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Low Molecular Weight Chitosan Accelerates Glucagon-like Peptide-1 Secretion in Human Intestinal Endocrine Cells via a p38-Dependent Pathway

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ABSTRACT: Chitosan is widely employed as a dietary supplement. Several studies have shown that chitosan possesses an antidiabetic effect. An important intestinal incretin hormone, glucagon-like peptide-1 (GLP-1), is also known to contribute to the amelioration of diabetes. This study investigated whether chitosan possesses an ability in GLP-1 synthesis and secretion in human intestinal cells. Low molecular weight chitosan (LMWC) significantly increases GLP-1 secretion in human intestinal endocrine cells (NCI-H716) in a dose-dependent manner. LMWC could also dose-dependently increase the mRNA expression of proglucagon, a GLP-1 precursor, but did not affect prohormone convertase 3 (PC 3) mRNA expression. LMWC effectively increased the phosphorylation of mitogen-activated protein kinases (MAPK)-p38 and c-Jun N-terminal kinases (JNK), but not extracellular-signal-regulated kinases (ERK). An inhibitor of p38, but not JNK and ERK, significantly reversed the LMWC-increased proglucagon expression. Taken together, LMWC accelerates proglucagon expression and GLP-1 secretion through a p38/MAPK-dependent signaling pathway. These findings suggest that LMWC may provide a strategy for diabetes therapy.

KEYWORDS: *chitosan, glucagon-like peptide-1, proglucagon, p38 MAPK, intestine*

INTRODUCTION

Chitosan is a biopolymer of glucosamine obtained from the deacetylation of chitin.[1] Chitosan presents a variety of biological and physicochemical characters that have been widely employed as a dietary supplement and used for pharmacological and biomedical applications.[2] Chitosan is not digestible by mammalian digestive enzymes and possesses hypolipidemic activities.[3,4] Recently, chitosan has also been found to alleviate diabetes-related disorders. Chitosan was demonstrated to have a hypoglycemic effect through the increase of secretory capacity of insulin[5] and reduced liver gluconeogenesis as well as enhancement of skeletal muscle glucose uptake[6] in type I diabetic rats. In addition, other animal studies indicate good efficacy of chitosan in suppression of lipid accumulation in liver and adipose tissues[7] or increase of fecal cholesterol[8] by interfering in intestinal micelle formation.[9] These studies suggested that chitosan possesses potential benefits to mitigate diabetic complications. Low molecular weight chitosan (LMWC; average MW about 2.0×10^4 Da) had shown a better hypolipidemic effect through two different approaches: first, LMWC could be absorbed through intestinal epithelial cells and then regulate lipid metabolism;[3,10] second, it showed good binding capacity with lipid and was directly excreted.[3,11] LMWC also displayed good antidiabetic ability in different animal models.[12,13] However, the molecular mechanism of the hypoglycemic effects of LMWC is still unclear and needs to be further clarified.

Glucagon-like peptide-1 (GLP-1) is known as a gut-derived incretin hormone coencoded carboxy terminal to glucagon in the proglucagon gene. GLP-1 released from intestinal L cells can stimulate the secretion of insulin, suppress the secretion of glucagon, inhibit gastric emptying, and reduce food intake.[14,15] The evidence has indicated that both peripheral and brain GLP-1 play a role in food intake regulation and glucose homeostasis.[16] Clinical studies have shown that blood glucose is lowered to near normal levels in type 2 diabetic patients under continuous administration of GLP-1 in both fasting and postprandial states.[17,18] The antidiabetic activities of GLP-1 receptor agonists (incretin mimetics) and dipeptidyl peptidase-4 (DPP-4) activity inhibitors (incretin enhancers) have been demonstrated in clinical studies.[19] Previous studies have also found that the mitogen-activated protein kinase (MAPK)-related signaling pathway is involved in the insulin-stimulated GLP-1 secretion from human enteroendocrine L cell NCI-H716 cells[20] and GLP-1-modulated β-cell chromatin structure.[21] However, it is unclear whether chitosan possesses ability in GLP-1 synthesis and secretion in human intestinal endocrine cells. Therefore, in the present study, we tried to investigate the effect and possible mechanism of LMWC on GLP-1 synthesis and secretion in human intestinal endocrine cells. The results showed that LMWC accelerates the expression of proglucagon and the secretion of GLP-1 through a p38 MAPK-dependent signaling pathway.

MATERIALS AND METHODS

Materials. The chitosan (high molecular weight chitosan, HMWC) was prepared from shrimp shell chitin, which was purchased from Taiwan Applied Chemistry Co. (Kohsiung, Taiwan). The LMWC was prepared from HMWC as described previously.[6] In brief, HMWC powder was dissolved in 8 N HCl with slight and gentle shaking, and

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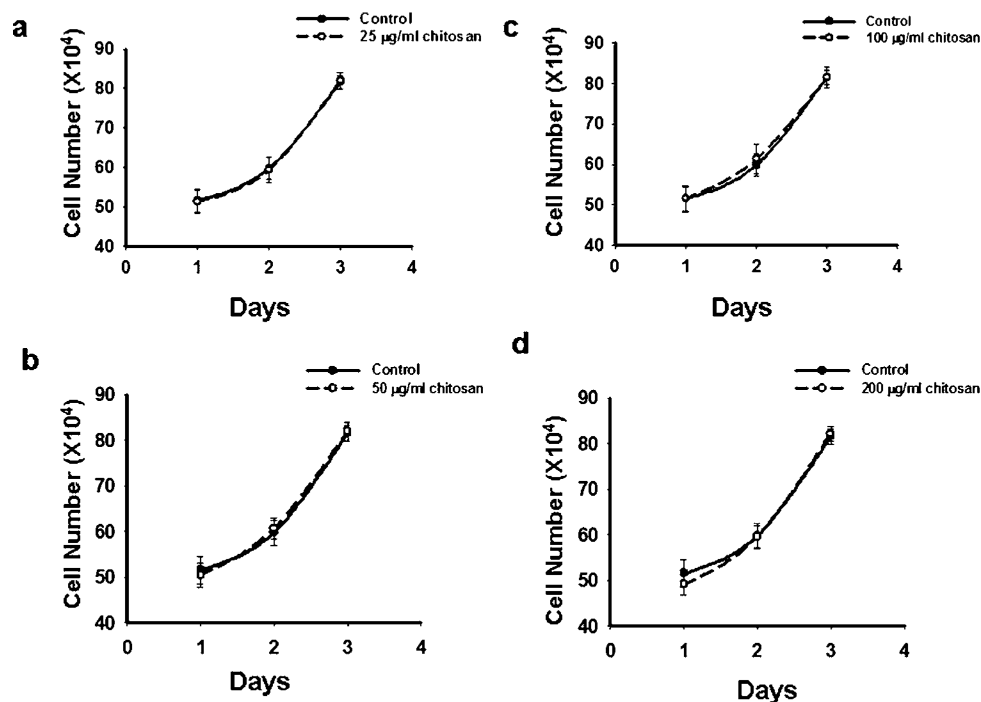

Figure 1. Effect of low molecular weight chitosan on cell viability in human intestinal endocrine cell line NCI-H716 cells. Cells were incubated with (a) 25, (b) 50, (c) 100, and (d) 200 μg/mL chitosan for the indicated time courses. Data are presented as means ± SEM for each group in the three independent experiments.

then the dissolved sample was hydrolyzed at 55 °C for 3 h. The reaction was stopped by cooling the solution to 0 °C and adding equal NaOH (8 N, 0 °C) to adjust the pH to 9−10. Finally, the reaction sample was washed by deionized water and dried at 50 °C. The average molecular weight and deacetylation degree were determined using high-performance liquid chromatography and Fourier transform infrared spectroscopy, respectively, as described by Chen and Liu.[22] A viscosity test was displayed by a Haake viscometer CV20 (Haake Mess-Technik GmbHu Co., Karlsruhe, Germany). Human intestinal endocrine cell line NCI-H716 was purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Chemicals SP600125, U0126, and SB203580 were purchased from (Sigma-Aldrich, St. Louis, MO, USA).

Cell Culture. NCI-H716 cells have been reported to potentially present intestinal endocrine function and autosecrete GLP-1,[23,24] and their characters were indicated to fit with the murine intestinal secretion model.[25,26] The cell culture procedure was followed per the manufacturer's instruction. Cells were cultured at 37 °C in 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 units/mL penicillin (Invitrogen), and 0.1 mg/mL streptomycin (Invitrogen).

Cell Viability Assay. NCI-H716 cells (2×10^5/well) were seeded in a 12-well plate containing with DMEM overnight. NCI-H716 cells were then incubated with chitosan (25−200 μg/mL) for 24−72 h and subsequently stained with trypan blue (Sigma-Aldrich) to count the viable cells under a microscope (IX71, Olympus America).

Detection of GLP-1 Secretion. NCI-H716 (2×10^5/well) cells were seeded in 12-well plates with RPMI medium (Invitrogen) overnight. Subsequently, the cells were transferred to the high-glucose DMEM and treated with chitosan (25−200 μg/mL) for 2 h. Then the cells were centrifuged for protein quantification, and the supernatant was collected for analysis of GLP-1 levels using an enzyme-linked immunosorbent assay (ELISA) kit (Linco Research, Charles, MO, USA).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay. Cells were collected and lysed by TRIzol solution (Life Technologies, Grand Island, NY, USA). The steps followed the manufacturer's instruction to extract total RNA. A total of 5 μg RNA was reverse transcribed in a reaction volume of 30 μL with a Promega reverse transcriptase reagent (Promega BioSciences, San Luis, CA, USA). The RT cDNA products were analyzed by PCR. The conditions were as follows. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; denature 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and run for 33 cycles): forward primer, 5′-GCTGAGAACGGGAAGCTTGT-3′; reverse primer, 5′-TCTCCA-TGGTGGTGAAGACG-3′. Prohormone convertase 3 (PC 3; denature 94 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 30 s, and run for 33 cycles): forward primer, 5′-CGCTGACCTGCACAATGACT-3′; reverse primer, 5′-CAGACA-ACCAGGTGCTGCAT-3′. Proglucagon (denature 94 °C for 30 s, annealing at 59 °C for 30 s, extension at 72 °C for 30 s, and run for 33 cycles): forward primer, 5′-GTAATGCTGGTACAAGGCAG-3′; reverse primer, 5′-TTATAAAGTCCCTGGCGGCA-3′. Finally, the PCR products were analyzed by 3% agarose gel electrophoresis.

Western Blotting Analysis. NCI-H716 (5×10^5/well) cells were seeded in 6-well plates. The cells were starved for 24 h and then treated with LMWC (25−200 μg/mL) for 24 h to collect the cells. The cells were centrifuged at 13000*g* for 20 min at 4 °C and then lysed by the RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The supernatant solution was determined by bicinchoninic acid (BCA) protein assay reagent (Thermo Fisher Scientific, Dreieich, Germany). Equal amounts of total proteins (30 μg per well) were loaded, followed by electrophoresis, transfer, and blocking. The primary antibodies p38, phosphorylated p38, ERK, phosphorylated ERK, JNK, phosphorylated JNK (Santa Cruz) were used. The membrane and primary antibodies (1:1000 dilution) were incubated in the 2% bovine serum albumin (BSA; Sigma-Aldrich) for 6 h at least. Secondary antibodies were incubated for 1 h and then reacted with enhanced chemiluminescence solution (Millipore Technology, Billerica, MA, USA). Quantification of the results was performed by densitometric analysis.

Statistical Analysis. The results were performed in triplicate. Data are presented as means ± SEM. Statistical analysis was performed using Student's *t* test to compare data in different groups. A *P* value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

LWMC Enhances GLP-1 Secretion and Proglucagon Expression. Whether chitosan influences GLP-1 expression is still unclear. We first observed the effect of chitosan on GLP-1 secretion in NCI-H716 cells. Chitosan derivatives have been shown to possess an antidiabetic effect on pancreatic islet cells using concentrations of 10−500 μg/mL.[27] Therefore, we first tested the effect of chitosan on NCI-H716 cells using concentrations of 25, 50, 100, and 200 μg/mL. These concentrations of chitosan did not affect NCI-H716 cell viability (Figure 1). As shown in Figure 2, after 24 h of treatment, chitosan (25−200 μg/mL) significantly increased the GLP-1 secretion from NCI-H716 cells in a dose-dependent manner.

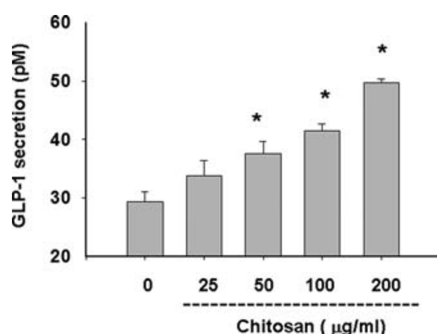

Figure 2. Effect of low molecular weight chitosan on GLP-1 secretion in NCI-H716 cells. Cells were incubated with 25−200 μg/mL chitosan for 2 h. A GLP-1 secretion assay was performed by an ELISA kit. Data are presented as means ± SEM for each group in the three independent experiments. (*) $P < 0.05$ versus control group.

Proglucagon is a precursor of either glucagon or GLP-1 in the pancreas, colon, and intestine.[15,28] We next tested whether chitosan-enhanced GLP-1 secretion was contributed by the proglucagon induction. As shown in Figure 3, chitosan (25−200 μg/mL) significantly elevated proglucagon mRNA expression in NCI-H716 cells in a dose-dependent manner. Moreover, proglucagon is known to be proteolyzed by the prohormone convertase 1/3 (PC 1/3; PC 3), resulting in the formation of GLP-1, in several endocrine tissues including intestine.[15,29] We next examined the effect of chitosan on the expression of PC 3 mRNA in NCI-H716 cells. As shown in Figure 4, chitosan did not modulate the PC 3 mRNA expression. These data indicated that chitosan enhances GLP-1 secretion through the up-regulation of proglucagon expression, but not PC 3 expression, in NCI-H716 cells.

LWMC Augments Proglucagon Expression via a p38 MAPK-Dependent Pathway. The MAPK signaling pathway has been demonstrated to be involved in the insulin-stimulated GLP-1 secretion from human enteroendocrine L cells.[20] Chitosan has also been reported to suppress the expressions of pro-inflammatory mediators in lipopolysaccharide-stimulated human umbilical vein endothelial cells[30] and microglial cells[31] via a MAPK signaling pathway. We next investigated the role of MAPK signals in chitosan up-regulated proglucagon expression. As shown in Figure 5, chitosan (25−100 μg/mL) significantly increased the phosphorylation of p38 in NCI-H716 cells in a dose-dependent manner. Chitosan could increase the JNK phosphorylation at higher concentrations (100 and 200 μg/mL) (Figure 6). Chitosan (25−100 μg/mL) did not affect the phosphorylation of ERK (Figure 7). Furthermore, p38 inhibitor

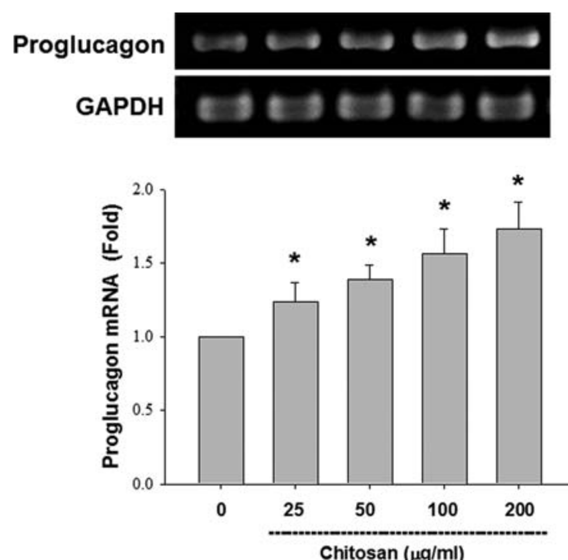

Figure 3. Effect of low molecular weight chitosan on proglucagon mRNA expression in NCI-H716 cells. Cells were incubated with 25−200 μg/mL chitosan for 24 h. Proglucagon mRNA expression was determined by the RT-PCR assay. Data are presented as means ± SEM for each group in the three independent experiments. (*) $P <$ 0.05 versus control group.

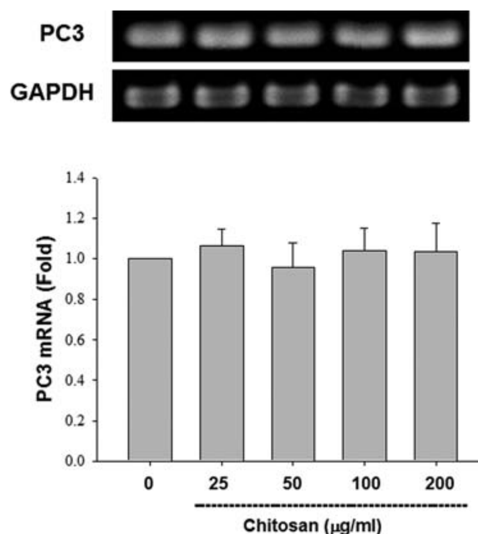

Figure 4. Effect of low molecular weight chitosan on prohormone convertase 3 (PC 3) mRNA expression in NCI-H716 cells. Cells were incubated with 25−200 μg/mL chitosan for 24 h. PC 3 mRNA expression was determined by the RT-PCR assay. Data are presented as means ± SEM for each group in the three independent experiments.

SB203580, but not JNK inhibitor SP600125 and ERK inhibitor U0126, significantly inhibited the increased proglucagon expression induced by chitosan (100 and 200 μg/mL) in NCI-H716 cells (Figure 8). Finally, chitosan-enhanced GLP-1 secretion was significantly attenuated by p38 inhibitor SB203580 (Figure 9). These data suggest that LWMC enhances the proglucagon expression and GLP-1 secretion in NCI-H716 cells via a p38-dependent pathway.

DISCUSSION

Chitosan is known as a versatile biomaterial. It is widely employed as a dietary supplement and used for biomedical and

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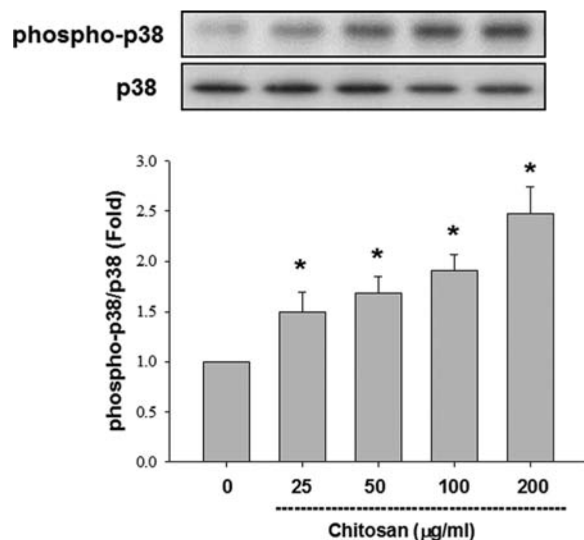

Figure 5. Effect of low molecular weight chitosan on p38 phosphorylation and protein expression in NCI-H716 cells. Cells were treated with 25−200 μg/mL chitosan for 2 h. The p38 phosphorylation and protein expression were performed by Western blotting. Data are presented as means ± SEM for each group in the three independent experiments. (∗) $P < 0.05$ versus control group.

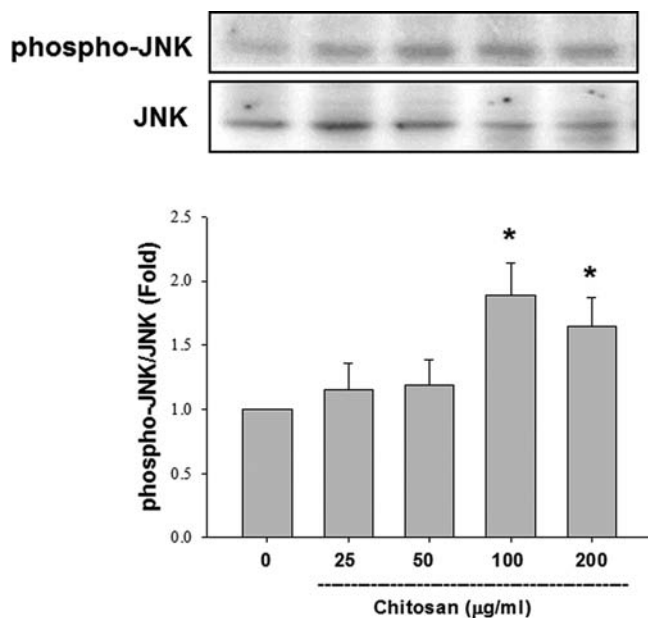

Figure 6. Effect of low molecular weight chitosan on JNK phosphorylation and protein expression in NCI-H716 cells. Cells were treated with 25−200 μg/mL chitosan for 2 h. JNK phosphorylation and protein expression were performed by Western blotting. Data are presented as means ± SEM for each group in the three independent experiments. (∗) $P < 0.05$ versus control group.

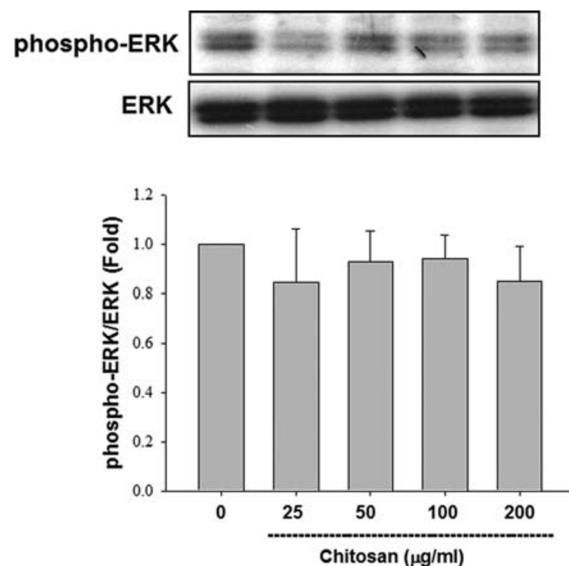

Figure 7. Effect of low molecular weight chitosan on ERK phosphorylation and protein expression in NCI-H716 cells. Cells were treated with 25−200 μg/mL chitosan for 2 h. ERK phosphorylation and protein expression were performed by Western blotting. Data are presented as means ± SEM for each group in the three independent experiments.

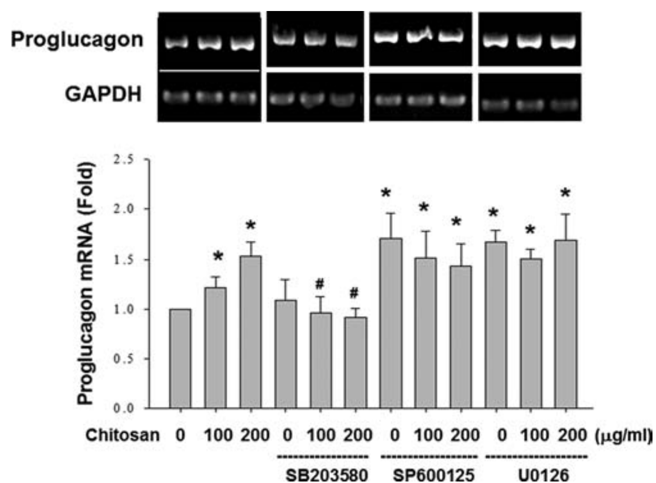

Figure 8. Effect of inhibitors of p38, JNK, and ERK on proglucagon mRNA expression in NCI-H716 cells. Cells were pretreated with inhibitors of p38, JNK, and ERK (SP600125, 10 μM; U0126, 10 μM; respectively) for 1 h and then treated with chitosan (100 and 200 μg/mL) for 24 h. Proglucagon mRNA expression was determined by RT-PCR assay. Data are presented as means ± SEM for each group in the three independent experiments. (∗) $P < 0.05$ versus control group; (#) $P < 0.05$ versus chitosan alone group.

pharmacological applications.[7,32,33] As exemplified by recent discoveries, chitosan presents beneficial functions by lowering overweight/obesity and triggering hypolipidemic/hypocholesterolemic as well as hypoglycemic activities.[3,12,34,35] Previous studies have demonstrated that LMWC possesses antidiabetic activity in several diabetic animal models.[6,12,13] The real mechanism of antidiabetic activity by LMWC is still unclear. Moreover, GLP-1, an intestinal autacoid, has been demonstrated to improve the hyperglycemia and diabetic complications.[17,36,37] These findings lead us to speculate whether chitosan could modulate GLP-1 secretion to improve the diabetic hyperglycemia. In the present study, we demonstrated for the first time that LWMC significantly increased proglucagon expression and GLP-1 secretion in human intestinal endocrine cells. LWMC did not affect PC 3 expression, indicating that the chitosan-enhanced GLP-1 secretion may be not mediated by the increase in cleavage of intestinal proglucagon. These results may partially integrate the connection between LWMC-enhanced GLP-1 secretion and

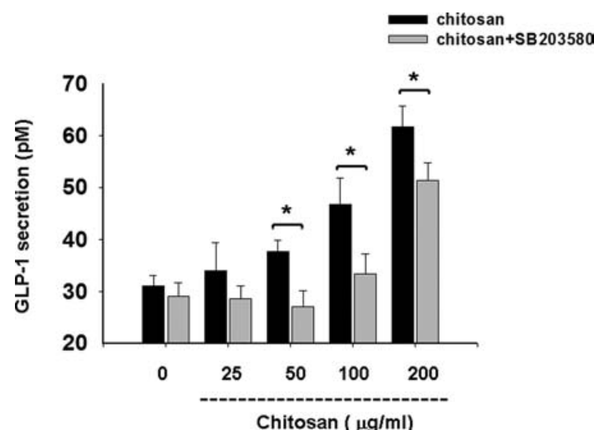

Figure 9. Effect of p38 inhibitor on GLP-1 secretion in NCI-H716 cells. Cells were pretreated with p38 inhibitor (SB203580, 10 μM) for 1 h and then treated with chitosan (25−200 μg/mL) for 2 h. GLP-1 secretion was performed by GLP-1 ELISA kit as described under Materials and Methods. Data are presented as means ± SEM for each group in the three independent experiments. (*) $P < 0.05$ versus control group.

improvement of diabetic hyperglycemia that provides a therapeutic strategy for diabetes.

Our previous study showed that feeding chitosan could effectively elevate the plasma GLP-1 level in diabetic rats.[7] Zeng et al. have indicated that the absorption of chitosan can be increased by the decrease of its molecular weight[10] and might enhance the decrease of lipid absorption in the intestinal epithelial cells or lipid metabolism.[3] Interesting, LMWC has potent gastric cytoprotective and ulcer-healing promoting functions in rats[38] and lowered the serum glucose level or improved the hypertriglyceridemia in diabetic mice.[12] Here, we demonstrated that LMWC could enhance the proglucagon expression and GLP-1 secretion in human intestinal endocrine cells. The previous and our findings suggested that LMWC might be absorbed to regulate endocrine secretion, like GLP-1, or nutrition metabolism in the intestine and alleviate the diabetes disorders.

MAPK-related signal transduction is important in cell proliferation, adaption, differentiation, and apoptosis.[39] It has been shown that cyclic AMP can regulate proglucagon gene expression in intestinal and pancreatic proglucagon-producing cell lines via a MAPK kinase-ERK signaling pathway.[40] Reimer has found that MAPK p38 and ERK 1/2 signals are involved in the meat hydrolysate and essential amino acid-triggered GLP-1 secretion in human enteroendocrine NCI-H716 cells.[41] Adachi et al. have also indicated that free fatty acid α-linolenic acid administered into the intestine could enhance GLP-1 secretion through an ERK MAPK signaling pathway.[42] As exemplified by the recent discoveries, MAPK signaling participated in the carboxymethylated chitosan-stimulated Schwann cell proliferation[43] and chitooligosaccharide-suppressed microglial inflammatory responses.[31] Chitooligosaccharide has also been shown to be capable of inhibiting the H_2O_2-activated MAPK (ERK, p38, and JNK) phosphorylation in neuronal cells that may contribute to its neuroprotective activity.[44] Our recent study has also shown that chitosan supplementation to streptozotocin-induced diabetic rats effectively inhibits the increased liver p38 MAPK phosphorylation, which further induces the mechanism to reduce liver gluconeogenesis.[6] These findings indicate that chitosan or its derivatives possess the ability to regulate the function of MAPK signaling pathway.

Several previous studies have indicated that chitosan has hypolipidemic or hypocholesterolemic properties,[3,45] which ameliorate insulin resistance (or sensitivity) in diabetic rats[7] and clinical obese subjects.[46] Interesting, long-term feeding with chitosan has also been shown to elevate plasma GLP-1 levels, reduce plasma adipocytokine (i.e., TNF-α and IL-6) levels, or induce peroxisomal proliferator-activated receptor-α (PPAR-α) expression.[7] These results hinted that chitosan may improve the insulin and GLP-1 abilities during diabetes disorders. In addition, our early work indicated that chitosan reversed the leptin diminishing expression in diabetic animals.[6] Lim et al. have also mentioned that insulin or leptin activates the downstream signal transduction, including phosphatidylinositide 3-kinases (PI3K)/AKT or MAPK, and then elevates GLP-1 secretion.[47] These results raised the possibility that chitosan may accelerate GLP-1 secretion through insulin and its downstream signaling pathways. However, it needs to be clarified in the future. In the present study, LMWC activated the phosphorylations of MAPK p38 and JNK, but not ERK, in NCI-H716 cells in a dose-dependent manner. The p38 inhibitor, but not JNK and ERK inhibitors, significantly inhibited the chitosan-enhanced proglucagon expression in NCI-H716 cells. These results indicate that MAPK signaling, especially p38 MAPK, is involved in the chitosan-enhanced proglucagon expression in human intestinal endocrine cells.

There are other signaling pathways for the mechanism of GLP-1 formation. The cAMP-related signaling pathway has been found to be involved in the proglucagon gene transcription and GLP-1 production.[48] Liu et al. have also shown that insulin stimulates the phosphorylations of β-catenin and cAMP response element-binding protein (CREB) in intestinal L cells in a phosphatidylinositol 3-kinase (PI3K)- and/or ERK MAPK-sensitive manner and then enhances proglucagon gene expression.[49] Cell division cycle 42 (Cdc42) has been demonstrated to regulate actin remodeling, activation of ERK MAPK and Cdc42-dependent p21-activated kinase-1 (PAK1), and GLP-1 secretion in response to insulin in intestinal endocrine L cells.[50] These pieces of empirical evidence provide other possible signaling pathways for LMWC-enhanced proglucagon gene expression and GLP-1 production in intestinal endocrine cells. It can be elucidated whether these signaling pathways are involved in the effect of chitosan in the future.

In conclusion, this study demonstrated for the first time that LMWC accelerates proglucagon expression and GLP-1 secretion in human intestinal endocrine cells via a p38 MAPK-dependent signaling pathway. These results may provide useful information for application of chitosan in the treatment of diabetes.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

GLP-1, glucagon-like peptide-1; LMWC, low molecular weight chitosan; PC 1/3, prohormone convertase 1/3; MAPK, mitogen-activated protein kinases; HWMC, high molecular weight chitosan; DPP-4, dipeptidyl peptidase-4

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