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Recombinant Mouse Osteocalcin Secreted by *Lactococcus lactis* Promotes Glucagon-Like Peptide-1 Induction in STC-1 Cells

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Abstract An osteoblastic protein, osteocalcin (OC), exists in vivo in two forms: carboxylated OC, and uncarboxylated or low-carboxylated OC (ucOC). ucOC acts as a hormone to regulate carbon and energy metabolism. Recent studies demonstrated that ucOC exerts insulinotropic effects, mainly through the glucagon-like peptide 1 (GLP-1) pathway. GLP-1 is an insulinotropic hormone secreted by enteroendocrine L cells in the small intestine. Thus, efficient delivery of ucOC to the small intestine may be a new therapeutic option for metabolic diseases such as diabetes and obesity. Here, we genetically engineered a lactic acid bacterium, *Lactococcus lactis*, to produce recombinant mouse ucOC. Western blotting showed that the engineered strain (designated NZ-OC) produces and secretes the designed peptide (rOC) in the presence of nisin, an inducer of the recombinant gene. Highly-purified rOC was obtained from the culture supernatants of NZ-OC using immobilized metal affinity chromatography. An in vitro assay showed that purified rOC promotes GLP-1 secretion in a mouse intestinal neuroendocrine cell line, STC-1, in a dose-

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dependent manner. These results clearly demonstrate that NZ-OC secretes rOC, and that rOC can promote GLP-1 secretion by STC-1 cells. Genetically modified lactic acid bacteria (gmLAB) have been proposed over the last two decades as an effective and low-cost mucosal delivery vehicle for biomedical proteins. NZ-OC may be an attractive tool for the delivery of rOC to trigger GLP-1 secretion in the small intestine to treat diabetes and obesity.

Introduction

Osteocalcin (OC) is a protein produced specifically in osteoblasts. Most OCs undergo vitamin K-dependent γ-carboxylation of three glutamate residues (positions 17, 21, and 24) in vivo to provide carboxylated OC (cOC). This modification facilitates the binding of OC to hydroxyapatite in bone matrix [5]. However, a small population of OC exists in the circulation in the uncarboxylated or low-carboxylated form (ucOC). Lee et al. reported that OC gene knockout mice are afflicted with hyperglycemia and obesity, accompanied by decreased serum insulin levels, insulin sensitivity, decreased β cell mass and proliferation, glucose intolerance, decreased serum adiponectin levels, and increased serum triglyceride levels [6]. Interestingly, their study also showed that mice lacking the osteotesticular protein tyrosine phosphatase (a membrane-bound enzyme that stimulates the carboxylation of OC) were hypoglycemic and protected from obesity and glucose intolerance. These results suggest that ucOC acts as a hormone to regulate glucose and energy metabolism. Subsequent studies showed that ucOC directly acts on β cells to stimulate insulin expression and β cell proliferation, or on adipocytes to stimulate adiponectin expression, and protected wild-type mice from type 2 diabetes and obesity [2, 3, 6]. Recent mechanistic studies revealed that the bioactivities of ucOC in these cells are probably mediated by G protein-coupled receptor family C member A (GPRC6A) proteins [11, 12].

Mizokami et al. demonstrated that ucOC induces the secretion of glucagon-like peptide-1 (GLP-1), an insulinotropic hormone produced by intestinal endocrine L cells, by using a mouse enteroendocrine cell line (STC-1) that expresses *Gprc6a* mRNA [8]. In addition, both oral and intraperitoneal administration of ucOC increased serum GLP-1 and insulin levels, suggesting an indirect mechanism for the insulinotropic effect of ucOC. Interestingly, the insulinotropic effect observed in that study seemed to be mediated largely by the GLP-1 pathway, given that serum insulin levels increased by ucOC treatment were reversed by the administration of GLP-1 receptor antagonist. Therefore, the oral delivery of ucOC to intestinal endocrine L cells may be one approach for treating glucose metabolism disorders. Indeed, it was demonstrated that long-term, intermittent oral administration of ucOC reduced fasting blood glucose levels, improved glucose tolerance, and increased the serum insulin levels of healthy mice [9]. However, the accessibility of orally administrated ucOC to the murine small intestine is extremely low (recovery rate of ucOC from the small intestine = less than 0.01%) [9].

In this context, we supposed that oral delivery of ucOC using genetically modified lactic acid bacteria (gmLAB) could be a valuable strategy for preventing or treating metabolic diseases such as diabetes and obesity. Efficient expression systems for recombinant genes, such as the nisin-controlled gene expression (NICE) system, have been established over the past two decades in several LAB strains with generally recognized as safe (GRAS) status [7, 23]. In addition, gmLAB engineered to produce or secrete biomedical proteins have been proposed as an effective and low-cost mucosal delivery vehicle for these proteins. The utility of this idea for treating various disorders, such as infections, allergies, and inflammatory diseases, is supported by numerous studies in model animals and in humans [1, 17, 22]. Indeed, a previous study demonstrated that oral delivery of bioactive heme oxygenase-1 to the murine intestine using gmLAB alleviates symptoms of acute colitis [19].

The aim of this study is to construct a gmLAB strain secreting OC and thus provide a tool for the development of an efficient oral delivery system for OC. We engineered *Lactococcus lactis* to secrete mouse OC (mOC) based on the NICE system and examined the bioactivity of the recombinant mOC (rOC) by assaying GLP-1 secretion in STC-1 cells.

Materials and Methods

Plasmids, Bacterial Strains, and Growth Conditions

A lactococcal plasmid, pNZ8148#2:SEC [18], was employed as the expression vector for the recombinant gene. A DNA sequence coding for mOC (DDBJ accession number: LC217844) was synthesized and cloned into pTAKN-2 by Eurofins Genomics (Tokyo, Japan) using codon optimization for *L. lactis* subsp. *cremoris* MG1363. *L. lactis* NZ9000 (NZ9000) was purchased from MoBiTec (Gottingen, Germany) and used as the host strain for recombinant gene expression. NZ9000 was grown at 30 °C in M17 broth (Becton, Dickinson and Company, Sparks, MD, USA) supplemented with 0.5% glucose (GM17) without shaking. NZ-VC [18], a derivative strain of NZ9000 harboring pNZ8148#2:SEC, was used as a vector

control strain. Genetically modified strains of NZ9000 (gmNZ9000) were grown at 30 °C in GM17 with 10 μg/mL chloramphenicol (GM17 cm) without shaking.

Construction of a gmNZ9000 Strain for mOC Gene Expression

General molecular cloning techniques were performed essentially according to previously described methods [16]. A DNA fragment of mOC was amplified by polymerase chain reaction (PCR) using KOD-Plus-Neo DNA polymerase (TOYOBO, Osaka, Japan) and a primer pair (*Bam*HI-OC F: 5′-CGCGGATCCTATTTAGGTGCATCT GTTCC-3′, *Hin*dIII-OC R: 5′-CGCCCAAGCTTTATGG TAATTCCGTAAATACG-3′) according to the manufacturer's instructions. The resulting fragment was digested with the restriction enzymes *Bam*HI and *Hin*dIII (TaKaRa BIO, Shiga, Japan), then ligated with *Bam*HI/*Hin*dIII-digested pNZ8148#2:SEC. The resulting plasmid (pNZ8148#2:SEC-OC) was analyzed by DNA sequencing (performed by Eurofins Genomics) and was consistent with the predicted sequence. pNZ8148#2:SEC-OC was introduced to electrocompetent NZ9000 cells by electroporation using a Gene Pulser Xcell electroporation system (Bio-Rad, Hercules, CA, USA). The resulting gmNZ9000 strain was designated NZ-OC.

Induction of Recombinant Gene Expression in gmNZ9000 Strains

gmNZ9000 strains were cultured in 2-, 50-, or 500-mL culture systems to induce recombinant gene expression. An overnight culture of gmNZ9000 was inoculated at 1/20 dilution into fresh GM17 cm broth and incubated until the turbidity reached an optical density at 600 nm (OD_{600}) of around 0.4. Then, the culture was supplemented with or without nisin (MoBiTec) at a final concentration of 1.25 ng/mL, and further incubated for 3 h (2- and 50-mL scale culture) or 6 h (500-mL scale culture). Bacterial cells were harvested from 50-mL scale cultures by centrifugation at 3000×*g* for 20 min, washed twice with Hanks' balanced salt solution (HBSS; Thermo Fisher Scientific), and suspended in 10 mL HBSS containing 1.25 ng/mL nisin. Each suspension was incubated at 30 °C for 1 h and immediately used for the co-culture assay with STC-1 cells (described below).

Preparation of Protein Samples from the gmNZ9000 Culture

After nisin induction, bacterial cells and culture supernatants were separated by centrifugation at 8000×*g* and 4 °C for 5 min. Bacterial cells from 2-mL cultures were washed once with 1 mL ice-cold Tris-buffered saline (TBS: 50 mM Tris, 138 mM sodium chloride, 2.7 mM potassium chloride, pH 8.0), then suspended with 400 μL ice-cold TBS containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The suspension was mixed with 0.4 g glass beads (0.2 mmφ, AS ONE, Osaka, Japan) and shaken using a bead beater (μT-12, TAITEC, Saitama, Japan). The soluble fraction was collected by centrifugation at 20,400×*g* and 4 °C for 15 min. Supernatant samples from 2-mL cultures were prepared using trichloroacetic acid precipitation as described elsewhere [18]. An equal volume of 2× Laemmli Sample Buffer (Wako Pure Chemical Industries, Osaka, Japan) was added to several samples and the mixtures were boiled for 5 min.

Detection of rOC

The protein samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using a 4–20% (v/v) gradient polyacrylamide gel. The bands of separated proteins were visualized by gel staining with Coomassie Brilliant Blue (Polysciences, Warrington, PA, USA) or transferred from the gel onto a polyvinylidene fluoride membrane (GE Healthcare, Buckinghamshire, UK). The protein blotted-membrane was blocked with skim milk, reacted with mouse anti-His-tag antibody (Ab) (1/1000) (652501; BioLegend, San Diego, CA, USA), followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG Ab (1/5000) (A4416; Sigma-Aldrich, St. Louis, MO, USA). The resulting blots were reacted with ECL Prime Western Blotting Detection Reagent (GE Healthcare) and detected using a lumino image analyzer (ImageQuant LAS 500, GE Healthcare).

Quantitation of rOC

The rOC concentrations of the cellular extracts, the supernatant samples, and the eluted fraction and the dialyzed solution obtained following purification (described below) were quantified by an enzyme-linked immunosorbent assay (ELISA) according to a previously established method [20]. Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with the prepared samples or recombinant green fluorescence protein (rGFP: 0–250 ng/mL) purified from *Escherichia coli* M15-GFP as a standard protein [20], then saturated with bovine serum albumin (Nacalai Tesque, Kyoto, Japan). The plate-immobilized proteins were reacted with anti-His-tag Ab (652501; BioLegend). The resulting complexes were further reacted with HRP-conjugated goat anti-mouse IgG Ab (A4416; Sigma-Aldrich), then visualized by a HRP-substrate (05298-80, Nacalai Tesque) reaction. The reaction was

stopped with 2 N sulfuric acid and absorbance was measured at 450 nm. In all ELISA experiments, we confirmed good correlation between the rGFP concentration and the absorbance at 450 nm ($R^2 > 0.95$).

Purification of rOC

Recombinant gene expression was induced by nisin in 500-mL cultures as described above, then each culture supernatant was collected by centrifugation at 3000×*g* and 4 °C for 20 min and an equal volume of 2× binding buffer (40 mM sodium phosphate, 1 M sodium chloride) was added. The pH was adjusted to 7.4 with sodium hydroxide and the solution was passed through a membrane filter (pore size = 0.45 μm, Merck Millipore, Billerica, MA, USA). The filtrate was loaded onto a HisTrap HP column (1 mL, GE Healthcare) equilibrated with 1 × binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.4), then the column was washed with 5 column volumes (CV) of 1× binding buffer. The column-absorbed proteins were then eluted with a linear gradient of 0–500 mM imidazole over 35 CVs at 1 mL/min using a fast protein liquid chromatography system (AKTA pure 25, GE Healthcare). The collected fractions (culture supernatant, flow-through, wash, and eluate) were analyzed by SDS-PAGE and western blotting as described above. The eluted fractions were dialyzed against phosphate-buffered saline (PBS; 137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM disodium hydrogen phosphate, 1.76 mM potassium dihydrogen phosphate, pH 7.4). The rOC concentration in the concentrate was determined by western blotting with anti-His-tag Ab, as described previously [20].

Culture Conditions for STC-1 Cells

The mouse intestinal neuroendocrine cell line STC-1 was purchased from the American Type Culture Collection (Manassas, VA, USA). STC-1 cells were maintained in 75 cm^2 cell culture flasks (TPP, Trasadingen, Switzerland) in Dulbecco's modified eagle medium (DMEM; Thermo Fisher Scientific, Rockford, IL, USA) containing 10% fetal bovine serum (GE Healthcare), 100 U/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque) (complete DMEM) at 37 °C in a humidified incubator supplied with 5% carbon dioxide. STC-1 cells were subcultured when the cells occupied 80–90% of the flask growth surface.

Bioactivity Assay of rOC

STC-1 cells (4×10^6) in 2 mL complete DMEM were plated onto a 12-well cell culture plate (Thermo Fisher Scientific). After 48-h incubation, the plate-adhered cells were washed twice with 2 mL HBSS and stimulated for 1 h with HBSS, HBSS containing 5 μM phorbol 12-myristate 13-acetate (PMA), or various concentrations of purified rOC. Mouse active GLP-1 concentrations in the culture supernatants were quantified using an ELISA kit (AKMGP-011; Shibayagi, Gunma, Japan) according to the manufacturer's instructions.

Co-culture Assay with Nisin-Induced gmNZ9000 and STC-1 Cells

STC-1 cells (2×10^6) were adhered onto a 6-well plate (Thermo Fisher Scientific) for 18 h and washed twice with HBSS. Then, the cells were stimulated with a 1-mL gmNZ9000 (1×10^8 CFU) culture prepared as mentioned above and diluted with HBSS. After 1-h incubation, mouse active GLP-1 concentrations in the culture supernatants were quantified using an ELISA kit (AKMGP-011; Shibayagi) according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using a statistical software package (GraphPad Prism7, GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by Dunnett's test was used to determine the significance of the differences. Differences were considered significant at $P < 0.05$. Values are expressed as mean ± standard deviation (SD).

Results

NZ-OC Produces and Secretes rOC Depending on Nisin Stimulation

An expression vector for the mOC gene (pNZ8148#2:SEC-OC) was introduced into NZ9000 (NZ-OC) cells to obtain a gmN9000 strain secreting mOC. Western blotting using anti-His-tag Ab showed bands corresponding to the signal peptide-conjugated form of rOC (11.8 kDa) and to the signal peptide-cleaved form of rOC (9.0 kDa) in the cellular extracts and supernatant samples, respectively, of nisin-induced NZ-OC (Fig. 1a, b). Bands detected in the cellular extracts and supernatant samples of nisin-induced NZ-VC were consistent with the signal peptide-conjugated and signal peptide-cleaved forms, respectively, of pNZ8148#2:SEC-coding peptide (Fig. 1a, b). No bands were detected in either the cellular extracts or supernatant samples from two non-induced gmNZ9000 strains (Fig. 1a, b). ELISA showed that the cellular extracts and supernatant samples from a 1-mL culture of nisin-induced NZ-OC contained 1797.3 ± 60.3 and 188.4 ± 2.9 ng, respectively,

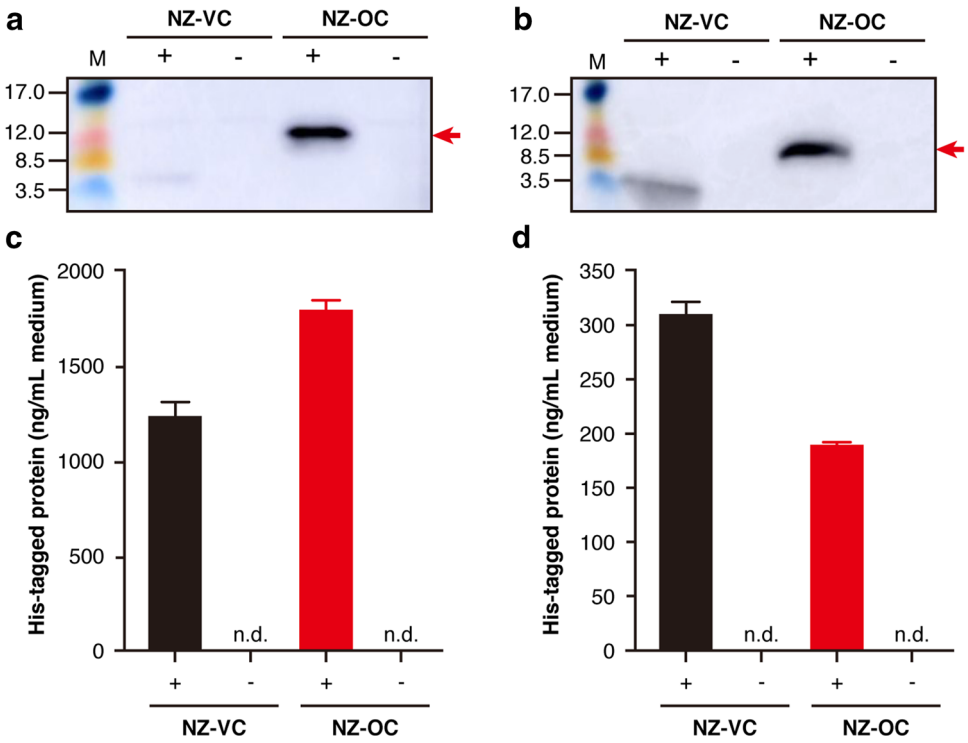

Fig. 1 Detection and quantitation of rOC. Gene expression was induced in NZ-VC and NZ-OC with (+) or without (−) nisin. Cellular extracts (**a**, **c**) and culture supernatants (**b**, **d**) were analyzed with western blotting (**a**, **b**) or ELISA (**c**, **d**) using an anti-His-tag Ab. **a**, **b** The red arrows indicate the signal peptide-conjugated form of rOC (11.8 kDa, **a**) or the signal peptide-cleaved form of rOC (9.0 kDa, **b**). M, molecular weight marker. **c**, **d** Data are expressed as mean ± SD of two independent experiments ($n = 3$). n.d., not detected (Color figure online)

of the His-tagged protein (Fig. 1c, d). The assay also detected the His-tagged protein in samples of nisin-induced NZ-VC (Fig. 1c, d). The concentrations of the His-tagged protein in samples prepared from nisin-non-induced strains were below the detection limit of the ELISA (Fig. 1c, d).

Purification of rOC

rOC was purified from the 500-mL culture supernatant of nisin-induced NZ-OC using immobilized metal ion affinity chromatography. The column-absorbed proteins were eluted using a linear gradient of imidazole and the eluate was collected in three fractions, as described in Fig. 2a. Western blotting showed that the column-absorbed rOC mainly eluted in fraction E-2 (Fig. 2b). In addition, SDS-PAGE analysis showed that rOC was the major protein component of E-2 (Fig. 2c). These results clearly demonstrated that rOC was purified from the culture supernatant of NZ-OC. The rOC concentration in fraction E-2 was 60 μg/mL.

rOC Produced by NZ-OC Promotes GLP-1 Secretion by STC-1 Cells

The ability of rOC to promote GLP-1 secretion in vitro was investigated by co-culturing STC-1 cells, an accurate model of intestinal L cells, with various concentrations of purified rOC or nisin-induced NZ-OC for 1 h. GLP-1 secretion by STC-1 cells was significantly stimulated with 12 or 120 ng/mL rOC (corresponding to 1.3 or 13 nM, respectively) when compared with the solvent control ($P < 0.01$, Fig. 3a). A significant increase in GLP-1 secretion was also induced by stimulation with 5 μM PMA used as a positive control ($P < 0.05$, Fig. 3a). Conversely, GLP-1 secretion by STC-1 cells stimulated with 1.2 or 600 ng/mL (corresponding to 0.13 or 65 nM, respectively) rOC were at baseline levels (Fig. 3a). GLP-1 secretion levels of STC-1 cultures stimulated with nisin-induced NZ-OC were significantly higher compared to those stimulated with nisin-induced NZ-VC (Fig. 3b).

Discussion

NICE in *L. lactis* is among the most common and efficient ways to obtain a desired recombinant protein and to develop a mucosal delivery system for protein using gmLAB [7]. In this study, we constructed a NICE-based secretion system for mOC in NZ9000 cells using a secretion vector (pNZ8148#2:SEC-OC) with an expression cassette for a fusion peptide comprising a lactococcal signal peptide, His-tag, and mOC, under the control of the nisin A promoter. Western blotting showed that pNZ8148#2:SEC-OC-transformed NZ9000 (NZ-OC) cells produce an intracellular His-tagged protein corresponding in size to the signal peptide-conjugated form of rOC (11.8 kDa) following nisin stimulation. The cells then secrete the produced protein using the cell's secretion

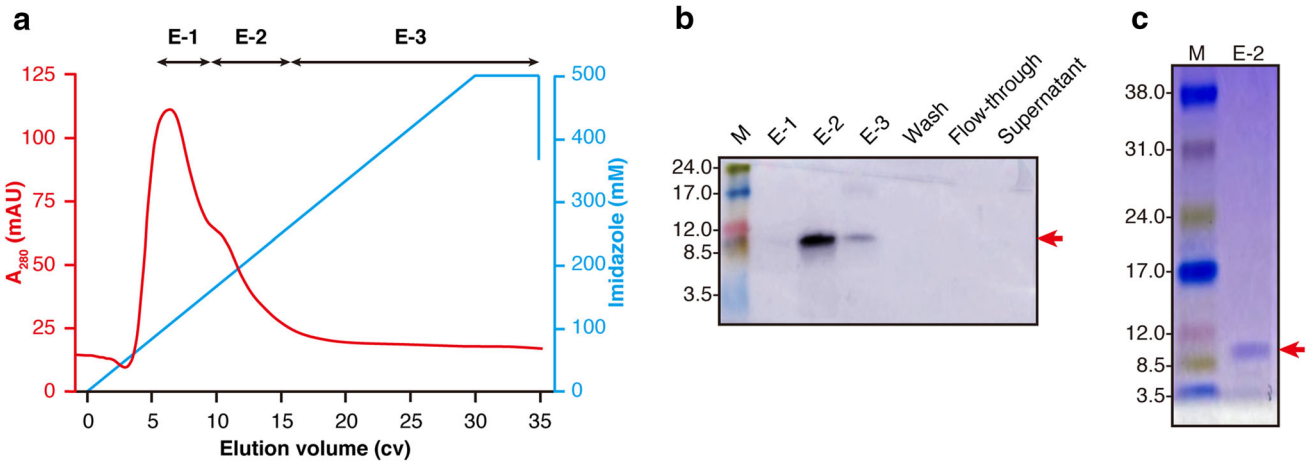

Fig. 2 Purification of rOC. rOC was purified from culture supernatants prepared from nisin-induced NZ-OC using an immobilized metal ion affinity chromatography technique. **a** A chromatogram of the elution phase of the chromatography step. The eluate was collected in three fractions (E-1 to E-3). **b** Fractions obtained following chromatography were analyzed with western blotting using an anti-His-tag Ab. **c** Fraction E-2 was analyzed by SDS-PAGE. **b**, **c** The red arrows indicate the signal peptide-cleaved form of rOC (9.0 kDa). M, molecular weight markers (Color figure online)

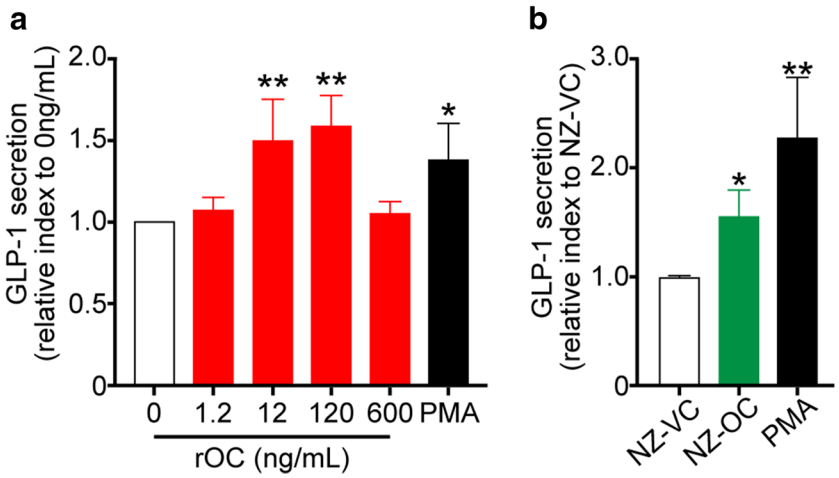

Fig. 3 Bioactivity of rOC in GLP-1 secretion by STC-1 cells measured by ELISA. STC-1 cells were stimulated with various concentrations of purified rOC (0–600 ng/mL) or 5 μM PMA for 1 h (**a**) or by NZ-OC in comparison with the control, NZ-VC (**b**). Data are expressed as relative values versus 0 ng/mL rOC (**a**) or NZ-VC (**b**) and as mean ± SD of three independent experiments ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ were considered significant

machinery, given that the western blot band observed from the culture supernatant corresponds to the signal peptide-cleaved form of rOC (9.0 kDa). ELISA quantified the production and secretion levels of rOC by nisin-induced NZ-OC as 1797.3 ± 60.3 and 188.4 ± 2.9 ng per 1-mL medium, respectively.

Wild-type NZ9000 possesses only one major secretory protein (Usp45) [21], and thus a highly pure recombinant protein can be obtained from the culture supernatant using a relatively simple purification method [10, 15]. We attempted the purification of rOC from a 500-mL culture supernatant of nisin-induced NZ-OC using an immobilized metal affinity chromatography technique and obtained highly-purified rOC by this single purification procedure.

The bioactivity of rOC towards GLP-1 secretion in vitro was examined by stimulating STC-1 cells with various concentrations of purified rOC. We found that 12 and 120 ng/mL rOC promote GLP-1 secretion by STC-1 cells, whereas 1.2 and 600 ng/mL rOC do not. This dose-dependent effect was consistent with a previous report describing the dose dependency of the bioactivity of ucOC in STC-1 cells [8]. Based on two lines of evidence, rOC produced by NZ-OC is probably in the uncarboxylated form. First, although there are a few exceptions, such as *Leptospira borgpetersenii* [14], most bacteria do not have a vitamin K-dependent carboxylase [13]. Therefore, rOC produced in gm-*E. coli* is in an uncarboxylated form [4] and has been used as ucOC in biological studies [2, 3, 6, 8, 9]. Second, it was demonstrated that only ucOC can promote GFP-1 secretion by STC-1 cells [8].

In conclusion, we successfully constructed a gmNZ9000 strain (designed NZ-OC) which secretes rOC based on the NICE system and we clearly demonstrated that rOC secreted by NZ-OC exhibits bioactivity in a dose-

dependent manner to promote GLP-1 secretion in the STC-1 mouse enteroendocrine cell line. These results suggest that NZ-OC may be an attractive tool for the oral delivery of OC to trigger GLP-1 secretion in the small intestine to treat diabetes and obesity. Further studies using model animals are needed to investigate these possibilities further.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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