Attenuating effect of casein glycomacropeptide on proliferation, differentiation, and lipid accumulation of in vitro Sprague-Dawley rat preadipocytes

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ABSTRACT

Food components with the ability to suppress preadipocyte proliferation and intracellular lipid accumulation may be helpful in the prevention of obesity, which is a worldwide health concern. Casein glycomacropeptide (GMP), which has pronounced gastric inhibitory activity, could potentially possess fat synthesis inhibition properties and an obesity-alleviating capacity. The objective of the present study was to investigate the effect of GMP on the proliferation and differentiation of preadipocytes as well as triglyceride accumulation and glycerol-3-phosphate dehydrogenase activity in preadipocytes isolated from Sprague-Dawley rats. Different dosages (0, 0.31, 0.625, 1.25, 2.5, and 5.0 mg/mL) of GMP were co-incubated with preadipocytes. The proliferation activity of preadipocytes significantly decreased in the GMP-treated group compared with that of the control group without GMP supplementation. The GMP exhibited an inhibitory effect against preadipocyte proliferation in a dose-dependent manner; the maximal antiproliferative effect was obtained with 2.5 mg/mL. The GMP also attenuated differentiation, as revealed by decreased lipid content, and the effect was more pronounced when cells were treated with GMP before or at the beginning of differentiation induction than at later stages of cell differentiation. Cultured preadipocytes treated with GMP accumulated fewer triglycerides and had lower glycerol-3-phosphate dehydrogenase activity than did the control cells without GMP supplementation. In conclusion, GMP can inhibit the proliferation, differentiation, and lipid accumulation of preadipocytes in vitro.

Key words: casein glycomacropeptide (GMP), proliferation, differentiation, Sprague-Dawley rat preadipocyte

INTRODUCTION

Obesity has become the leading metabolic disease in both developed and developing countries during recent years. A pathologic increase in the size and number of adipocytes and accumulation of fat are its main characteristics at the cellular level (Furuyashiki et al., 2004; Garaulet et al., 2006). The amount of adipose tissue can be regulated by the inhibition of adipogenesis and fat deposition (Rahman et al., 2008). It is well established that obesity can be attenuated not only by reduction of food intake due to the satiety-induction signal, but also by diminishing fat accumulation. Therefore, food components with the ability to suppress preadipocyte proliferation, differentiation, and intracellular lipid accumulation may be beneficial to body weight control and prevention of obesity.

Some researches have shown that flavonoids and phenolic acids (Hsu et al., 2006; Hsu and Yen, 2007), naringenin and hesperetin (Morikawa et al., 2008), and chitosan oligosaccharides (Rahman et al., 2008) inhibit the proliferation and adipogenesis of 3T3-L1 adipocytes. The ability of proteins and their hydrolysates to positively inhibit fat accumulation has been demonstrated in many in vitro experimental and animal models (Pilvi et al., 2007; Martinez-Villaluenga et al., 2009; Rajic et al., 2010). Soy protein hydrolysates enriched in β -conglycinin decreased lipid accumulation in 3T3-L1 adipocytes in vitro (Martinez-Villaluenga et al., 2009). An enriched bioactive fraction from whey protein isolate has been shown to inhibit adipocyte differentiation and lipid accumulation using both in vitro and in vivo models (Rajic et al., 2010). Whey-derived protein sources, particularly β -lactoglobulin-enriched fractions, have the ability to decrease both body weight gain and the adiposity index (Pichon et al., 2008). In addition, a high-calcium diet containing whey protein has shown to attenuate body weight and adipose tissue gain induced by a high-fat diet in an obese C57Bl/6J mice model (Pilvi et al., 2007). However, very little literature exists about the function of milk proteins and their hydrolysates on the proliferation and differentiation of preadipocytes.

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Dairy products and dairy components are known to suppress short-term food intake, increase subjective satiety, and stimulate the mechanisms known to signal satiety and satiety (Aziz and Anderson, 2007). Milk protein proteolysis products that are relevant to food intake regulation include casomorphins, casein glycomacropeptide (**GMP**), and the branched-chain amino acid leucine (Anderson and Moore, 2004). Casein glycomacropeptide is the C-terminal AA fragment of κ -casein released by chymosin by cleavage of the Phe105-Met106 peptide bond. Current research is investigating its proposed physiological activities, such as blood pressure lowering (Miguel et al., 2007), bacterial and viral adhesion inhibition (Brück et al., 2006), and bifidobacterial growth promotion (Azuma et al., 1984). The effect of GMP on the secretion of cholecystokinin (**CCK**) and induction of satiety has been reported (Burton-Freeman, 2008; Lam et al., 2009). For women, oral GMP may stimulate the release of CCK, a satiety-inducing peptide, and slow down gastric emptying (Yvon et al., 1994; Burton-Freeman, 2008). There is also an indication that whey is more satiating than is casein (Hall et al., 2003). Casein glycomacropeptide could inhibit gastric acid secretion in calves (Guilloteau et al., 1994). It also has been shown to be associated with reduced fat mass in Wistar rats (Royle et al., 2008). In addition, consumption of recombinant human GMP decreased the body weight gain, as well as liver, kidney, and adipose tissue weight of rats fed a high-fat diet (Kim et al., 2005). However, a study showed that GMP did not affect postprandial CCK concentrations, and the extent of GMP glycosylation had no influence on the results (Keogh et al., 2010). This controversy still needs to be settled.

Although GMP has been shown to stimulate the release of CCK, which may promote satiety and, thereby, decrease body weight and body fat mass, no data have shown whether GMP can regulate proliferation, differentiation, and adipogenesis of preadipocytes and adipocytes until now. We hypothesized that GMP could possess the particular property of fat synthesis inhibition and, thereby, decrease body weight. To further understand the contribution of GMP to body weight control, in the present study, we examined the inhibitory properties of GMP on the proliferation and differentiation, as well as lipid accumulation and glycerol-3-phosphate dehydrogenase (**GPDH**) activity of rat preadipocytes.

MATERIALS AND METHODS

Reagents and Materials

Reagents for preadipocyte culture, including Dulbecco's Modified Eagle Medium (**DMEM**), fetal

bovine serum (**FBS**), HEPES, Ham's F-12 medium, penicillin, and streptomycin, were obtained from Gibco BRL Life Technologies Inc. (Grand Island, NY). Dexamethasone, 1-methyl-3-isobutylxanthine (**MIX**), insulin, L-glutamine, glucose, sodium pyruvate, Oil Red O, formalin, and 3-(4,5)-dimethylthiazolium(-z-y1)-3,5-diphenyltetrazolium bromide (**MTT**) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Triglyceride (**TG**) and GPDH assay kits were acquired from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Casein glycomacropeptide with a purity of over 90% was separated from cheese whey as described previously (Ma et al., 2008).

Cell Line and Cell Culture

Primary rat preadipocytes were isolated from epididymal fat pads of male Sprague-Dawley rats as described previously (Forest et al., 1983). After collagenase digestion, the cell suspension was filtered through a 150- μ m nylon mesh filter to remove undigested tissue. The pellet obtained after centrifugation was resuspended in DMEM supplemented with 10% of FBS. The obtained rat preadipocytes can differentiate into adipocytes and increase lipid droplets in the cytoplasm following adipogenic stimulation as described previously (Torii et al., 2003). Preadipocytes were subcultured in DMEM with 10% of fetal bovine serum at 37°C in a 5% CO₂ atmosphere until 2 d postconfluence.

Determination of Cell Proliferation and Viability by MTT Assay

For cell proliferation, DMEM and Ham's F-12 medium (pH 7.2; containing 25 mmol/L HEPES) were used in a 1:1 ratio as the culture medium, and 4 mmol of L-glutamine/L, 1.5 g of sodium bicarbonate/L, 4.5 g of glucose/L, 1.0 mmol of sodium pyruvate/L, 100 U of penicillin/mL, 100 μ g of streptomycin/mL, and 8% of FBS were added to the cell culture. To assess whether GMP could inhibit the proliferation of rat preadipocytes in a dose-responsive manner, preadipocytes were treated with GMP at concentrations of 0 (control group), 0.31, 0.625, 1.25, 2.5, or 5.0 mg/mL. In order to evaluate the effect of treatment duration, preadipocytes were co-incubated with GMP at a concentration of 2.5 mg/mL for 0, 24, 48, 72, and 96 h. Cell growth was determined by an MTT assay according to the method as described previously (Mosmann, 1983). Briefly, cells were continuously cultured in the same medium with 0.1 mg of MTT/mL for 4 h. After the culture medium was removed, cells were incubated in 0.1 mL of lysis solution. The MTT activity in each well was determined at a wavelength of 570 nm. Antiproliferative activity was calculated using the following equation:

$$\text{Antiproliferative activity (\%)} = \left(1 - \frac{\text{OD value of treated sample}}{\text{OD value of control}} \right) \times 100\%,$$

where OD is the optical density.

Differentiation of Rat Preadipocytes

Cell differentiation was induced using DMEM with 10% of fetal bovine serum supplemented with MDI solution (0.5 mM 1-methyl-3-isobutylxanthine, 1 μ M dexamethasone, and 10 μ g of insulin/mL) as described previously (Guo et al., 2000). The GMP was added to cells at a concentration of 2.5 mg/mL to analyze the effect of GMP addition time on the differentiation of rat preadipocytes. Lipid droplets were visible by phase-contrast microscopy 2 d after MDI treatment.

Lipid Quantification in Differentiated Preadipocytes by Oil Red O Assay

In order to observe the effect of GMP on adipocyte differentiation and lipid accumulation, cells were further treated with GMP at a concentration of 2.5 mg/mL. Cultures were washed twice with PBS after 8 d of differentiation and fixed with 2 mL of 10% formalin (in PBS) for 1 h. Cells were then washed twice with double distilled water and stained with 0.5% of Oil Red O for 15 min at room temperature (Wu et al., 1996). Absorbance at 510 nm was measured using a spectrophotometer. Inhibition of lipid accumulation in adipocytes was calculated using the following equation:

$$\text{Lipid accumulation inhibitory activity (\%)} = \frac{(\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}})}{\text{absorbance}_{\text{control}}} \times 100.$$

Quantification of GPDH Activity and TG Content

At the beginning of the differentiation period, GMP was added to preadipocytes at concentrations of 0, 0.31, 0.625, 1.25, 2.5, and 5.0 mg/mL, respectively. After 8 d of differentiation, the cells were harvested and washed twice with ice-cold PBS. The GPDH activity was determined according to the procedure as described previously (Wise and Green, 1979). Protein concentration was measured using the Lowry method (Lowry et al., 1951). One unit of enzyme activity was defined as the amount of protein required for oxidation of 1 nmol of reduced nicotinamide adenine dinucleotide per minute. Triglyceride content was measured using

a commercial available kit according to the manufacturer's instructions. The GPDH-attenuating activity and TG-decreasing activity were calculated according to the following equations:

$$\text{GDPH-attenuating activity (\%)} = \frac{(\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}})}{\text{absorbance}_{\text{control}}} \times 100$$

and

$$\text{TG-decreasing activity (\%)} = \frac{(\text{absorbance}_{\text{control}} - \text{absorbance}_{\text{sample}})}{\text{absorbance}_{\text{control}}} \times 100.$$

Statistical Analysis

Results are expressed as mean $\pm$ standard deviation of 3 replicated cultures. Data were analyzed using one-way ANOVA, followed by Duncan's multiple comparison test. All analyses were performed using SPSS version 13.0 software (SPSS Inc., Chicago, IL). The statistical significance level was set at $P < 0.05$.

RESULTS

Effect of GMP Concentration on Preadipocyte Proliferation

The ability of GMP to inhibit preadipocyte proliferation is shown in Figure 1. Preadipocytes receiving GMP showed higher proliferation-decrease responses in comparison to those of the control cells (i.e., no GMP addition; $P < 0.05$). The proliferation activity of preadipocytes treated with GMP was significantly decreased at all examined GMP concentrations in a dose-dependent manner up to 2.5 mg/mL. The maximal antiproliferative concentration was 2.5 mg/mL, which achieved $22.02 \pm 1.35\%$ inhibition when compared with that of the control (0 mg of GMP/mL). When GMP concentration was greater than 2.5 mg/mL, the antiproliferative activity did not increase significantly ($P > 0.05$).

Effect of Incubation Duration on the Antiproliferative Activity of GMP

Casein glycomacropeptide was co-incubated with rat preadipocytes for 0, 24, 48, 72, and 96 h. The antiproliferative activity of GMP increased when the length of incubation with preadipocytes was increased up to 72

Figure 1. Effect of various concentrations of casein glycomacropeptide (GMP) supplementation on rat preadipocyte proliferation, expressed as antiproliferative activity (%). The values are presented as means $\pm$ SD of 3 replicated cultures. Columns that do not share the same letter are significantly different from each other at $P < 0.05$. Preadipocytes were treated with GMP at concentrations of 0 (control group), 0.31, 0.625, 1.25, 2.5, or 5.0 mg/mL. The 3-(4,5)-dimethylthiazoliazolo(-z-y1)-3,5-di-phenyltetrazoliumromide (MTT) activity was determined at a wavelength of 570 nm.

h (Figure 2). The antiproliferative activity was greatest when co-incubation time was 72 h. The GMP co-incubation time (72 h) was used for further antiproliferative activity studies.

Effect of GMP Addition Time on Preadipocyte Proliferation

The proliferation curve of preadipocytes without GMP is shown in Figure 3A. The growth of preadipocytes during the first 3 d was slow, which was termed the lag phase. After 3 d and until 6 d, the growth of preadipocytes became rapid. This was termed the logarithmic growth phase. After 6 d, the growth of preadipocytes became very slow, and was termed the stable phase. At different growth phases (i.e., lag, logarithmic, and stable phase), GMP was added to preadipocytes and co-incubated for 72 h. The antiproliferative activity of GMP added at each of these phases is shown in Figure 3B. Adding GMP at the lag phase had the highest antiproliferative activity ($22.77 \pm 1.27\%$) compared with that of the logarithmic and stable phases ($12.04 \pm 0.35\%$ and $6.34 \pm 0.22\%$, respectively; $P < 0.05$). This demonstrated that the antiproliferative activity of GMP depended on the phase of its addition, and treatment of preadipocytes with GMP at the early phase

of cell proliferation (lag phase) markedly decreased preadipocyte proliferation.

Effect of GMP Addition Time on Adipocyte Lipid Accumulation

The natural differentiation curve of adipocytes without GMP addition is shown in Figure 4A. The defined stages are stage 1 (from 2 d before differentiation induction; i.e., d -2 to d 0), stage 2 (from d 0 to 2), stage 3 (from d 2 to 6), stage 4 (from d 6 to 10), and stage 5 (from d 10 to 14). The effect of GMP treatment during these stages on the lipid content of adipocytes is shown in Figure 4B. A decrease in lipid content was found for all GMP treatments, which was $36.38 \pm 1.27\%$, $38.92 \pm 0.35\%$, $30.53 \pm 0.42\%$, $28.27 \pm 0.36\%$, and $15.33 \pm 0.34\%$ when GMP was added at stages 1 to 5, respectively, which suggests that adding GMP before or at the beginning (stage 1 and 2) of cell differentiation caused greater inhibition of lipid accumulation compared with that caused by GMP addition at later stages.

Effect of GMP Concentration on Intracellular TG Content and GPDH Activity

The effect of GMP concentration on differentiated adipocyte intracellular TG accumulation was determined, as shown in Figure 5A. Casein glycomacropeptide reduced intracellular TG accumulation in a dose-dependent manner between 0 and 5.0 mg/mL. Maximal TG reduction ($33.28 \pm 2.36\%$) was achieved with 2.5

Figure 2. Decrease of rat preadipocyte proliferation (antiproliferative activity, %) in response to casein glycomacropeptide (GMP) treatment at incubation time between 0 and 96 h. The 3-(4,5)-dimethylthiazoliazolo(-z-y1)-3,5-di-phenyltetrazoliumromide (MTT) activity was determined at a wavelength of 570 nm. The values are presented as means $\pm$ SD of 3 replicated cultures. Columns that do not share the same letter are significantly different from each other at $P < 0.05$.

mg of GMP/mL supplementation. The addition of GMP also resulted in a marked decrease in GPDH activity in a similar dose-dependent manner (Figure 5B), which was $35.35 \pm 1.27\%$ when adding GMP at a concentration of 1.25 mg/mL.

DISCUSSION

The present work indicates that proliferation of preadipocytes significantly decreases with GMP treatment in a dose-dependent manner. The exposure of preadipocyte to GMP also significantly attenuated adipocyte differentiation in vitro, as revealed by decreased adipocyte lipid content, TG content, and GPDH activity, which indicated the inhibition of adipogenesis and adipocyte differentiation by GMP supplementation. Our results indirectly suggest that GMP supplementation may decrease the number and size of adipocytes. Casein glycomacropeptide has been shown to be associated with reduced fat mass in Wistar rats and has significant additional influence on fat accumulation when combined with whey protein isolate (WPI; Kim et al., 2005). Wang and Jones (2004) proposed that decreased preadipocyte proliferation and adipocyte lipogenesis are mechanisms of antiobesity. Whether GMP can attenuate fat accumulation induced by a high-fat diet of obese rats in vivo is still under further study in our laboratory.

This study shows that GMP addition at 2.5 mg/mL possesses the highest inhibition activity of preadipocyte proliferation and differentiation, as well as attenuating lipid accumulation. Chitosan oligosaccharides at the concentration of 4 mg/mL could significantly inhibit the differentiation of 3T3-1 adipocytes (Rahman et al., 2008). A high-protein diet (300 g/kg) of whey protein concentrate was more effective than was red meat in reducing body weight gain and body fat content of rats when energy intakes were comparable (Belobrajdic et al., 2004). Carcass fat mass and body weight gain of Wistar rats fed WPI plus 200 g of GMP/kg were significantly lower than that of rats fed WPI alone or casein-fed animals (Kim et al., 2005). Supplementation of recombinant human GMP lowered the body weight gain of male Sprague-Dawley rats fed a high-fat diet by 8.4 and 19.1% at doses of 0.5 and 1%, respectively (Keogh et al., 2010). Therefore, the effective dosage of GMP remains to be clarified by rat models or human clinical trials.

Adipocytes are the primary sites for lipid accumulation and storage. Fat deposition in adipose tissue can be lowered by reducing lipid uptake in adipocytes via suppressing lipoprotein lipase or reducing lipid synthesis through inhibiting fatty acid synthase (FAS) (Li et al., 2008). Fatty acid synthase is an important enzyme in

Figure 3. The proliferation curve of preadipocytes without casein glycomacropeptide (GMP) supplementation (A; OD = optical density). Casein glycomacropeptide (2.5 mg/mL) was added to preadipocyte culture at different phases of cell growth, and the decrease in rat preadipocyte proliferation was measured after incubation for 72 h (B). The lag phase was from d 0 to 3, the logarithmic phase from d 3 to 6, and the stable phase from d 6 to 8. The values are presented as means $\pm$ SD of 3 replicated cultures. Columns that do not share the same letter are significantly different from each other at $P < 0.05$.

the pathway of fatty acid synthesis. Fatty acid synthase inhibitors have been reported to provide a potential pathway to target obesity therapy (Kuhajda et al., 2005). The present work shows that GMP can inhibit the differentiation of rat preadipocytes as well as attenuate lipid accumulation, which may indirectly mean that GMP can act as a FAS inhibitor. Glycerol-3-phosphate dehydrogenase, a kind of terminal differentiation marker, is essential for glycerol-3-phosphate synthesis to induce the formation of TG (Lien et al., 2007). The

Figure 4. The natural differentiation curve of adipocytes without casein glycomacropeptide (GMP) supplementation (A; OD = optical density). Casein glycomacropeptide (2.5 mg/mL) was added to cell cultures at different stages: stage 1 (from 2 d before differentiation induction; i.e., d -2 to d 0), stage 2 (from d 0 to 2), stage 3 (from d 2 to 6), stage 4 (from d 6 to 10), and stage 5 (from d 10 to 14). The effect on lipid accumulation was quantified (B). Results are presented as means $\pm$ SD of 3 replicated cultures. Columns that do not share the same letter are significantly different from each other at $P < 0.05$.

reduction of GPDH activity was also observed in the cultures treated with GMP in the present study. All of these may indirectly demonstrate that GMP possesses FAS-inhibiting activity.

It is now accepted that not only the lipid content, but also the number of adipocytes, is variable in adult mammals. Apoptosis plays an important role in the regulation of adipose mass. Adipocyte number may be increased by differentiation of preadipocytes, a

process which has been widely studied (Cornelius et al., 1994; Prins and O'Rahilly, 1997). The decrease of lipid accumulation was more evident for treatments that consisted of incubating the 3T3-L1 cells with xanthohumol, a *Humulus lupulus* L. prenylflavonoid, from confluence (d -2 until d 2 of differentiation; Mendes et al., 2008). Cultures treated with activin A, a member of the transforming growth factor- β superfamily, during the late phase was less effective than the treatment

Figure 5. Effect of casein glycomacropeptide (GMP) supplementation at concentrations from 0 to 5.0 mg/mL on glycerol-3-phosphate dehydrogenase (GPDH) activity and triglyceride production of preadipocytes. Results are expressed as means $\pm$ SD of triplicate experiments. Columns that do not share the same letter are significantly different from each other at $P < 0.05$.

during the early phases (Hirai et al., 2005). The present study shows that the inhibition activity of GMP to the proliferation and differentiation of rat preadipocytes was dependent on the phase of GMP addition. Adding GMP at the lag phase of preadipocyte proliferation, as well as before or at the beginning of differentiation, was more efficient than addition at other phases. This means that GMP addition time is very important and the antidifferentiation effect of GMP occurs in the early differentiation stages.

CONCLUSIONS

The proliferation, differentiation, and lipid accumulation of rat preadipocytes in vitro is significantly inhibited

after being treated with GMP. The effect was more pronounced with a treatment time of 72 h than were other co-incubation periods. In addition, the decrease of lipid accumulation was the most effective for treatment of preadipocytes with GMP from 2 d before differentiation induction to within 2 d after differentiation began. Casein glycomacropeptide not only decreased the proliferation and differentiation of preadipocytes, but also attenuated lipid accumulation and the GPDH activity of adipocytes. These results demonstrate that GMP supplementation efficiently suppresses adipogenesis in rat preadipocytes and adipocytes. Therefore, further research can focus on the potential antiobesity effect of GMP on obese rats and human subjects induced by a high-fat diet.

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