**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 weeks old) underwent a 10-wk 60% HFD intervention. GMP was absent in the control low-fat diet and HFD WPI (-GMP) groups. HFD WPI (1xGMP) contained a standard amount of GMP while HFD WPI (2xGMP) 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 (2xGMP) might adversely affect tissue obesity-related pathologies. Together, dietary GMP given in a whey protein matrix during a HFD intervention does not alter weight gain, insulin resistance, glucose intolerance, and sphingolipid accumulation in the liver and skeletal muscle.

Keywords: 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 1 β (IL-1β), interleukin 6 (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, nonalcoholic fatty liver disease (NAFLD). 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, like 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 a high-fat diet.

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, and/
or COPD) (Zhou et al., 2015). Likewise, in rodents, whey protein given along with a 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 ~20–25% of the whey protein fraction. GMP is a 64-amino acid long peptide and ~50% of the GMP is glycosylated to different extents (e.g., mono-, di-, tri-, or tetrasaccharides). To date, 14 different glycosylated forms of GMP have been identified (Neelima et al., 2013). GMP 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). GMP has also been revealed to increase fecal excretion of lipids when given during a 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 a 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 if GMP contributes to anti-diabetic outcomes. We hypothesized that GMP would offset weight gain, muscle inflammation, insulin resistance, and skeletal muscle and liver ceramide accumulation during an obesogenic diet.

METHODS

Animals and general experimental design

Male C57BL/6J mice aged 9 weeks old were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) 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-week intervention (Figure 1). After the dietary intervention and additional repeated measures testing (below), mice were fasted for ~4h and euthanized under isoflurane followed by cervical dislocation. Gastrocnemius muscle, liver, heart, and fat depots were dissected, weighed, and frozen in liquid nitrogen or optimal cutting temperature (OCT) (Fisher Scientific 23–730–571, Waltham, MA, USA) in isopentane, and stored at −80°C. All animal procedures were conducted in agreement with standards set by the University of Utah Institutional Animal Care and Use Committee (IACUC).

Experimental diets (Table 1 and 2)

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 nearly 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 (1xGMP) (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.

Figure 1. Overview of experimental design where 10-week-old mice underwent a 10-week dietary intervention of a high-fat diet with different amounts of GMP within the whey protein fraction. GMP was eliminated from protein fractions of the control diet as well as from HFD (-GMP). HFD (1xGMP) contained a commonly found amount of GMP within the whey protein fraction, whereas HFD (2xGMP) contained twice the amount of GMP.
The HFD WPI (2xGMP) (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 kcals), diets contained (% kcal) 0.14%, 0.49%, 4.6%, and 7.7% GMP for control, HFD WPI (-GMP), HFD WPI (1xGMP), and HFD WPI (2xGMP), respectively (Table 1). To maintain a similar protein content in the 2xGMP experimental diet (~19%), some whey protein isolate was removed and replaced with GMP. All dietary protein content and GMP were provided by Glanbia Nutritionals (Twin Falls, ID, USA). Complete formulated diets for experimental treatments were assembled and provided by Envigo (Indianapolis, IN, USA). GMP content in manufactured diets was tested by high-performance liquid chromatography (HPLC) and amino acids content was tested and determined using a Waters Corporation (Milford, MA, USA) kit (system guide 71500129702/Revision B) and ultra-performance liquid chromatography (UPLC), all performed by Glanbia Nutritionals.

### Table 1. Dietary composition

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HFD WPI (-GMP)</th>
<th>HFD WPI (1xGMP)</th>
<th>HFD WPI (2xGMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>10.2</td>
<td>60.0</td>
<td>60.0</td>
<td>60.1</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>70.8</td>
<td>21.2</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>
<td>18.7</td>
</tr>
<tr>
<td>GMP</td>
<td>0.1</td>
<td>0.5</td>
<td>4.6</td>
<td>7.7</td>
</tr>
<tr>
<td>kcal/g food</td>
<td>3.7</td>
<td>5.1</td>
<td>5.1</td>
<td>5.1</td>
</tr>
</tbody>
</table>

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

### Body composition and physical function testing

A nuclear magnetic resonance (NMR) instrument (Bruker Minispec MQ20 NMR analyzer, Rheinstetten, German) 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, USA). The speed began at 0.1rpm and increased by 0.3rpm/s increments until it reached 50rpm. 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 (GTT) were performed by injection of intraperitoneal glucose of 5μL/g body weight of 20% dextrose (Sigma-Aldrich G8769l; 1mg glucose per gram 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 ~10min to clot before centrifugation at 12,500xg for 10min 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, USA) at OD505. For serum insulin analyses, a low-range ultra-sensitive mouse insulin ELISA kit (Crystal Chem 90080) was used (using manufacturer guidelines) and read using an EPOCH plate reader (Take3, BioTek, Winooski, VT, USA) at OD450/630. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as previously described (Bowe et al., 2014).

### Table 2. Breakdown of individual amino acids per experimental diet

<table>
<thead>
<tr>
<th></th>
<th>HFD WPI (-GMP)</th>
<th>HFD WPI (1xGMP)</th>
<th>HFD WPI (2xGMP)</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>
<td>3.9</td>
<td>4.5</td>
<td>5.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.8</td>
<td>6.7</td>
<td>8.7</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.9</td>
<td>1.7</td>
<td>1.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.4</td>
<td>2.7</td>
<td>2.1</td>
</tr>
<tr>
<td>Valine</td>
<td>5.8</td>
<td>6.1</td>
<td>6.7</td>
</tr>
</tbody>
</table>

Values are shown as a percentage of the total amount of protein in the diet.
Metabolic measurements using CLAMS

To measure numerous metabolic parameters, mice were placed in metabolic cages (CLAMS; Columbus Instruments Comprehensive Lab Animal Monitoring System (Columbus, OH, USA, serial# 180072)) for 72h before the end of the experimental intervention. Mice were single-housed and acclimated for 48h before taking measurements and collecting data for 24h (light and dark phase). Respiratory exchange ratio (RER) was calculated from VCO₂ production and VO₂ consumption. 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, while an RER closer to 0.7 indicates fats are used as a fuel source. Additionally, energy expenditure was calculated by dividing heat production (kcal/hr) by body weight. 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 10μm 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 (CSA), sections were blocked in Mouse (M.O.M.) (Vector Laboratories, MKB-2212-1) for 1h. 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) (in 1x PBS) overnight. Next, slides were incubated in secondary antibody AMCA (Vector Laboratories CI-1000) at a concentration of 1:250 in 1x PBS for 1h. Stained sections were imaged on a Zeiss Slide Scanner Axio Scan.Z1 (Carl Zeiss Inc., Oberkochen, Germany) with the 20x objective lens. Images were analyzed for the total area covered by lipid droplets using ImageJ software.

Liver. After dissecting, but before freezing, a piece of liver tissue was placed in 4% paraformaldehyde for 24h, after which it was stored in 70% ethanol. Images were adjusted for contrast and brightness using ImageJ software and analyzed using semi-automatic muscle analysis using segmentation of histology: a MATLAB application (SMASH) (Smith and Barton, 2014). An average of 2,006 ± 143 SEM fibers were analyzed.

Gene expression

RNA was isolated from liver (~10mg) and gastrocnemius (~35mg) 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, USA), 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 5min, 46°C 20min, 95°C 1min, 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) TTCTCTGTCACAATGTCAA, and (Reverse) GGCTTTTTCGTTTCTTATAGGA. The following primers were purchased from Bio-Rad: Tumor necrosis factor (Tnf, qMmuCED0004141), Interleukin 6 (Il6, qMmuCED0045760), Interleukin 1β (Ilb, qMmuCED0045755).

Tissue triglyceride and sphingolipid detection and analysis

Triglycerides. To investigate triglyceride content in the gastrocnemius, ~70mg 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 BCA protein assay kit (ThermoFisher 23227) measured by an EPOCH spectrophotometer (Take3, BioTek, Winooski, VT, USA).

Lipid extractions. Frozen gastrocnemius and liver (10–15mg) were homogenized in 225μL of MeOH containing internal standard (IS) and 188μL 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 MeOH without tissue homogenate. 750μL MTBE was added to sample tubes, which were incubated on ice for
60min, with brief vortexing every 15 min. Samples were centrifuged for 10 min at 15,000xg 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:MeOH:ddH2O, as mentioned above, and organic and aqueous fractions were evaporated and resuspended for 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); dihydroceraamide (d18:0/18:1) (44.2 pmol/sample); glucosylceramide (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)-15:0) (850.4 pmol/sample); 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 Agilent 1290 Infinity pump, and Agilent 6490 triple quadrupole (QQQ) mass spectrometer. Sphingolipids were detected using dynamic multiple reaction monitoring (dMRM) in positive ion mode. Source gas temperature was set to 175°C, with a gas (N2) flow of 12L/min and a nebulizer pressure of 30psi. Sheath gas temperature was 250°C, sheath gas (N2) flow of 12L/min, capillary voltage was 3500V, nozzle voltage was 500V, high-pressure RF 190V, and low-pressure RF 120V. Injection volume was 3μL and the samples were analyzed in a randomized order with the pooled QC sample injected 8 times throughout the sample queue. Mobile phase A consisted of ACN:H2O (60:40 vol/vol) in 10mM ammonium formate and 0.1% formic acid, and mobile phase B consisted of IPA:ACN: H2O (90:9:1 vol/vol/v) in 10mM 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 1.4min, increased to 80% B from 1.4 to 7.0min, and increased to 99% B from 7.0 to 7.14min, where held until 9.45 min before returned to starting conditions at 9.8min. Post-time was 3.5 min and the flow rate was 0.4mL/min throughout.

**LC-MS data processing.** For data processing, Agilent MassHunter (MH) Workstation and software 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 was evaluated using Excel where initial lipid targets were parsed based on the following criteria. Only lipids with relative standard deviations (RSD) less than 30% in QC samples are used for data analysis. Additionally, only lipids with background AUC 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 ± SEM. 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 GTT 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, USA) was utilized for all statistical analyses and figure assembly.

**RESULTS**

**Tissue composition and physical function**

As expected, body weight, lean mass, and fat mass increased following a 60% HFD (Newsom et al., 2017) but these outcomes were not prevented by GMP (Figure 2A-C). Alternately, 2xGMP subtly increased fat mass further compared with the -GMP group. Regarding physical function, grip strength decreased when normalized to lean mass and was further reduced in the 2xGMP group (vs -GMP), while time on the rotorod decreased in all groups compared with control (Figure 2E, F). Finally, the 1xGMP 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 body weight 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 (Figure 3B-D). All HFD interventions decreased glucose clearance, and increased fasting levels of glucose and insulin, resulting in an increased HOMA-IR index. Additionally, we subjected mice to indirect calorimetry at the end of the 10-week dietary intervention, to investigate their metabolic health by measuring respiratory exchange ratio (RER), energy expenditure, and physical activity levels. As expected (White et al., 2015), the ability to switch between fats and carbohydrates for fuel was altered with a HFD and unchanged with the addition of GMP (compared with control) (Figure 4A, B). Absolute energy expenditure was increased during the light phase for all HFD groups (Figure 4C) and energy expenditure normalized to body weight and ambulatory activity were not different between all groups (Figure 4D, E). 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 (Figure 5C-F). However, liver weight was higher in the 2xGMP (vs -GMP). Gastrocnemius weights were also increased following HFD, likely due to an increase in body weight, but this occurred without an increase in fiber size (Figure 5A, B). Interestingly, there was 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 since sphingolipids, such as ceramides, are drivers of insulin resistance and tissue inflammation following HFD (Holland et al., 2011) (Figure 6, 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

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**Figure 2.** 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-week intervention. Body composition measured by NMR shown as absolute (B) lean mass and (C) fat mass. (D) Food intake as an average of kilocalories per day of the entire 10-week 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.
were not affected by different amounts of GMP in the diet. We also noted mild local inflammation in skeletal muscle after the high fat diet and this was found only after the 2xGMP dietary intervention compared with -GMP (Figure 6C-E).

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 a HFD in the 1xGMP group compared with control (Figure 7B, C). There was a tendency for all HFD groups to increase TNF-α mRNA expression with 2xGMP significantly increasing TNF-α expression compared with control (Figure 7D). Similarly, IL-6 gene expression was increased only in the 2xGMP group following a 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 inflam-

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**Figure 3.** Whole-body glucose intolerance and markers of insulin resistance were unaffected by the addition of GMP. (A) Glucose tolerance test at the 10-week time point, measurements taken after intraperitoneal glucose injections of 5μL/g body weight of 20% dextrose. Fasting levels of serum (B) insulin and (C) glucose, with which (D) Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated. (A) A 2-way ANOVA with Tukey’s multiple comparisons test was used to determine significance. (B-D) A one-way ANOVA with Tukey’s post-hoc analysis was used to determine significance. (A) All groups significantly different from control (10% kcal from fat and no GMP) (*), HFD WPI (1xGMP) significantly different from control (**), $P < 0.05$. (B-D) Significantly different from control (10% kcal from fat and no GMP) (*), $P < 0.05$. n = 8–11/group.
mation, and ceramide accumulation in liver and skeletal muscle tissue after a 10-week obesogenic diet. The main findings of this study were that GMP did not prevent weight gain or reduce adiposity during diet-induced obesity. In addition, GMP did not prevent diet-induced obesity-related tissue ceramide accumulation or insulin resistance compared with a dietary intervention nearly 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.

Figure 4. Altered substrate utilization due to a HFD is unaffected with the addition of GMP. (A) Respiratory exchange ratio (RER) (V\text{CO}_2/\text{VO}_2) over a 24-h period (light and dark cycle (indicated by the gray filled area)). (B) Average RER per light and dark cycle from the last 24h of the metabolic chambers. (C) Average absolute energy expenditure per hour, (D) average energy expenditure per hour normalized to body weight, 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.
in 10-week-old mice. More so, we did not observe a GMP protective effect against high fat diet-induced tissue ceramide accumulation, 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-week 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-week high fat dietary 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 therapeutic value. In fact, mice consumed more GMP per day (data not shown) in our experiment than given in prior studies. Our interest was to better understand the anti-inflammatory properties of dietary whey protein and therefore we wanted to test if 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 above. 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 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-amino acid 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 GMP has the potential to reach circulation in some capacity. Though it is unclear if GMP reaches the circulation and metabolically active tissues fully or partially intact, GMP directly/indirectly seems to have some effects on metabolically active tissues during an obesogenic diet as found in our study and others. In fact, GMP when provided in the diet within a whey protein matrix has a modest adverse response when given in high enough concentrations since we saw an increase in liver weight, adiposity and select pro-inflammatory readouts in the liver and muscle, and a decrease in grip strength when doubling the dietary GMP (2xGMP). In this circumstance, we cannot rule out that GMP may be hydrolyzed to its individual amino acids 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 amino acids when GMP is delivered by a bolus. In contrast, continuously elevated amino acids in the blood by the repeated hour by hour consumption of GMP may be counterproductive to the healthy maintenance of cellular function. Indeed, high

Figure 5. GMP reduced the HFD WPI (-GMP) increase in epididymal adipose tissue. (A) Gastrocnemius muscle weight along with (B) average gastrocnemius fiber cross-sectional area. 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.
levels of branched-chain amino acids have been shown to increase reactive oxygen species (ROS) production 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 pro-inflammatory 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 amino acids 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 diet-induced obesity-mediated ceramide accumulation in the liver and skeletal muscle halted. Future studies are needed to investigate the mechanism by which GMP impacts metabolic outcomes.

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Conflicts of interest EDB is employed by Dairy West and LSW and BLP are employed by Glanbia Nutritionals.

Contributions NMMPdH and MJD conceived and designed experiments. NMMPdH, JJP, RJN, EMY, LvO, MJL, PEB, and PJF performed experiments and data acquisition. NMMPdH, JJP, and RJN analyzed data. EDB, LSW, and BLP provided and tested protein content for diets. NMMPdH and MJD designed final figures and created the manuscript. All authors approved the final version of the manuscript.

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