A Whey Protein Hydrolysate Promotes Insulinotropic Activity in a Clonal Pancreatic \( \beta \)-Cell Line and Enhances Glycemic Function in \( ob/ob \) Mice\(^1\text{-}^3 \)

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Abstract

Whey protein hydrolysates (WPHs) represent novel antidiabetic agents that affect glycemia in animals and humans, but little is known about their insulinotropic effects. The effects of a WPH were analyzed in vitro on acute glucose-induced insulin secretion in pancreatic BRIN-BD11 \( \beta \) cells. WPH permeability across Caco-2 cell monolayers was determined in a 2-tiered intestinal model. WPH effects on insulin resistance were studied in vivo following an 8-wk oral ingestion (100 mg/kg body weight) by \( ob/ob \) (OB-WPH) and wild-type mice (WT-WPH) compared with vehicle control (OB and WT groups) using a \( 2 \times 2 \) factorial design, genotype \( \times \) treatment. BRIN-BD11 cells showed a robust and reproducible dose-dependent insulinotropic effect of WPH (from 0.01 to 5.00 g/L). WPH bioactive constituents were permeable across Caco-2 cell monolayers. In the OB-WPH and WT-WPH groups, WPH administration improved glucose clearance after a glucose challenge (2 g/kg body weight), as indicated by differences in the area under curves (AUCs) \((P \leq 0.05)\). The basal plasma glucose concentration was not affected by WPH treatment in either genotype. The plasma insulin concentration was lower in the OB-WPH than in the OB group \((P \leq 0.005)\) but was similar between the WT and WT-WPH groups; the interaction genotype \( \times \) treatment was significant \((P \leq 0.005)\). Insulin release from pancreatic islets isolated from the OB-WPH group was greater \((P \leq 0.005)\) than that from the OB group but did not differ between the WT-WPH and WT groups; the interaction genotype \( \times \) treatment was not significant. In conclusion, an 8-wk oral administration of WPH improved blood glucose clearance, reduced hyperinsulinemia, and restored the pancreatic islet capacity to secrete insulin in response to glucose in \( ob/ob \) mice. Hence, it may be useful in diabetes management. J. Nutr. doi: 10.3945/jn.113.174912.

Introduction

Diabetes can be defined as an inability to produce or optimally respond to insulin. It has been classified into 2 types, which differ in their clinical presentation. Type 1 diabetes, an autoimmune condition, is characterized by the destruction of pancreatic \( \beta \) cells, which results in reduced or complete loss of insulin secretion. Type 2 diabetes is characterized by insulin resistance in peripheral tissues (skeletal muscle, liver, and adipose tissue), which can evolve into deficiencies of insulin secretion and \( \beta \)-cell dysfunction in more severe cases \((1)\). Incidence figures for diabetes worldwide are alarming; according to the WHO, type 2 diabetes is estimated to increase to 366 million by 2030 \((2)\). These figures probably underestimate the problem. Beside diabetes, impaired glucose metabolism, hyperinsulinemia together with dyslipidemia, central obesity, and hypertension have also been associated with the development of the metabolic (insulin resistance) syndrome \((1,3)\). Management of diabetes and insulin resistance syndrome involves strategies including modifications in lifestyle, physical activity, and a balanced diet \((1)\), leading to an overall reduction in energy intake; a decrease in the consumption of saturated fats, trans fats, and cholesterol; and an increase in the consumption of low-fat dairy products, vegetables, and grains \((4)\).
Milk proteins and milk-derived peptides have been associated with various health-promoting and disease risk-reducing agents, notably in the modulation of diverse conditions associated with metabolic syndrome (5–7). The consumption of dairy products, particularly low-fat dairy products, has been linked with a reduction in the risk of type 2 diabetes (4,8). Milk proteins, milk-derived peptides, and free amino acids have been demonstrated to regulate postprandial glycemia and insulin secretion in normoglycemic and type 2 diabetic individuals (3,9,10). In vitro studies using pancreatic β-cell lines or primary islet cells have highlighted strong, acute, insulinotropic effects of various amino acids (11,12). Amino acids (Leu, Arg, Ile, Phe, and Ala) or milk-derived peptides ingested in combination with carbohydrates have been shown to increase the secretion of insulin in vivo (13–15). Optimal bioactive peptide mixtures could beneficially promote insulin secretion and thus blood glucose concentration, thereby altering known risk factors for diabetes, leading to modified metabolic control in insulin-resistant or type 2 diabetic patients.

The objective of this study was to assess the potential for enzymatically generated whey protein (WP)12-derived peptides to beneficially modulate insulin secretion in vitro and insulin action and glucose homeostasis in vivo.

Materials and Methods

Generation of the WP hydrolysate. A WP substrate (0.88 kg protein/kg WP, Carbery Milk Products) from bovine milk was hydrolyzed with a pancreatic enzyme preparation as described in Nongonierma and Flirt-Gerald (16). The WP and WP hydrolysate (WPH) samples were freeze-dried (FreeZone 18L, Labconco) and maintained at −20°C until utilization. The enzymatic hydrolysis reaction was carried out on 2 independent occasions to confirm the reproducibility of the hydrolysis process (trials 1 and 2).

Reverse-phase HPLC and molecular mass distribution profiles of peptides and proteins in WPH and WP. WP and WPH were analyzed by reverse-phase-HPLC with a liquid chromatograph (Waters) essentially as described by Spellman et al. (17). The molecular mass profiles of the proteins and peptides were determined by gel permeation chromatography (GPC)-HPLC essentially as described by Spellman et al. (17). Each sample was analyzed in duplicate.

Determination of free amino acids in WPH. Free amino acid analysis was performed as per Mounier et al. (18). Certain amino acids, including Trp, Gln, and Asn, could not be determined using this method.

Membrane processing of the WPH. WPH was fractionated using an ultrafiltration (UF) unit (Sartorff Alpha filtration system, Sartorius). A volume of 1.3 L of WPH at 50 g/L was placed in the feed tank to carry out the UF at 30°C. The UF unit was mounted with a 5-kDa and subsequently a 2-kDa modified cellulose acetate UF cassette (Sartorius). The feed pressure was set at 0.20 MPa and the retentate at 0.10 MPa, which corresponded to a trans-membrane pressure of 0.15 MPa. The 2-kDa permeate was subsequently UF treated using a Minimate-TFF System (Pall) mounted with a 0.65-kDa polyethersulfone membrane (Pall) at room temperature (21°C). The inlet pressure was set at 0.28 MPa and the retentate at 0.25 MPa (transmembrane pressure, 0.26 MPa). The different fractions collected were freeze-dried and maintained at −20°C until utilization.

Cell culture. BRIN-BD11 pancreatic β cells were cultured as described by Kiely et al. (19) with the addition of 0.1% l-glutamine. Caco-2 cells were purchased from the American Type Culture Collection and cultured in DMEM (Invitrogen) supplemented with 10% FBS, 1% (v/v) nonessential amino acids, 1% sodium pyruvate, and penicillin (100 U)/streptomycin (100 mg/L) (all from Sigma Aldrich). Cells were maintained in oxygenated 75-cm² flasks in a humidified cell culture incubator with 5% CO₂ and 95% air at 37°C.

In vitro measurement of acute insulin secretion. Cells were seeded and washed as described by Kiely et al. (19) before being incubated in Krebs-Ringer bicarbonate buffer at pH 7.4 containing 1.1 mmol/L glucose. After 40 min, the buffer was removed and replaced by a Krebs-Ringer bicarbonate buffer at pH 7.4 containing 16.7 mmol/L glucose and WP or WPH at different concentrations. After a 20-min incubation, the supernatant was removed and used to measure the insulin secretion level by ELISA (ultrasensitive kit, Mercodia). All chemicals were purchased from Sigma Aldrich.

In vitro measurement of intestinal permeation. Caco-2 cells (3 × 10⁵ cells) were seeded onto 12-mm polycarbonate Transwell inserts (Corning Costar) and grown for 21 d. On d 20 of the Caco-2 Transwell culture, BRIN-BD11 cells (1 × 10⁶ cells) were seeded on culture plates to adhere overnight. On d 21, the Caco-2 Transwell support was inserted into the plates (Supplemental Fig. 1). Both apical and basolateral chambers were equilibrated with prewarmed Krebs-Ringer buffer, after which WPH (10 g/L) or buffer control was added to the apical chamber and the transmucosal flux was measured at time 0 and after 1, 2, 3, and 4 h by detecting changes in insulin secretion in the basolateral BRIN-BD11 cells, a surrogate marker of intestinal permeation. The experimental procedure did not allow for repeated sampling; therefore, each well was analyzed once for each treatment and sampling time tested. The insulin concentration peaked after 3 h and decreased after 4 h, possibly due to exhaustion of the secretion capacities of the pancreatic β cells. Transepithelial electric resistance (TEER) of monolayer was measured with an epithelial voltmeter with a chopstick electrode set (World Precision Instruments). The TEER value was calculated by subtracting the resistance of a blank filter from the monolayer, normalized as cm², and expressed as percent change in TEER measured prior to the addition of test agent. TEER was monitored throughout the transport experiment to ensure that the WPH did not significantly alter the integrity of the intestinal monolayers.

Mice and housing. Heterozygous ob/ob wild-type mice (Jackson Laboratories) were bred and littermates were genotyped by analyzing the presence of the ob mutation by PCR using DNA from ear pieces (RedExtract-N-Amp Tissue PCR kit, Sigma Aldrich) followed by a DdeI restriction reaction (Roche Diagnostics). Homozygous ob/ob mice and their wild-type littermates were used for all experiments. Mice were maintained in a 12-h-light/24-h-dark cycle and consumed food [Harlan Teklad no. 2018 rodent diet (20)] and water ad libitum. All experimental procedures described herein were conducted in accordance with the regulatory guidelines of the University College Dublin and were approved by the Ethics Committee.

WPH administration. Ten-week-old ob/ob mice (n = 10; body weight, 52 ± 4 g) and their wild-type littermates (n = 10; body weight, 25 ± 2 g) were administered with the WPH aqueous solution at a concentration of 100 mg/kg body weight by once daily oral gavage for 8 wk. Body weight was monitored once per week and at the end of the treatment period (63 ± 3 g for ob/ob mice and 27 ± 3 g for wild-type mice). Control ob/ob and wild-type mice received a similar volume of vehicle (water) following the same protocol. Wild-type and ob/ob mice treated with WPH were labeled as WT-WPH and OB-WPH, respectively, and groups treated with vehicle were labeled as WT and OB, respectively.

Oral glucose tolerance test. At the end of the 8-wk treatment period, food was removed for 12 h and the blood glucose concentration from all 4 groups of mice (OB-WPH, WT-WPH, OB, and WT) was measured from the tail vein using a glucometer (Abbott Diagnostics). Mice received glucose at 2 g/kg by oral gavage and their blood glucose concentration was monitored every 15 min during a period of 240 min.

12 Abbreviations used: GPC, gel permeation chromatography; IRS, insulin resistance syndrome; OB, ob/ob mice treated with vehicle; OB-WPH, ob/ob mice treated with the whey protein hydrolysate; TEER, transepithelial electric resistance; UF, ultrafiltration; WP, whey protein; WPH, whey protein hydrolysate; WT, wild-type mice treated with vehicle; WT-WPH, wild-type mice treated with the whey protein hydrolysate.
Measurement of metabolic markers in blood. At the end of the 8-wk treatment period, food was removed and mice were killed by CO₂ asphyxiation and cervical dislocation on the following day. Blood was withdrawn by cardiac puncture and centrifuged at 4000 × g for 15 min. Glucose was measured using a colorimetric assay (Glucose Liquid color kit, Omega), insulin was measured by ELISA (Mercodia), and adiponectin was also measured by ELISA (R&D Systems). Insulin resistance was calculated using a HOMA-IR by dividing the basal plasma insulin (mU/L) × basal blood glucose (mmol/L) by 22.5.

Measurement of chronic insulin secretion from isolated pancreatic islets. Pancreas was harvested from all 4 groups of mice (OB-WPH, WT-WPH, OB, and WT) and islets were separated as described by Krause et al. (21) before being individually picked and cultured at 37°C in a humidified atmosphere for 24 h with RPMI-1640 culture medium supplemented with 11.1 mmol/L glucose and 2 mmol/L glutamine. Chronic (24 h) insulin release was determined by ELISA as described above.

Statistical analyses. All data are presented as mean ± SEM. A 1-way ANOVA was performed for the in vitro insulin secretion by BRIN-BD11 cells treated with different samples, followed by a Fisher’s paired least significant difference post hoc test. A 2-way ANOVA was used to assess the differences in the intestinal permeation measured in cultured pancreatic β cells. Treatment and time were tested as the main effects and the interaction treatment × time was studied. Fisher’s paired least significant difference post hoc test was performed following ANOVA. A 2-way ANOVA was performed on the plasma glucose, plasma adiponectin, and chronic insulin concentrations secreted from isolated pancreatic islets, with genotype and treatment as the main effects tested. Interactions between genotype and treatment were studied. Fisher’s paired least significant difference post hoc test was performed following ANOVA when effects or interactions were found. Due to unequal variances between groups for plasma insulin, the ANOVA was conducted on log-transformed data. A repeated-measures ANOVA with one within factor (time) and 2 between factors (genotype and treatment) was performed on the blood glucose concentration measured during the oral glucose tolerance test. Analysis of the between-subject effects allowed study of the effects of genotype, treatment, and their interaction. Fisher’s paired least significant difference post hoc test was performed following ANOVA when effects or interactions were found. All analyses were performed with GraphPad Prism version 6 (GraphPad Software).

Results

Physicochemical characteristics of WP and WPH. The reverse-phase-HPLC profile of the unhydrolyzed WP was determined (Supplemental Fig. 2A). In agreement with information provided by the manufacturer, the WP substrate contained the major individual WPs typically found in cheese whey (i.e., caseinomacropeptide, BSA, α-lactalbumin, and β-lactoglobulin). Corresponding protein peaks were found in the GPC profile of WP (data not shown). Following 4-h hydrolysis, the individual proteins present in WP were substantially degraded, leaving small peaks corresponding to residual α-lactalbumin and β-lactoglobulin (Supplemental Fig. 2B). The other individual WPs (caseinomacropeptide and BSA) could not be detected in the profile of WPH, indicating that they were digested by the enzyme preparation during incubation. The GPC results (Supplemental Fig. 3) indicated that the largest proportion of the peptide/protein material present in WPH had a molecular mass <2 kDa, indicating that the hydrolysate was composed mainly of a mixture of short peptides (<20 amino acid residues). In agreement with the molecular weight cutoff of the membranes used to fractionate the WP, permeates mostly contained peptides smaller than the membrane cutoff, whereas larger molecular mass peptides were concentrated in the retentate (Supplemental Fig. 3). The total concentration of free amino acids was 138 g/kg in WPH compared with 1 g/kg in WP.

The gel permeation results (Supplemental Table 1) indicate that the hydrolysis process was reproducible and allowed the manufacture of hydrolysates with similar physicochemical characteristics in terms of protein breakdown and peptide profile. The hydrolysates obtained during both reactions were tested in BRIN-BD11 cells. The amount of insulin secreted following administration of the 2 WPH samples did not significantly differ (data not shown), suggesting that the bioactivity of both products was also comparable.

In vitro insulinotropic effect of WPH and associated UF fractions. The addition of WP or WPH to BRIN-BD11 cells did not affect cell viability within the range of concentrations tested (data not shown). Therefore, BRIN-BD11 cells were treated with concentrations of WPH ranging from 0.01 to 5.00 g/L. The addition of WPH led to a concentration-dependent increase in the concentration of insulin secreted (Fig. 1). Insulin secretion was higher with WPH than with the WP control (P ≤ 0.005). No modification in insulin secretion was seen with WP compared with the Krebs-Ringer buffer.

An insulinotropic effect was seen in all UF fractions, except in the 5-kDa permeate, suggesting that bioactivity was due to peptides having a range of different physicochemical characteristics. Therefore, we concluded that proteins peptides in WPH having different masses displayed a significant insulinotropic effect. However, the insulinotropic activity of the UF fractions tested was always lower than that of WPH. Combinations of the 5-kDa retentate and permeate were associated with an in vitro insulinotropic response, which was higher (P ≤ 0.005) than that of the 5-kDa permeate per se (Table 1).

Assessment of the intestinal permeability of constituents of WPH in a 2-tiered transport model. Results from intestinal permeation of WPH over time are presented in Figure 2. At time zero, the basal insulin concentration in the basolateral compartment was similar prior to addition of WPH and the Krebs-Ringer buffer control. A higher concentration of insulin was secreted when intestinal cells received WPH compared with the control. After 1 h of incubation, treatment with WPH led to a higher insulin secretion compared with the control (P ≤ 0.005). Insulin secretion in the control was stable for 4 h and did not significantly differ from the concentration of insulin found in the absence of Caco-2 cells, suggesting that Caco-2 cells did not secrete insulinotropic constituents during the course of the transport experiment.

FIGURE 1 Insulin secretion from BRIN-BD11 cells treated with Krebs-Ringer buffer or Krebs-Ringer buffer supplemented with WP or a WPH. Values are means ± SEMs, n = 3. Means without a common letter differ, P ≤ 0.05. WP, whey protein; WPH, whey protein hydrolysate.
TABLE 1 In vitro insulin secretion by BRIN-BD11 cells treated with UF fractions of a WPH\(^1\)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Insulin secretion</th>
<th>Increase % of WP</th>
</tr>
</thead>
<tbody>
<tr>
<td>WP</td>
<td>0.18 ± 0.01 (\text{a}^L)</td>
<td>0</td>
</tr>
<tr>
<td>WPH</td>
<td>0.49 ± 0.04 (\text{b}^L)</td>
<td>108</td>
</tr>
<tr>
<td>WPH 5-kDa ret</td>
<td>0.37 ± 0.01 (\text{a}^L)</td>
<td>66</td>
</tr>
<tr>
<td>WPH 5-kDa perm</td>
<td>0.19 ± 0.02 (\text{b}^L)</td>
<td>0</td>
</tr>
<tr>
<td>WPH 5-kDa ret:WPH 5-kDa perm (1:3)</td>
<td>0.41 ± 0.03 (\text{c}^L)</td>
<td>91</td>
</tr>
<tr>
<td>WPH 5-kDa ret:WPH 5-kDa perm (1:1)</td>
<td>0.51 ± 0.01 (\text{b}^L)</td>
<td>125</td>
</tr>
<tr>
<td>WPH 5-kDa ret:WPH 5-kDa perm (3:1)</td>
<td>0.41 ± 0.02 (\text{d}^L)</td>
<td>91</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SEMs, \(n = 3\). Means without a common letter differ, \(P \leq 0.05\). Perm, permeate; ret, retentate; UF, ultrafiltration; WP, whey protein; WPH, whey protein hydrolysate.

**Chronic administration of WPH to ob/ob mice improves glucose clearance following a glucose load.** After receiving glucose at 2 g/kg, the blood glucose concentration of the mice was monitored for 2 h (Fig. 3). Although the basal blood glucose concentration (time 0) was not affected by WPH treatment, the OB-WPH group had a better clearance of the glucose load than the OB group at 60 min (\(P \leq 0.005\)) (Fig. 3). Overall differences in the blood glucose concentration between the OB-WPH and OB groups could be seen by calculating the AUC, which was lower in the OB-WPH group (16 ± 2 mmol \(\cdot\) L\(^{-1}\) \(\cdot\) min) than in the OB group (19 ± 1 mmol \(\cdot\) L\(^{-1}\) \(\cdot\) min) (\(P \leq 0.005\)). Administration of WPH also had a significant effect on blood glucose clearance in wild-type mice compared with the vehicle (Fig. 3). Fifteen minutes following glucose administration, the WT-WPH group had a lower (\(P \leq 0.005\)) blood glucose concentration than the WT group. Beyond 30 min, the WT-WPH and the WT groups had similar blood glucose concentrations. As a result, the AUC was lower in the WT-WPH group (14 ± 1 mmol \(\cdot\) L\(^{-1}\) \(\cdot\) min) than in the WT group (16 ± 1 mmol \(\cdot\) L\(^{-1}\) \(\cdot\) min) (\(P \leq 0.01\)).

**WPH reduces hyperinsulinemia in ob/ob mice.** The effects of 8-wk WPH administration on plasma markers related to insulin resistance in wild-type and ob/ob mice are shown in Table 2. The plasma glucose concentration was slightly elevated for the OB group but was not affected by WPH treatment in both groups of mice (Table 2). The plasma insulin concentration was lower in the OB-WPH group than in the OB group (\(P \leq 0.005\)), but it did not differ between the WT-WPH and WT groups (\(P \geq 0.05\)) (Table 2). As a consequence, the HOMA-IR index was lower in the OB-WPH group (140 ± 14) than in the OB group (320 ± 23), indicating a lower insulin resistance (\(P \leq 0.005\)). It did not differ between the WT (16 ± 2) and WT-WPH (19 ± 5) groups (\(P \geq 0.05\)). The plasma adiponectin concentration, a metabolic indicator involved in glucose regulation secreted by adipose tissue, was not affected by treatment with WPH in either type of mice (Table 2).

**WPH restores pancreatic islet capacity to secrete insulin in ob/ob mice.** In line with the in vitro data (Fig. 1), pancreatic islets from the OB-WPH group had higher levels of insulin secretion over 24 h than the OB group (320 ± 23), indicating a lower insulin resistance (\(P \leq 0.005\)). It did not differ between the WT (16 ± 2) and WT-WPH (19 ± 5) groups (\(P \geq 0.05\)). The plasma adiponectin concentration, a metabolic indicator involved in glucose regulation secreted by the islets during 24 h compared with administration of the vehicle (\(P \geq 0.05\)) (Table 2).

**Discussion**

The insulinotropic action of various amino acids on pancreatic \(\beta\) cells has been extensively studied. Among others, Ala, Arg, Leu, and Gln have been reported to stimulate insulin secretion (11,12,22). Furthermore, endogenous peptides, including gastro-inhibitory polypeptide and structural analogs of gastro-inhibitory polypeptide, promote insulin secretion in both BRIN-BD11 cells and ob/ob mice (23,24). The insulinotropic properties of milk-derived components have been well documented, including the insulin-regulating effects of casein-derived hydrolysates (3,9,14) and WPs or WPHs. The insulinotropic action of intact and hydrolyzed WPs has been demonstrated in studies with normoglycemic (25,26) and diabetic subjects (10,15,27). Furthermore, human studies have shown that WPHs had higher insulinotropic effects in vivo than unhydrolyzed proteins (26,28). The enhanced insulinotropic activity of hydrolysates has been correlated with higher rates of intestinal absorption of amino acids and short peptides (14). Consumption of hydrolysates has been associated with an increase in the plasma concentration of specific insulinotropic amino acids such as Leu, Phe, Arg, and Tyr (15), and BCAA dipeptides (28). The in vitro data presented herein describe a particularly potent insulinotropic action of WPH, in agreement with literature findings. Furthermore, intact WP did not alter the insulin secretion by BRIN-BD11 cells, indicating that protein breakdown leading to...

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**FIGURE 2** Insulin secretion measured across a 2-tiered intestinal transport model from BRIN-BD11 cells after treatment of Caco-2 cells with Krebs-Ringer buffer or Krebs-Ringer buffer supplemented with a WPH at 10 g/L. Values are means ± SEMs, \(n = 3\). Means without a common letter differ, \(P \leq 0.05\). WPH, whey protein hydrolysate.

**FIGURE 3** Blood glucose concentration during an oral glucose tolerance test in OB-WPH, WT-WPH, OB, and WT mice. Values are means ± SEMs, \(n = 10\) group. Means without a common letter differ, \(P \leq 0.05\). OB, ob/ob mice treated with vehicle; OB-WPH, ob/ob mice treated with the whey protein hydrolysate; WT, wild-type mice treated with vehicle; WT-WPH, wild-type mice treated with the whey protein hydrolysate.
the release of peptides may be a prerequisite for the insulinotropic effect (Supplemental Table 1; Fig. 1).

It has been proposed that the combined activity of free BCAAs and whey-derived peptides was responsible for the insulinotropic activity of WPHs (13). This observation is supported by the insulinotropic effects observed when mixtures of peptides and amino acids containing different ratios of the 5-kDa permeate and retentate (1:3, 1:1, and 3:1) of WPH were presented to BRIN-BD11 cells (Table 1). We expected that fractionation would lead to an enrichment in insulinotropic peptides in the 5-kDa permeate or retentate; however, this was clearly not the case (Table 1). These results confirm the observation that a range of components (amino acids and peptides) is responsible for the promotion of insulin secretion.

The bioavailability and efficacy of compounds with systemic targets require their transport across the gut following oral ingestion. Factors affecting the absorption of intact peptides include their digestibility in the gastrointestinal tract and their permeability through the intestinal mucosa (29). Therefore, intestinal permeability of WPH was measured in a 2-tiered transport model consisting of Caco-2 monolayers on the basis of the 5-kDa permeate and retentate (1:3, 1:1, and 3:1) of WPH were transported across Caco-2 monolayers on the basis of the increase in insulin concentration in the basolateral compartment (Fig. 2). Permeation was not found with higher molecular weight candidate fractions with insulinotropic action (data not shown). Although the delivery of peptides by the oral route may be restricted by poor intestinal permeability, there is evidence of intestinal transport of intact peptides in vivo. Di- and tripeptides are transported by intestinal transporters such as human peptide transporter-1 (hPepT1). A study on the ingestion of a lacto-tripeptide-enriched yogurt beverage containing Ile-Pro-Pro demonstrated that this peptide could reach the circulation without being degraded (31). Dipeptides (Leu-Trp, Phe-Tyr, and Ile-Tyr) were also detected in plasma following ingestion of the lacto-tripeptide-enriched yogurt beverage (31). Fractionation studies showed that WPH had active constituents that were <650 Da and thus the WPH mixture contained peptides with <7 amino acid residues along with free amino acids. The bioactivity observed in our 2-tiered model herein (Fig. 2) may arise from permeation of these amino acids and short peptides.

The ability to not only pass through the intestinal epithelium while retaining stability in serum and avoiding degradation and elimination before reaching the target cells can only be successfully determined in vivo. It was observed that wild-type mice ingesting WPH for 8 wk had a better capacity to regulate a glucose load than the control group of mice (Fig. 3). A similar effect was observed in ob/ob mice after oral ingestion of WPH for 8 wk (Fig. 3). This indicates that bioactive peptides and amino acids within WPH may be able to stimulate insulin release from the pancreas and potentiate its effect on target tissues to regulate the blood glucose concentration. The differences between the insulin secretion responses to WPH observed in vivo and those observed in vitro (Table 2) in the pancreatic cells isolated from the wild-type mice may be related to other insulin secretion modification mechanisms such as WP-mediated inhibition of dipeptidyl peptidase IV (32,33). The effect of WPs on metabolic responses induced by the administration of a high-fat diet in C57BL/6j mice was recently reported (34). The mice fed for 11 wk a high-fat diet supplemented with WPs had an improved glucose tolerance and insulin sensitivity. Administration (by oral gavage) of a WPH to diabetic KKAY mice was reported to lead to an improvement in glucose tolerance (35). Similar results were previously reported for the tetrapeptide, Leu-Ser-Glu-Leu. In a study involving several animal models of insulin resistance, this peptide displayed antidilabetics effects, with a suppression in the elevation of blood glucose during an oral glucose tolerance test (36). In a recent study, Todebusch et al. (37) analyzed the postprandial effects of intact (WP) and hydrolyzed WPs (WPH) in Wistar rats. Higher serum Leu and insulin concentrations were observed after a 60-min gavage with WPH compared with WP. In contrast with the study herein, gavage of the rats was conducted in a postabsorptive phase (3 h after food removal) for 30 d. However, similar overall results were obtained, indicating an insulinotropic activity of WPH. The results herein additionally demonstrate that WPH reduced hyperinsulinemia in ob/ob mice and restored insulin secretion capacity from isolated pancreatic islets (Table 2). Although the basal blood glucose concentration in ob/ob mice was not affected by WPH administration, our results indicate that WPH stimulates insulin release from pancreatic β cells. This may protect β cells from dysfunction/destruction, which is a condition usually associated with obese and diabetic mice. In addition, the reduction in insulin resistance observed in ob/ob mice (based on HOMA-IR index values) suggests that WPH administration resulted in a more efficient action of insulin on peripheral target tissues. No weight loss was observed in the ob/ob mice group treated with WPH. Furthermore, no modification was observed in the serum adiponectin concentration in ob/ob mice receiving WPH, which could be related to lower insulin resistance in adipose tissue. The results herein indicate the potential role of WPH as a functional food ingredient in the management of glycemic function and diabetes.

### Table 2: Plasma glucose, insulin, and adiponectin concentrations and chronic (24 h) insulin secretion measured ex vivo from isolated pancreatic islets from OB-WPH, OB, WT-WPH, and WT mice

<table>
<thead>
<tr>
<th>Mouse group</th>
<th>OB</th>
<th>OB-WPH</th>
<th>WT</th>
<th>WT-WPH</th>
<th>Genotype</th>
<th>Treatment</th>
<th>Genotype × treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mmol/L</td>
<td>16 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2390 ± 164&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
<td>0.50</td>
<td>0.79</td>
</tr>
<tr>
<td>Insulin, pmol/L</td>
<td>15 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1500 ± 152&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Adiponectin, mg/L</td>
<td>10 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>272 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 ± 1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.002</td>
<td>0.39</td>
<td>0.84</td>
</tr>
<tr>
<td>Ex vivo insulin, fmol / L&lt;sup&gt;-1&lt;/sup&gt; · islet&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>10 ± 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>297 ± 20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>18 ± 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21</td>
<td>0.001</td>
<td>0.11</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± SEMs, n = 10/group. Means in a column without a common letter differ, P ≤ 0.05. OB, ob/ob mice treated with vehicle; OB-WPH, ob/ob mice treated with the whey protein hydrolysate; WT, wild-type mice treated with vehicle; WT-WPH, wild-type mice treated with the whey protein hydrolysate.

<sup>b</sup> Chronic insulin secretion was measured ex vivo from isolated pancreatic islets resuspended in Krebs-Ringer buffer containing 11.1 mmol/L glucose and 2 mmol/L glutamine.

<sup>2</sup> Statistical analyses for plasma insulin concentrations were conducted on log-transformed data due unequal variance between groups.
In conclusion, enzymatic hydrolysis of bovine WPs produces a combination of peptides and amino acids that induce a significant insulinotropic response when presented to a pancreatic β cell line in an acute manner in combination with glucose. Hydrolysate components, or their enzymatic breakdown products, appeared to be able to cross the intestinal epithelium in an in vitro Caco-2 cell model of intestinal permeation. Oral administration of WPH led to improved blood glucose control, reduced hyperinsulinemia, and a restoration of pancreatic islet insulin secretion capacity in obese and diabetic (ob/ob) mice. The WPH may be a suitable functional food ingredient to improve glycemic control and insulin action in humans.

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Literature Cited