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# Oral administration of osteocalcin improves glucose utilization by stimulating glucagon-like peptide-1 secretion

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ABSTRACT

Uncarboxylated osteocalcin (GluOC), a bone-derived hormone, regulates energy metabolism by stimulating insulin secretion and pancreatic β-cell proliferation. We previously showed that the effect of GluOC on insulin secretion is mediated largely by glucagon-like peptide-1 (GLP-1) secreted from the intestine in response to GluOC exposure. We have now examined the effect of oral administration of GluOC on glucose utilization as well as the fate of such administered GluOC in mice. Long-term intermittent or daily oral administration of GluOC reduced the fasting blood glucose level and improved glucose tolerance in mice without affecting insulin sensitivity. It also increased the fasting serum insulin concentration as well as the β-cell area in the pancreas. A small proportion of orally administered GluOC reached the small intestine and remained there for at least 24 h. GluOC also entered the general circulation, and the serum GLP-1 concentration was increased in association with the presence of GluOC in the intestine and systemic circulation. The putative GluOC receptor, GPRC6A was detected in intestinal cells, and was colocalized with GLP-1 in some of these cells. Our results suggest that orally administered GluOC improved glucose handling likely by acting from both the intestinal lumen and the general circulation, with this effect being mediated in part by stimulation of GLP-1 secretion. Oral administration of GluOC warrants further study as a safe and convenient option for the treatment or prevention of metabolic disorders.


## Introduction

Osteocalcin is a bone-derived hormone that, in the uncarboxylated form, plays an important role in glucose and energy metabolism, male fertility, and brain function [1–5]. Analysis of various genetically modified animals has revealed that an increase in the circulating concentration of osteocalcin prevents obesity and glucose intolerance [1–3,6]. These effects of osteocalcin are likely mediated by its putative receptor GPRC6A [4,7] and achieved through stimulation of insulin secretion and the proliferation of pancreatic β-cells [7–9] and consequent promotion of glucose homeostasis in muscle and adipose tissue [9,10]. The potential therapeutic relevance of osteocalcin has been demonstrated in mice, in which continuous administration of uncarboxylated osteocalcin (GluOC) via a subcutaneous osmotic pump [9] or its intermittent intraperitoneal injection lowered blood glucose and increased β-cell mass, insulin secretion, and insulin sensitivity [9,10]. Moreover, daily intraperitoneal injection of GluOC for 14 weeks resulted in full recovery of the liver in mice with steatosis induced by a high-fat diet [10]. For therapeutic and, more especially, preventive purposes, however, intraperitoneal injection is not a preferable route of drug administration.

We recently showed that oral administration of GluOC was as effective as intraperitoneal administration in increasing the serum concentration of insulin in mice. This effect of the ingested GluOC was mediated in large part through stimulation of glucagon-like peptide-1 (GLP-1) secretion from intestinal endocrine cells, as revealed by its inhibition by the GLP-1 receptor antagonist exendin(9-39) [11]. Furthermore, we found that orally administered GluOC is absorbed into the general circulation [11], suggesting that this method of application allows GluOC to trigger the secretion of GLP-1 by acting at both apical and basolateral membranes of intestinal endocrine cells. GLP-1 is one of the incretin hormones that are secreted from the intestine on ingestion of various nutrients to stimulate insulin secretion from pancreatic β-cells in a glucose-dependent manner [12,13]. After its secretion, GLP-1 undergoes degradation by dipeptidyl peptidase-4 and is thereby

*Abbreviations:* DAPI, 4′,6-diamidino-2-phenylindole; EIA, enzyme-linked immunoassay; GLP-1, glucagon-like peptide-1; GluOC, uncarboxylated osteocalcin; IPGTT, intraperitoneal glucose tolerance test; OGTT, oral glucose tolerance test; PCNA, proliferating cell nuclear antigen.

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rapidly inactivated [14]. In addition to its insulinotropic action, GLP-1 exerts various biological effects such as inhibition of food intake, amelioration of fatty liver, and prevention of cardiovascular disease [12,13]. Incretin-related drugs such as GLP-1 receptor agonists and dipeptidyl peptidase-4 inhibitors are thus under investigation for clinical use to achieve better glycemic control in individuals with type 2 diabetes [15].

Whereas, for most drugs, oral administration is the most convenient and safest route of delivery, such administration of protein or peptide agents risks their degradation by proteinase activities in the digestive tract and consequent limited absorption into the general circulation. In the case of GluOC, however, the presence of the intact peptide in the intestine might be expected to promote GLP-1 secretion by acting at the apical membrane of intestinal endocrine cells. In the present study, we examined whether long-term oral application of GluOC might be effective in promoting glucose tolerance in mice, and we determined the distribution of the administered drug in the alimentary canal and systemic circulation.

## Materials and methods

### *Preparation of recombinant GluOC*

Recombinant mouse GluOC was prepared as described previously [11]. In brief, a glutathione *S*-transferase (GST)–GluOC fusion protein was isolated from bacteria by consecutive exposure to a buffer containing 0.1% Triton X-114 (Sigma-Aldrich, St. Louis, MO) and the same buffer without detergent in order to remove endotoxin. The GluOC moiety was cleaved from GST with the use of thrombin, which was then removed from the mixture with the use of a Benzamidine Sepharose 4 Fast Flow column (GE Healthcare, Little Chalfont, UK). The purity of the GluOC preparation was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis with a Tris–Tricine buffer system followed by staining with Coomassie brilliant blue, and the concentration and integrity of the purified protein were determined with an enzyme-linked immunoassay (EIA) kit for mouse GluOC (Takara Bio, Shiga, Japan).

### *Application of GluOC for metabolic assessment*

All animal experiments were approved by the animal ethics committee of Kyushu University. C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were maintained in a specific pathogen-free facility under a 12-h-light, 12-h-dark cycle and were fed with normal chow (CRF-1; Oriental Yeast, Osaka, Japan) or a high-fat, high-sucrose diet (F2HFHSD, Oriental Yeast) ad libitum. Recombinant mouse GluOC (3 ng/g) was administered orally to the mice in 100 μl of physiological saline three times a week (at 0800 h on monday, wednesday, and friday) for 13 weeks, beginning at 3 weeks of age (immediately after weaning). Alternatively, it was administered orally each day at a dose of 10 ng/g (at 0800 h) for 4 weeks beginning at 5 weeks of age. In the experiments to examine the involvement of GLP-1, the antagonist, exendin(9-39) at 25 nmol/kg was injected subcutaneously 15 min before application of GluOC. Control mice were injected with saline alone. A glucose tolerance test was performed after the animals had been deprived of food for 22 h; glucose (2 or 1 g/kg for mice fed a normal diet or a high-fat, high-sucrose diet, respectively) was administered intraperitoneally (for an intraperitoneal glucose tolerance test, or IPGTT) or orally (for an oral glucose tolerance test, or OGTT), and the blood glucose concentration was measured at various times thereafter with the use of FreeStyle Lite Blood Glucose test strips (Abbott Laboratories, Abbott Park, IL). An insulin tolerance test were performed after the mice had been deprived of food for 4 h; insulin (Humulin R, 0.5 or 1.5 U/kg for mice fed a normal diet or a high-fat, high-sucrose diet, respectively; Eli Lilly, Indianapolis, IN) was injected intraperitoneally, and the blood glucose concentration was measured at the indicated times thereafter. For assessment of insulin secretion in response to glucose stimulation, glucose (2 g/kg) was administered intraperitoneally or orally in mice that had been deprived of food for 22 h. Serum was then isolated from blood collected from the orbital plexus at the indicated times and was assayed for insulin with an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden). A pyruvate tolerance test was performed as an index of gluconeogenesis as described previously [16]; mice deprived of food for 22 h were injected intraperitoneally with sodium pyruvate (1.5 g/kg, Sigma-Aldrich), and the blood glucose concentration was measured at the indicated times thereafter. All metabolic assessments were performed 48 to 54 h after the last administration of GluOC or saline.

### *Pancreas histomorphometry*

The pancreas was fixed in 10% neutral formalin, embedded in paraffin, and sectioned at a thickness of 6 μm. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) at 121 °C in an autoclave for 5 min after tissue rehydration. The sections were then incubated for 10 min with 3% $H_2O_2$ to quench endogenous peroxidase activity. For insulin staining, sections were exposed to 10% goat serum in phosphate-buffered saline to block nonspecific binding sites and were then incubated overnight at 4 °C with rabbit antibodies to insulin (1:1000 dilution of #4590; Cell Signaling Technology, Boston, MA). Immune complexes were detected with biotinylated goat antibodies to rabbit immunoglobulin G (1:1000 dilution; Vector Laboratories, Burlingame, CA) and an ABC Elite Kit plus ImmPact DAB (Vector Laboratories). For staining of proliferating cell nuclear antigen (PCNA), nonspecific binding sites were blocked with the use of a Histofine mouse staining kit (Nichirei, Tokyo, Japan) and the sections were then incubated overnight at 4 °C with mouse antibodies to PCNA (1:1000 dilution of #307901; BioLegend, San Diego, CA). Immune complexes were visualized with the use of the Histofine kit. All sections were counterstained with Mayer's hematoxylin (Muto Pure Chemicals, Tokyo, Japan), and the β-cell area was calculated as the surface positive for insulin immunostaining divided by the total pancreatic surface. Islets were also photographed at 400×, and the number of PCNA-positive β-cells was manually counted. At least three specimens from each mouse were analyzed with the use of a BZ-II Analyzer (Keyence, Osaka, Japan), and 10 to 15 mice were analyzed per group.

### *Analysis of GluOC absorption and GLP-1 secretion in vivo*

The indicated doses of recombinant GluOC in saline were administered orally to C57BL/6J mice at 10 weeks of age. For analysis of GluOC absorption, the animals were anesthetized with sevoflurane, and blood (not exceeding 10% of the total blood volume) was collected from the orbital plexus at various times after GluOC administration. Given that the serum osteocalcin concentration undergoes circadian oscillation, showing a small increase from 0900 to 1200 h, declining to the basal level by 1400 h, and then maintaining a stable level until the next morning [17], we initiated the analysis of serum GluOC at 1500 h. For measurement of GLP-1 secretion, mice deprived of food for 24 h were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg) before the onset of blood sampling from the portal vein at the indicated times after GluOC administration. A dipeptidyl peptidase-4 inhibitor (Merck-Millipore, Billerica, MA) was added to the blood samples at a final concentration of 100 μM. Serum prepared from blood samples was assayed for GluOC or GLP-1 with the use of respective EIA (Takara Bio) and ELISA (Shibayagi, Gunma, Japan) kits.

### *Distribution of orally administered GluOC in the alimentary canal*

Recombinant GluOC or saline was administered orally to C57BL/6J mice at 10 weeks of age. At 3, 6, and 24 h after the administration, the animals were anesthetized with pentobarbital (40 mg/kg) and the