sphingolipid Detection and Analysis

Triglycerides. To investigate triglyceride content in the gastrocnemius, ~70 mg of tissue was homogenized in 5% Nonidet P-40 (NP-40) in H₂O and analyzed for triglyceride content using a triglyceride assay kit (Abcam ab65336), using manufacturer procedures and an Epoch plate reader (OD₅70). Results were normalized to protein content determined by a Pierce bicinchoninic acid protein assay kit (ThermoFisher 23227) measured 24 h, after which it was stored in 70% ethanol. Samples were submitted to Associated Regional and University Pathologists (ARUP) laboratories at the University of Utah and the Department of Pathology for hematoxylin and eosin staining on sections, as previously described (Mahmassani et al., 2020). Briefly, liver was embedded in paraffin, sectioned at 5-μm thickness, and stained for hematoxylin and eosin to visualize lipid droplets. Slides were imaged on a Zeiss Slide Scanner Axio Scan.Z1 (Carl Zeiss Inc., Oberkochen, Germany) with the 20× objective lens. Images were analyzed for the total area covered by lipid droplets using Fiji (ImageJ) software (version 2.9.0/1.53t, National Institutes of Health).
by an Epoch spectrophotometer (Take3, BioTek, Winooski, VT).

**Lipid Extractions.** Frozen gastrocnemius and liver (10–15 mg) were homogenized in 225 μL of methanol containing internal standard and 188 μL of PBS. An aliquot of homogenate was collected from each sample for protein quantification. A process blank was included in the assay, which contained equal amounts of methanol without tissue homogenate. A total of 750 μL of methyl tert-butyl ether (MTBE) was added to sample tubes, which were incubated on ice for 60 min, with brief vortexing every 15 min. Samples were centrifuged for 10 min at 15,000 × g and 4°C, and the organic top layer containing lipid was transferred and evaporated to dryness. Lipids were extracted for a second time in MTBE: methanol:double-distilled water, as mentioned above, and organic and aqueous fractions were evaporated and resuspended for liquid chromatography (LC)-MS/MS analysis.

**Lipid Standards.** Sphingolipid internal standard stocks were obtained from Avanti Polar Lipids. Standards for tissue were prepared in methanol with the following lipid species and concentrations: sphingomyelin (d18:1/16:1)-d9 (74 pmol/sample); sphingomyelin (d18:1/18:1)-d9 (47 pmol/sample); sphingomyelin (d18:1/20:1)-d9 (23 pmol/sample); sphingomyelin (d18:1/22:1; 44 pmol/sample); sphingomyelin (d18:1/24:1; 64 pmol/sample); ceramide (d18:1-d7/16:0; 367 pmol/sample); ceramide (d18:1-d7/18:0; 349 pmol/sample); ceramide (d18:1-d7/24:0; 228.3 pmol/sample); ceramide (d18:1-d7/24:1; 305 pmol/sample); dihydroceramide (d18:0/18:1; 44.2 pmol/sample); glucosylerceramide (d18:1/17:0; 280.1 pmol/sample); sphingosine (d18:1-d7; 32.6 pmol/sample); sphingosine-1-phosphate-d7 (2.6 pmol/sample); sphinganine-1-phosphate-d7 (2.6 pmol/sample); triacylglycerol (15:0–18:1(d7)-15:0; 615.5 pmol/sample); diacylglycerol (15:0–18:1(d7); 850.4 pmol/sample); and phosphatidylcholine (15:0–18:1(d7); 663.9 pmol/sample).

**LC-MS Analysis.** Lipid extracts were separated on an Acquity UPLC CSH C18 column (2.1 × 100 mm; 1.7 μm) coupled to an Acquity UPLC CSH VanGuard precolumn (5 × 2.1 mm; 1.7 μm; Waters, Milford, MA) maintained at 65°C connected to an Agilent HPLC 1290 Sampler, Agilent 1290 Infinity pump, and Agilent 6490 triple quadrupole mass spectrometer. Sphingolipids were detected using dynamic multiple reaction monitoring in positive ion mode. Source gas temperature was set to 175°C, with a gas (N2) flow of 15 L/min and a nebulizer pressure of 30 psi (206,843 Pa). Sheath gas temperature was 250°C, sheath gas (N2) flow of 12 L/min, capillary voltage was 3,500 V, nozzle voltage was 500 V, high-pressure radio frequency 190 V, and low-pressure radio frequency 120 V. Injection volume was 3 μL, and the samples were analyzed in a randomized order with the pooled quality control (QC) sample injected 8 times throughout the sample queue. Mobile phase A consisted of acetonitrile:H2O (60:40 vol/vol) in 10 mM ammonium formate and 0.1% formic acid, and mobile phase B consisted of isopropyl alcohol:acetonitrile:H2O (90:9:1 vol/vol/vol) in 10 mM ammonium formate and 0.1% formic acid. The chromatography gradient started at 15% mobile phase B, increased to 30% B over 0.7 min, increased to 60% B from 0.7 to 1.4 min, increased to 80% B from 1.4 to 7.0 min, and increased to 99% B from 7.0 to 7.14 min, where held until 9.45 min before returned to starting conditions at 9.8 min. Post-time was 3.5 min and the flow rate was 0.4 mL/min throughout.

**LC-MS Data Processing.** For data processing, Agilent MassHunter (MH) Workstation and software (version B.08) packages MH Qualitative and MH Quantitative were used. The pooled QC (n = 8) and process blank (n = 4) were injected throughout the sample queue to ensure the reliability of acquired lipidomics data. Data exported from MH Quantitative were evaluated using Excel where initial lipid targets were parsed based on the following criteria. Only lipids with relative standard deviations less than 30% in QC samples are used for data analysis. Additionally, only lipids with background area under the curve counts in process blanks that were less than 30% of QC were used for data analysis. The parsed Excel data tables were normalized based on the ratio to class-specific internal standards, then to sample protein amount.

**Statistical Analyses**

All data are shown as mean ± standard error of the mean. To determine differences between dietary groups, a one-way ANOVA with Tukey’s multiple comparisons test was conducted. To determine differences between groups across different time points during the glucose tolerance tests or across different sphingolipid species, a 2-way ANOVA with Tukey’s post hoc test was conducted. Statistical significance was set to P < 0.05. GraphPad Prism Version 9.5.1 (La Jolla, CA) was used for all statistical analyses and figure assembly.

**RESULTS**

**Tissue Composition and Physical Function**

As expected, BW, lean mass, and fat mass increased following a 60% HFD (Newsom et al., 2017), but these outcomes were not prevented by GMP (Figures 2A, 2B, and 2C). Alternately, 2×GMP subtly increased fat mass further compared with the —GMP group. Regard-
ing physical function, grip strength decreased when normalized to lean mass and was further reduced in the 2×GMP group (vs. −GMP), and time on the rotarod decreased in all groups compared with control (Figures 2E and 2F). Finally, the 1×GMP diet decreased the average kilocalories of food intake (Figure 2D) throughout the intervention, as similarly shown by others (Xu et al., 2013), but did not affect BW or body composition. Together, GMP in the diet did not prevent adiposity or loss of physical function. In contrast, doubling the GMP content may further increase fat mass and induce whole-body weakness.

**Insulin Sensitivity and Metabolic Function**

We next examined the effects of GMP on glucose tolerance (Figure 3A) and hepatic insulin resistance (Figures 3B, 3C, and 3D). All HFD interventions decreased glucose clearance, and increased fasting levels of glucose and insulin, resulting in an increased homeostatic model assessment of insulin resistance index. Additionally, we subjected mice to indirect calorimetry at the end of the 10-wk dietary intervention, to investigate their metabolic health by measuring RER, energy expenditure, and physical activity levels. As expected

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*Figure 2.* Glycomacropeptide (GMP) does not prevent whole-body adiposity nor a decrease in muscle function testing. (A) Body weight at the day of sacrifice after the 10-wk intervention. Body composition measured by nuclear magnetic resonance shown as absolute (B) lean mass and (C) fat mass. (D) Food intake as an average of kilocalories per day of the entire 10-wk intervention. Muscle function tested by (E) rotarod and (F) grip strength (normalized to lean mass). A one-way ANOVA with Tukey’s post hoc analysis was used to determine significance: significantly different from control (10% kcal from fat and no GMP; *) and significantly different from HFD WPI (−GMP) group (#), \( P < 0.05 \). \( n = 8–11 \)/group. Data are shown as mean ± SEM. Individual data points are shown as circles, triangles, diamonds, and squares for control, HFD WPI (−GMP), HFD (1×GMP), and HFD (2×GMP), respectively. HFD = high-fat diet; WPI = whey protein isolate; − = absent; 1× = normal amount; 2× = twice the normal amount.
(White et al., 2015), the ability to switch between fats and carbohydrates for fuel was altered with an HFD and unchanged with the addition of GMP (compared with control; Figures 4A and 4B). Absolute energy expenditure was increased during the light phase for all HFD groups (Figure 4C) and energy expenditure normalized to BW and ambulatory activity were not different between all groups (Figures 4D and 4E). These data show that GMP does not alter substrate utilization, glucose intolerance, and insulin resistance induced by an obesogenic diet.

**Tissue Mass, Lipids, and Tissue Ceramide Content**

We investigated 2 tissues that contribute to the development of insulin resistance in obesity: liver and skeletal muscle (Samuel and Shulman, 2016). An increase in most tissue weights was observed after the HFD intervention, and these were not affected by GMP (Figures 5C, 5D, 5E, and 5F). However, liver weight was higher in the 2×GMP (vs. −GMP). Gastrocnemius weights were also increased following HFD, likely due to an increase in BW, but this occurred without an increase in fiber size (Figures 5A and 5B). Interestingly, we observed a trend for GMP to decrease epididymal adipose tissue accumulation (√P = 0.0633 and 0.0926) compared with −GMP. We next analyzed ceramide lipid accumulation in response to GMP in gastrocnemius and liver tissue because sphingolipids, such as ceramides, are drivers of insulin resistance and tissue inflammation following HFD (Holland et al., 2011; Figures 6 and 7). We found that triglyceride content was elevated in all HFD groups in gastrocnemius (Figure 6A). Similarly, total ceramide content was elevated in response to HFD and was primarily a result of increased the muscle dominant C18 ceramide lipid species (Figure 6B). These responses were not affected by different amounts of GMP in the diet. We also noted mild local inflammation in skeletal muscle after the HFD and this was found only after the 2×GMP dietary intervention compared with −GMP (Figures 6C, 6D, and 6E).

In liver tissue, we also detected an increase in ceramide accumulation, driven by the liver dominant C22 ceramide lipid species (Figure 7A). In addition, we detected an increase in lipid droplet accumulation following an HFD in the 1×GMP group compared with control (Figures 7B and C). We detected a tendency for all HFD groups to increase TNF-α mRNA expression, with 2×GMP significantly increasing TNF-α expression compared with control (Figure 7D). Similarly, IL-6 gene expression was increased only in the 2×GMP group following an HFD (Figure 7E), whereas IL-1β was only increased in the HFD that was absent of GMP (−GMP) compared with control (Figure 7F). Together, these data indicate that GMP does not offset ceramide accumulation and inflammation in the liver.

**DISCUSSION**

Obesity and insulin resistance are accompanied by heightened tissue inflammation and lipid accumulation, in particular the toxic sphingolipid species, ceramides (Holland et al., 2011; Ciaraldi et al., 2016). In the present study, we tested whether GMP, when delivered in the diet in its normal whey protein matrix, was capable of preventing weight gain and obesity-related inflammation, and ceramide accumulation in liver and skeletal muscle tissue after a 10-wk obesogenic diet. The main findings of this study were that GMP did not prevent weight gain or reduce adiposity during DIO. In addition, GMP did not prevent DIO-related tissue ceramide accumulation or insulin resistance compared with a dietary intervention approximately absent of GMP. In contrast, doubling the amount of dietary GMP might adversely affect obesity-related outcomes. Together, these data suggest that GMP, when given within the whey protein matrix in the diet during an obesogenic diet, is ineffective in reducing whole-body adiposity, insulin resistance, glucose intolerance, and ceramide accumulation in liver and skeletal muscle tissue.

In contrast to our hypothesis, GMP provided in the diet did not offset weight gain, metabolic dysfunction, or lipid accumulation during an obesogenic diet in mice aged 10 wk. More so, we did not observe a protective effect of GMP against tissue ceramide accumulation induced by a high-fat diet, even though previous studies indicated a potential suppressing effect of GMP on lipid synthesis in Sprague-Dawley rats (Kim et al., 2005; Xu et al., 2011). Given these outcomes, our findings are surprisingly different from prior GMP rodent studies. For example, liver steatosis and liver triglyceride content were significantly reduced following daily intragastric hydrolyzed GMP administration during an 8-wk HFD in C57BL/6J mice (Song et al., 2018). Additionally, using the same GMP administration approach but with the addition of sucrose to a 12-wk HFD intervention, insulin sensitivity was improved, and circulating and liver inflammation was reduced with the addition of GMP in C57BL/6 mice (Sauvé et al., 2021). We believe the discrepancy between these studies and ours may be because of the mode of GMP delivery, not due to an insufficient amount of GMP to offer therapeuentic value. In fact, mice consumed more GMP per day (data not shown) in our experiment than in prior studies. Our interest was to better understand the anti-inflammatory properties of dietary whey protein, and therefore we wanted to test whether the whey protein dietary benefits were driven at least partly by GMP.
Our study design provided GMP in the diet within its naturally occurring protein matrix. As a result, mice were consuming GMP in smaller boluses during their awake hours instead of a single large bolus daily as in the prior studies mentioned. In addition, the GMP used in our study was not hydrolyzed as in the prior studies, which might diminish the biological potency of GMP on whole-body insulin resistance and weight...
Figure 4. Altered substrate utilization due to a high-fat diet (HFD) is unaffected with the addition of glycomacropeptide (GMP). (A) Respiratory exchange ratio (RER; $\frac{V_{CO2}}{V_{O2}}$) over a 24-h period (light and dark cycle, dark indicated by the gray filled area). (B) Average RER per light and dark cycle from the last 24 h of the metabolic chambers. (C) Average absolute energy expenditure per hour, (D) average energy expenditure per hour normalized to BW, and (E) ambulatory activity counts from the last light and dark cycle. (B–D) A 2-way ANOVA with Tukey’s multiple comparisons test was used to determine significance; significantly different from control (10% kcal from fat and no GMP; *), $P < 0.05$. n = 3–6/group. Data are shown as mean ± SEM. Individual data points are shown as circles, triangles, diamonds, and squares for control, HFD WPI (−GMP), HFD (1×GMP), and HFD (2×GMP), respectively. WPI = whey protein isolate; − = absent; 1× = normal amount; 2× = twice the normal amount.
Figure 5. Glycomacropeptide (GMP) reduced the high-fat diet (HFD) WPI (−GMP) increase in epididymal adipose tissue. (A) Gastrocnemius muscle weight along with (B) average gastrocnemius fiber cross-sectional area (CSA). Whole-body adipose weights are separated by (C) epididymal fat and (D) inguinal fat. (E) Liver weight and (F) heart weight. A one-way ANOVA with Tukey’s post hoc analysis was used to determine significance; significantly different from control (10% kcal from fat and no GMP; *) and significantly different from HFD WPI (−GMP) group (#), P < 0.05. n = 7–11/group. Data are shown as mean ± SEM. Individual data points are shown as circles, triangles, diamonds, and squares for control, HFD WPI (−GMP), HFD (1×GMP), and HFD (2×GMP), respectively. WPI = whey protein isolate; − = absent; 1× = normal amount; 2× = twice the normal amount.
gain in C57BL/6 mice (Sawin et al., 2016). These findings suggest that the administration method and the hydrolyzation of GMP are important factors to unmask the potential bioactive properties.

Currently, it is not known in what form GMP arrives in the circulation and target tissues when orally consumed. The 64-AA-long GMP is primarily composed of threonine, glutamate, proline, isoleucine, and valine (Neelima et al., 2013). Recent work found parts of the GMP chain in jejunal fluids after oral ingestion (Koh et al., 2022), insinuating that GMP has the potential to reach circulation in some capacity. Although it is unclear whether GMP reaches the circulation and metabolically active tissues fully or partially intact, GMP directly or indirectly seems to have some effects on metabolically active tissues during an obesogenic diet, as found in our study and others. In fact, when provided in the diet within a whey protein matrix, GMP has a modest adverse response when given in high enough concentrations, as we saw an increase in liver weight, adiposity, and select pro-inflammatory readouts in the liver and muscle, and a decrease in grip

Figure 6. Skeletal muscle sphingolipids accumulated in response to a high-fat diet (HFD). Gastrocnemius (A) triglyceride and (B) ceramide content. Gene expression in gastrocnemius tissue of (C) tumor necrosis factor α (TNF-α), (D) IL-6, and (E) IL-1β. (A, C–E) A one-way ANOVA with Tukey’s post hoc analysis was used to determine significance. (B) A 2-way ANOVA with Tukey’s multiple comparisons test was used to determine significance. Significantly different from control (10% kcal from fat and no glycomacropeptide (GMP); *) and significantly different from HFD WPI (−GMP) group (#), P < 0.05. Graphs are shown as a fold change from control (10% kcal from fat and no GMP; indicated with the dotted line) after normalization to housekeeping gene, L32. (A, C–E) n = 7–11. (B) n = 5/group. Data are shown as mean ± SEM. Individual data points are shown as circles, triangles, diamonds, and squares for control, HFD WPI (−GMP), HFD (1×GMP), and HFD (2×GMP), respectively. WPI = whey protein isolate; − = absent; 1× = normal amount; 2× = twice the normal amount.
strength when doubling the dietary GMP (2×GMP). In this circumstance, we cannot rule out that GMP may be hydrolyzed to its individual AA before entering the circulation. Perhaps positive GMP outcomes in the prior studies (Song et al., 2018; Yuan et al., 2020; Sauvé et al., 2021) may be a function of a brief but heightened increase in AA when GMP is delivered by a bolus. In contrast, continuously elevated AA in the blood by the repeated hour-by-hour consumption of GMP may be counterproductive to the healthy maintenance of cellular function. Indeed, high levels of branched-chain AA have been shown to increase the production of reactive oxygen species and the activation of inflammatory pathway nuclear factor of kappa light polypeptide gene enhancer in B cell (NF-κB) in circulating blood cells, which could lead to proinflammatory outcomes in other tissues (Zhenyukh et al., 2017). As an outcome of normalizing protein content across the experimental diets, we found that the cystine content in the GMP groups was lower compared with the −GMP diet. The 2 cysteine AA linked with a disulfide bond play an important role in the production of glutathione (Stipanuk et al., 1992). A decrease in glutathione production may lead to a decrease in the ability to reduce reactive oxygen species (Mailloux et al., 2013), eventually leading to an increase in inflammation (Barbieri and Sestili, 2012). Therefore, it is possible that the lower cystine amount as a result of controlling for the amount of protein content across the experimental dietary interventions could have blunted possible beneficial gains induced by the GMP in the diet.

In conclusion, we show that GMP given within the whey protein matrix during an obesogenic dietary intervention does not mitigate weight gain or adipose mass. Furthermore, insulin resistance was not improved, nor was DIO-mediated ceramide accumulation in the liver and skeletal muscle halted. Future studies are needed to investigate the mechanism by which GMP affects metabolic outcomes.
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