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Dietary vitamin D_3 improves postprandial hyperglycemia in aged mice

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ABSTRACT

Type 2 Diabetes is closely associated with our daily diets and has become a global health problem with an increasing number of patients. Recent observational and randomized studies on vitamin D_3 suggested that higher plasma 25-hydroxyvitamin D_3 [25(OH)D_3] concentrations and more vitamin D_3 intake are associated with lower risk of type 2 diabetes, which is characterized by postprandial hyperglycemia due to inappropriate glucose stimulated insulin secretion (GSIS) and its age-dependent increase of onset. However, rapid action of dietary vitamin D_3 on the postprandial glucose profile has not been analyzed. When vitamin D_3 is orally ingested in mice aged 12–14 weeks during an oral glucose tolerance test (OGTT), the serum glucose profile was not changed. In contrast, when OGTT was performed with old mice aged 30–34 weeks, the glucose profile was dramatically improved with increased insulin secretion, suggesting that orally ingested vitamin D_3 potentiated GSIS in aged mice. Interestingly, there was also a significant increase in plasma GLP-1 in these aged mice. Our results suggest that orally ingested dietary vitamin D_3 in aged mice improves glucose metabolism as a GLP-1 enhancer.

1. Introduction

Type 2 diabetes is a global health problem with a 2014 prevalence of more than 387 million cases worldwide [1]. Many epidemiological studies have shown an association of low plasma vitamin D_3 level with increased risk of type 2 diabetes [2–4]. It is known that a low serum level of 25-hydroxyvitamin D_3 (25(OH)D_3), an indicator of vitamin D status, is a risk factor for type 2 diabetes, while vitamin D_3 supplementation improves insulin sensitivity [5–7], suggesting involvement of vitamin D_3 in glucose metabolism. These protective effects of vitamin D_3 against diabetes are mediated through the regulations of several components such as calcium homeostasis [8] and the immune system [9–11].

The most widely recognized cellular mechanism of action of vitamin D_3 is through activation of the nuclear vitamin D_3 receptor (VDR). In addition, it is also widely accepted that vitamin D_3 is capable of acting through both the membrane-associated receptor to initiate nongenomic effects and through the regulation of gene expression. Vitamin D_3 has been shown to exhibit a rapid insulinotropic effect on pancreatic β cells, suggesting nongenomic signal transduction through membrane-associated VDR [12]. It is well documented that caveolae are the source of many rapidly responding signal transduction pathways [13]. Interestingly, VDR has been shown to localize to the lipid-raft caveolae micro-domain in many different cell types [14]. It is noted that the binding property of membrane associated VDR is likely to differ from that of the nuclear VDR [15]. When the VDR is associated with plasma membrane caveolae, the secosteroid hormone can activate a variety of rapid signal transduction pathways that may include kinases, phosphatases or ion channels, indicating that nongenomic signaling that affects gene expression is often termed crosstalk. In addition to VDR, rapid response steroid binding protein (1, 25-D_3 MARRS) has been identified in the plasma membrane of chick intestinal membrane [16]. Collectively, the mechanism of membrane-associated rapid action of vitamin D_3 has not been fully understood.

Vitamin D_3 (cholecalciferol) is formed from the precursor steroid 7-dehydrocholesterol (7-DHC), which is concentrated in the plasma membrane of the basal keratinocytes in the skin [17]. Upon

Abbreviations: GSIS, glucose stimulated insulin secretion; RT-PCR, reverse transcription-polymerase chain reaction; OGTT, oral glucose tolerance test; VDR, vitamin D_3 receptor; 1,25(OH)$_2$$D_3$, 1-25 dihydroxy-vitamin D_3; 25(OH)D_3, 25-hydroxyvitamin D_3; GLP-1, glucagon like peptide-1; GIP, gastric inhibitory polypeptide; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration.

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exposure of sunlight, 7-DHC is converted to vitamin D_3, which in turn converts to 25 hydroxy-vitamin D_3 (25(OH)D_3, calcidiol) in the liver. This successively changes to 1-25 dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$, calcitriol), a full active form of vitamin D_3 for nuclear VDR, in peripheral tissues including kidney by 1α-hydroxylase. The serum 25(OH)D_3 level is the sum of dietary/supplementary intake and endogenous synthesis [18]. Although the major source of vitamin D_3 for humans is endogenous synthesis initiated by sun exposure, dietary intake of vitamin D_3 is responsible for approximately 20% of the serum 25(OH)D_3 level and is also important for maintaining the serum vitamin D_3 level [19].

Vitamin D_3 is naturally contained in foods including oily fish such as salmon, mackerel, and herring. It is noted that fish oil also contains polyunsaturated free fatty acid, which is known to have protective effects on type 2 diabetes through the mechanism of amelioration of gut incretin secretion [20]. However, it remains unclear whether or not vitamin D_3 in foods, before being activated, is involved in these beneficial effects. While it is widely accepted that vitamin D_3 requires activation to exert its function, the physiological role of dietary vitamin D_3 remains to be clarified.

In this study, we investigated the role of dietary vitamin D_3 on glucose metabolism through its membrane-associated rapid responses. We found that dietary vitamin D_3 showed an insulinotropic action in aged mice, but not in young mice, through the glucagon-like peptide-1 (GLP-1) secretion by enteroendocrine cells. In addition, we showed that vitamin D_3 evoked a rapid mobilization of intracellular Ca^{2+} in NCI–H716 cells, the human GLP-1 secreting enteroendocrine cells. Our results suggest an important role for dietary vitamin D_3 in the glucose metabolism as a GLP-1 enhancer in aged mice.

2. Materials and methods

2.1. Animals and reagents

Male BALB/c mice were obtained from Kyudo Animal Laboratory (Kumamoto, Japan). These mice were fed *ad libitum* with normal food (CRF-1; Oriental Yeast, Tokyo, Japan) and kept under a light–dark cycle of 12 h. All mouse procedures and protocols were in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committees on Animal Experimentation (Kyushu University, Graduate School of Medicine, Japan). All-*trans*-retinol (vitamin A) was purchased from LTK Laboratories, Inc. (St. Paul, MN, USA), activated 7-dehydrocholesterol (vitamin D_3: cholecalciferol), DL-α-tocopherol (vitamin E), and vitamin K were purchased from Nacalai Tesque (Kyoto, Japan). Aprotinin solution from bovine lung was obtained from Wako (Osaka, Japan) and dipeptidyl peptidase IV (DPP- IV) inhibitor was obtained from Linco Research (Billerica, MA, USA).

2.2. Glucose tolerance tests

Mice were fasted for 18 h and baseline blood glucose levels in the tail-vein were measured using a Glutest Sensor (Sanwa Kagaku Inc., Nagoya, Japan). Glucose (2 g/kg glucose or 2 g/kg glucose plus each vitamin) in sterile phosphate-buffered saline (PBS) was given orally by gavage or intraperitoneal injection and blood glucose levels were assayed after 15, 30, 60 and 90 min. Plasma insulin concentration was determined using a standard rat/mouse insulin enzyme-linked immunoabsorbent assay (ELISA) kit (Morinaga Institute of Biological Science, Tokyo, Japan). For measurement of active gastric inhibitory polypeptide (GIP) and GLP-1 levels, a 100 μl sample of blood was immediately mixed with EDTA and aprotinin and centrifuged for 10 min at 4 °C. Plasma was then collected in tubes with DPP-IV inhibitor. The concentration of active GLP-1 (7–36) and GIP (1–42) were measured by GIP ELISA kit (Sibayagi, Gunma, Japan) and GLP-1 ELISA kit (Wako, Osaka, Japan), respectively.

2.3. Analyses with NCI–H716 cells

Human enteroendocrine NCI–H716 cells were maintained in suspension culture. Two days before experiments, cells were seeded into 12-well culture plates pre-coated with Matrigel (BD Biosciences, Bedford, MA, USA) as described [21]. On the day of the experiments, supernatants were replaced by PBS containing 1 mM $CaCl_2$ and DPP-IV inhibitor. The solutions were adjusted to pH 7.2. Cells were incubated for 15 min at 37 °C in glucose solutions with or without vitamin D_3. GLP-1 was measured by GLP-1 ELISA Kit (Sibayagi) and normalized to protein content. Measurement of the intracellular calcium concentration change was performed according to the protocol of Calcium Kit II-Fluo4 (Dojindo, Kumamoto, Japan). Briefly, cells were plated onto Matrigel-coated glass bottom 35 mm dishes and were loaded with fluo4 at 37 °C. Following the 1 h loading period, cells were washed twice with HKRB buffer (20 mM HEPES, 103 mM NaCl, 4.77 mM KCl, 0.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 15 mM glucose, pH 7.3). Confocal imaging was performed using the LSM 700 Laser Scanning Microscope (Zeiss) using a 60 × oil objective. Images were acquired and processed with ZEN 2012 software (Zeiss).

2.4. Histological analysis and biochemical assay in the gut

Each portion of the gut, including the duodenum, jejunum, ileum, and colon, was excised, fixed in 4% (w/v) paraformaldehyde overnight at 4 °C, dehydrated in 100% ethanol, and embedded in paraffin. For immunostaining of GLP-1, sections were incubated with the primary antibody mouse anti GLP-1 (ab26278; 1:200, Abcam, Taiwan), at 4 °C for 12–16 h, followed by 1 h incubation with Alexa Flour 568 goat anti-rabbit IgG (A11011; 1:200, Invitrogen, OR, USA) as the secondary antibody. The cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The tissue specimens were homogenized and extracted with ethanol/acid (100% ethanol: sterile water: 12 N HCl 74:25:1 v/v) solution (5 ml/g tissue). Then the homogenates were centrifuged (2000 g for 20 min) and supernatants were collected and diluted 50-fold for GLP-1 measurement. All tissue extracts were assayed in triplicate.

2.5. RNA analysis

Total RNA was isolated from the intestine (TRIzol Reagent; Life Technologies, Carlsbad, CA, USA) and purified (SV Total RNA Isolation System; Promega Corporation, Madison, WI, USA). Total RNA (500 ng) was reverse-transcribed (QuantiTect Reverse Transcription Kit; Qiagen, Hilden, Germany), and quantitative real-time polymerase chain reaction (PCR) undertaken using 2× Power SYBR Green PCR Master Mix and an ABI Prism 7000 sequence detection system (Life Technologies). The PCR conditions were: 95 °C for 10 min, then 40 cycles at 95 °C for 15 s and 60 °C for 45 s. The primer sequences used were as follows: VDR: 5′-GAGGTGTCTGAAGCCTGGAG-3′/5′-ACCTGCTTTCCTGGGTAGGT-3′; 1, 25-D_3 MARRS: 5′-AGCAGGACCAGCTTCAGTTC-3′/5′-TGCTGGCTGCTTTTAGGAAT-3′; GAPDH: 5′-ATGCCCCCATGTTTGTGATG-3′/5′-CCATCACTGCCACCCAGAAG-3′. Relative gene expression *vs*. control was normalized to the expression of GAPDH genes.

2.6. Western blot analysis

Cells were homogenized and protein content measured using the BCA protein assay kit (Thermo, Rockford, IL, USA). Rabbit anti

phospho-PLCβ3 (Ser537) was used as a primary antibody (#2481s; 1:1000; Cell Signaling Technology, Danvers, MA, USA). The secondary antibody was peroxidase-conjugated (1:1000; Cell Signaling Technology, Danvers, MA, USA) and the internal control was peroxidase-conjugated GAPDH antibody (1:1000; Cell Signaling Technology). Protein bands were visualized with an ECL Plus Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

2.7. Statistical analysis

All data are expressed as mean ± SD. Analysis of the significance of differences was performed using Student's *t* test in Microsoft Excel software (Mac 2011 version 14.3.5: Microsoft Japan, Tokyo, Japan). A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Oral vitamin D_3 improves glucose tolerance and insulin secretion in aged mice

In order to clarify the physiological role of dietary vitamin D_3 on glucose metabolism, oral glucose tolerance tests (OGTTs) were performed. Since glucose tolerance is known to decline with age, the first experiment was performed with mice aged ≥30 weeks. Surprisingly, the mice orally ingested with 2 g/kg body weight (BW) of glucose together with 10 ng/kg BW of dietary vitamin D_3 showed improved glucose tolerance compared to the mice ingested with glucose alone as shown in Fig. 1A. The supplemented dose of vitamin D_3 (10 ng/kg BW) corresponds to 1 kg of salmon eaten in one meal by a person. Mice treated with Vitamin D_3 showed a reduction in plasma glucose level after 30 min and up to 90 min later. Consistent with this, insulin levels were significantly higher in mice that received vitamin D_3 at 30 min (Fig. 1B). There was no difference in glucose and insulin levels at 15 min (Fig. 1A, B), indicating that absorption of glucose by the gut and the subsequent first phase of glucose stimulated insulin secretion (GSIS) are not altered in the presence of vitamin D_3.

Intraperitoneal glucose tolerance tests were also performed with glucose (2 g/kg BW) alone or with added vitamin D_3 (10 ng/kg BW). As shown in Fig. 1C and D, both glucose and insulin profiles were similar in the two groups, indicating that the insulinotropic effect of vitamin D_3 is exerted via the gastrointestinal tract.

3.2. Dietary vitamin D_3 shows insulinotropic effect in an age-dependent manner

In order to investigate if the effects seen in the 30–34-week-old mice can be reproduced in young mice, the same experiments were carried out with 12–14-week-old mice. As shown in Fig. 1E and F, lower glucose and insulin values were observed in young mice in comparison with those seen in older mice. However, there was no difference in the glucose profiles and plasma insulin levels between the two groups, indicating that the vitamin D_3 exhibited its insulinotropic effect only in aged mice. In order to address whether this insulinotropic action is exclusive to vitamin D_3, other liposoluble vitamins such as vitamin A, E, and K were orally given during OGTT to aged mice. As shown in Fig. 1G and H, the improvement of the glucose profile was not observed with other liposoluble vitamins, suggesting that vitamin D_3 may employ its specific mechanism to exert the insulinotropic effect, although this is yet to be determined.

3.3. Dietary vitamin D_3 increases GLP-1 secretion

Insulin secretion from pancreatic β cells is categorized into two phases. The early phase takes up to several minutes and is a response to glucose. The second phase continues for more than 30 min and is influenced by the incretins secreted by the enteroendocrine cells in the gut epithelium. The time frame of the changes observed in the plasma insulin concentration during OGTT suggests that the second phase of insulin secretion might be affected by the intestinal incretins. Therefore, we measured the plasma concentration of GIP and GLP-1. As shown in Fig. 2A, there was no stimulating effect of vitamin D_3 on the secretion of GIP. While GLP-1 levels significantly increased by orally ingesting vitamin D_3, this may explain, at least in part, the insulinotropic effects of vitamin D_3 (Fig. 2B).

3.4. Vitamin D_3 potentiates glucose-stimulated GLP-1 in NCI–H716 cells through rapid membrane-associated signal transduction

To test the effect of vitamin D_3 on GLP-1 secretion *in vitro*, we used the human enteroendocrine NCI–H716 cells that have an ability of glucose-responsive GLP-1 secretion [21]. The NCI–H716 cells were stimulated with 11.2 mM glucose alone or together with vitamin D_3. As shown in Fig. 3A and 26 μM vitamin D_3 significantly increased GLP-1 secretion at 15 min after stimulation.

The dose of vitamin D_3 applied to the cells was estimated as being close to the actual luminal concentration of dietary vitamin D_3 in the intestinal lumen. Therefore, it is likely that dietary vitamin D_3 potentiates GSIS through the mechanisms of GLP-1 secretion from the neuroendocrine cells, and thereby improves postprandial hyperglycemia. Since the effect of vitamin D_3 was seen within several minutes, secretion of GLP-1 is likely to be regulated by rapid membrane-associated mechanisms involving signal transduction pathways that may include kinase, phosphatases or ion channels [15]. Phospholipase C (PLC) plays an important role in the membrane-bound receptor signaling pathway. Upon stimulation, PLC cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). IP3 is released as a soluble factor into the cytosol and leads to an increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Since PLCβ3 has shown to be activated by $1,25(OH)_2D_3$ in rat chondrocytes [22], its activation was analyzed by western blot. As shown in Fig. 3B, application of vitamin D_3 in the presence of 11.2 mM glucose for 15 min significantly increased the phosphorylation of PLCβ3 in NCI–H716 cells. We then determined whether vitamin D_3 could evoke Ca^{2+} mobilization in the NCI–H716 cells that had been previously loaded with the $[Ca^{2+}]_i$ indicator dye Fluo-4. Application of vitamin D_3 evoked a $[Ca^{2+}]_i$ rise and reached a peak within 25 s in NCI–H716 cells as shown in Fig. 3C. In contrast, no rise of $[Ca^{2+}]_I$ was observed by vitamin A, consistent with *in vivo* data shown in Fig. 1G and H. These results suggest that vitamin D_3 potentiates GLP-1 secretion through the rapid membrane-associated signal transduction.

3.5. GLP-1 content in the gut was significantly decreased in aged mice

An interesting finding was that vitamin D_3 could exhibit a beneficial effect on the glucose profile in aged mice. Taking this into account, we analyzed the tissue content of GLP-1 in duodenum, jejunum, ileum, and colon of young and old mice. Interestingly, there was a marked decrease in GLP-1 content in all segments of the gut from the old mice (Fig. 4A). However, the immunohistochemical study demonstrated that the number of

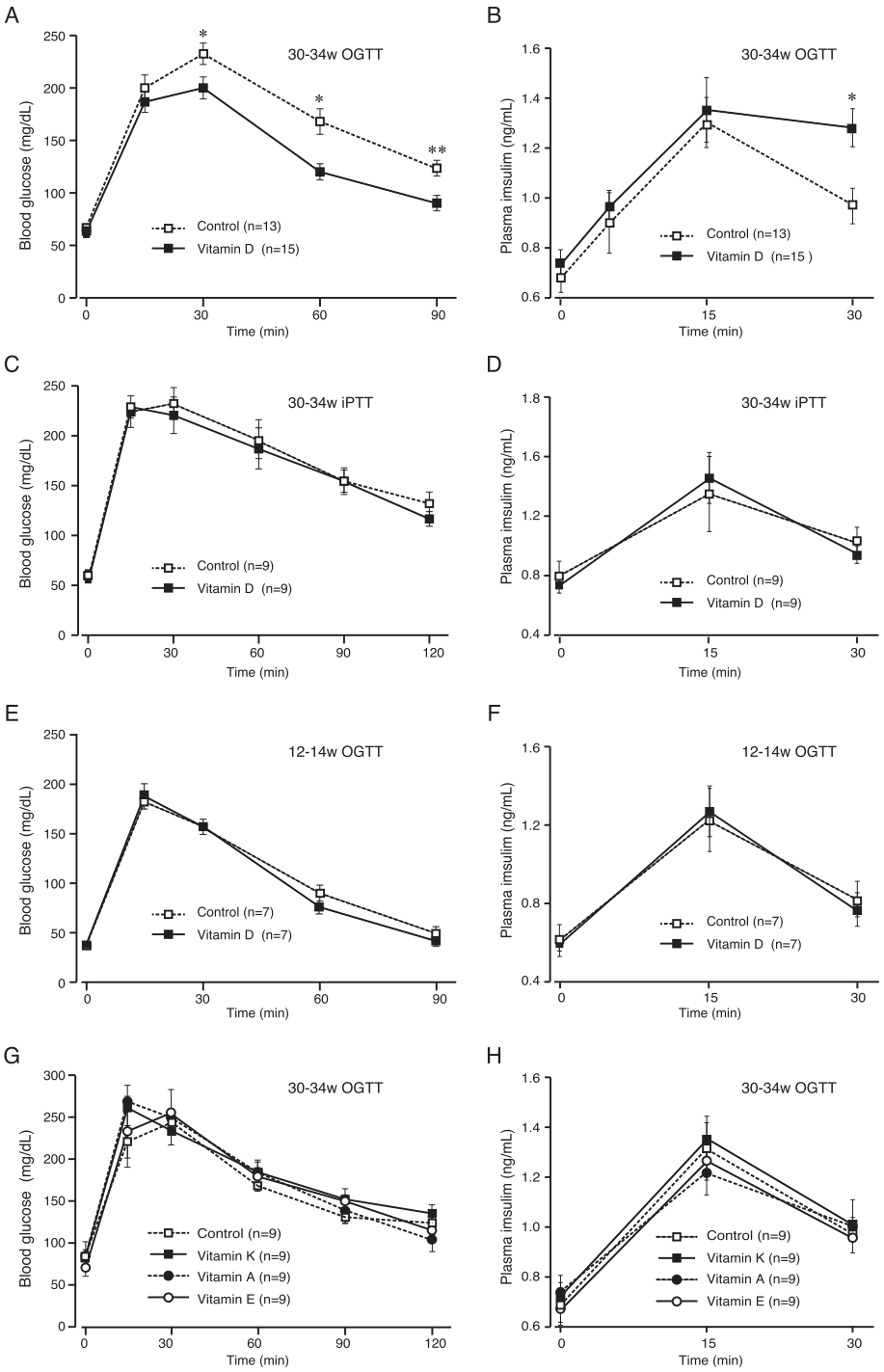

Fig. 1. Oral ingested vitamin D_3 improves glucose tolerance and insulin secretion in mice aged 30–34 weeks. (A–D) Oral glucose tolerance test (OGTT; A, B) and intraperitoneal glucose tolerance test (iPGTT; C, D) in old mice aged 30–34 weeks. Control group ingested 2 g/kg body weight (BW) of glucose and the vitamin D group ingested 2 g/kg BW of glucose plus 10 μg/kg BW of cholecalciferol. (E, F) OGTT in young mice aged 12–14 weeks. (G, H) OGTT in old mice aged 30–34 weeks. Each group ingested 52 μg/kg BW of vitamin K, 46 μg/kg BW of all-*trans*- retinol, or 1060 μg/kg BW of DL-α-tocopherol together with 2 g/kg BW of glucose. Blood glucose (A, C, E, G) and plasma insulin (B, D, F, H) concentrations are shown. OGTT: oral glucose tolerance test; iPTT: intraperitoneal glucose tolerance test. Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$.

GLP-1 positive cells in aged mice was comparable to that in young mice (Fig. 4B), suggesting that cellular content of GLP-1 was reduced in aged mice. To explore the mechanism underlying age dependent responsiveness of vitamin D_3, the expressions of known receptors for vitamin D_3 in the gut were also analyzed. However, there was no difference found in the expression of VDR and MARRS in the gut between the ages.

4. Discussion

The present study demonstrates that dietary vitamin D_3, cholecalciferol, can improve postprandial hyperglycemia in aged mice. Other lipophilic vitamins including vitamins A, E and K have no such effect, indicating a specific action of vitamin D_3. Furthermore, our results suggest that vitamin D_3 has an insulinotropic

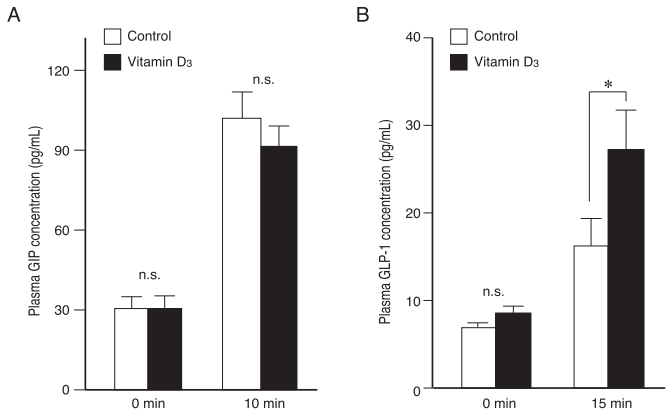

Fig. 2. Changes in plasma GIP and GLP-1 after oral administration of vitamin D_3. (A) Plasma GIP concentration before and 10 min after the administration of glucose alone or with vitamin D_3. (B) Plasma GLP-1 concentration before and 15 min after the administration of glucose alone or with vitamin D_3. Control group (n = 15) ingested 2 g/kg BW of glucose. Vitamin D_3 group (n = 14) ingested 2 g/kg BW of glucose plus 10 ng/kg BW of cholecalciferol. Data are expressed as mean ± SD. n.s. represents no significant difference, $^*P < 0.05$.

action by affecting secretion of GLP-1 by enteroendocrine cells. Interestingly, this insulinotropic action of orally administered vitamin D_3 was observed in aged mice. We demonstrated that the content of intestinal GLP-1 peptide declined with age, indicating an age dependent alteration in enteroendocrine cells.

We also demonstrated a direct effect of vitamin D_3 on GLP-1 secretion using a human intestinal L cell line, NCI–H716 cells. Vitamin D_3 induced GLP-1 secretion within 15 min using this cell line. This induction of GLP-1 secretion occurs too rapidly to be simply explained via a traditional VDR-mediated genomic response, which generally takes several hours to be fully apparent. We showed that vitamin D_3 treatment led to PLC activation and Ca^{2+} mobilization, further supporting our view that nongenomic rapid signal transduction responses by vitamin D_3 increase GLP-1 secretion in enteroendocrine cells.

In addition to an insulinotropic action, GLP-1 has various other beneficial effects, such as promoting β-cell proliferation, suppressing glucagon release and food intake, slowing gastric emptying, cardiovascular protection and cognitive function [23,24]. Therefore, restoration of GLP-1 secretion by intestinal L cells could be an important new therapeutic option not only for the management of type 2 diabetes but also for improvement of well-being.

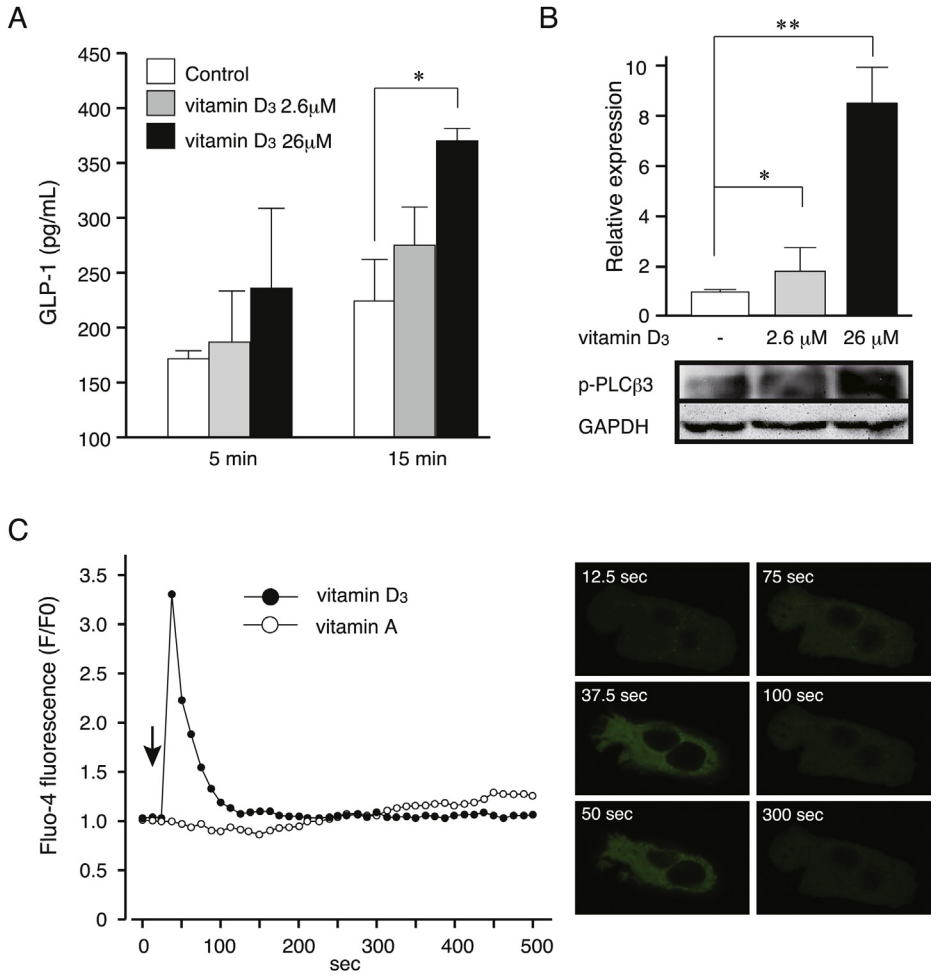

Fig. 3. Effects of Vitamin D_3 on GLP-1 secretion by NCI–H716 cells. (A) NCI–H716 cells were incubated with 0, 2.6 or 26 μM vitamin D_3 in the presence of 11.2 μM glucose. GLP-1 level in the culture medium was measured after 5 and 15 min (B) NCI–H716 cells were immunoblotted for phosphorylated PLCβ3, and the protein band intensities were quantified and normalized to GAPDH band intensities. (C) Ca^{2+} mobilization in response to vitamin D_3. Ca^{2+} indicator Fluo-4/AM loaded NCI–H716 cells were exposed to 26 μM vitamin D_3 or 26 μM vitamin K (arrow). The Fluo-4 fluorescence change was recorded every 12.5 s for 500 s (n = 3). Ca^{2+} mobilization was measured as described in Materials and Methods and the images at indicated time points are shown on the right panels. Data are expressed as mean ± SD. n = 3, $^*P < 0.05$, $^{**}P < 0.01$.

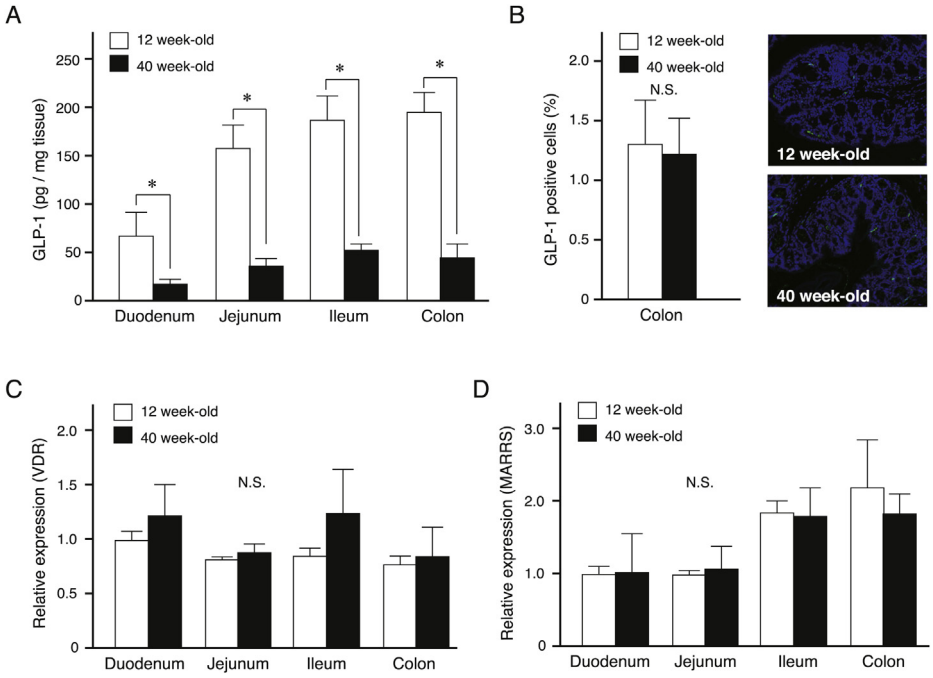

Fig. 4. GLP-1 content in the gut is reduced in aged mice. (A) GLP-1 contents in duodenum, jejunum, ileum, and colon of 12- (n = 5) and 40-week-old (n = 5) mice. (B) Expression of GLP-1 (green color) in the colon of 12- and 40-week-old mice determined using immunofluorescent staining. The cell nuclei were counterstained with DAPI (blue color). The number of GLP-1 positive cells was counted and shown as a percentage against the total cell number. (C, D) Comparison of the mRNA expression of VDR (C) and MARRS (D) in duodenum, jejunum, ileum, and colon in mice aged between 12 and 40 weeks. Relative expression to that in duodenum of 12-week-old mice is shown. Data are expressed as mean ± SD. n = 5, $^*P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In summary, we demonstrated that vitamin D_3, cholecalciferol, stimulates GLP-1 secretion both *in vivo* and *in vitro*. These results represent a possible new therapeutic approach for enhancing incretin action in the treatment of elderly people with type 2 diabetes.

Conflict of interest

The authors declare that they have no conflict of interest.

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