ORIGINAL ARTICLE

Dipeptidyl peptidase 4 inhibitor anagliptin ameliorates hypercholesterolemia in hypercholesterolemic mice through inhibition of intestinal cholesterol transport

Moritaka Goto[1]* , Shinji Furuta[1], Satoko Yamashita[1], Hiroyuki Hashimoto[1], Wataru Yano[2], Noriyuki Inoue[2], Noriaki Kato[1], Kohei Kaku[3]

[1]Pharmaceutical Research Laboratories, Sanwa Kagaku Kenkyusho Co., Ltd., Mie, [2]Tokyo New Drug Research Laboratories, Kowa Co., Ltd., Tokyo, and [3]Department of General Internal Medicine 1, Kawasaki Medical School, Okayama, Japan

ABSTRACT

Aims/Introduction: Recent data showed that dipeptidyl peptidase 4 (DPP-4) inhibitors exert a lipid-lowering effect in diabetes patients. However, the mechanism of action is not yet clearly understood. We investigated the effect of anagliptin on cholesterol metabolism and transport in the small intestine using non-diabetic hyperlipidemic animals, to clarify the mechanisms underlying the cholesterol-lowering action.

Materials and Methods: Male apolipoprotein E (ApoE)-deficient mice were orally administered anagliptin in the normal chow. Serum cholesterol levels and lipoprotein profiles were measured, and cholesterol transport was assessed by measuring the radioactivity in the tissues after oral loading of ^{14}C-labeled cholesterol (^{14}C-Chol). In additional experiments, effects of exendin-4 in mice and of anagliptin in DPP-4-deficient rats were assessed. Effects on target gene expressions in the intestine were analyzed by quantitative polymerase chain reaction in normal mice.

Results: The serum total and non-high-density lipoprotein cholesterol concentrations decreased after anagliptin treatment in the ApoE-deficient mice. The cholesterol-lowering effect was predominantly observed in the chylomicron fraction. The plasma ^{14}C-Chol radioactivity was significantly decreased by 26% at 2 h after cholesterol loading, and the fecal ^{14}C-Chol excretion was significantly increased by 38% at 72 h. The aforementioned effects on cholesterol transport were abrogated in rats lacking DPP-4 activity, and exendin-4 had no effect on the ^{14}C-Chol transport in ApoE-deficient mice. Furthermore, significant decreases of the intestinal cholesterol transport-related microsomal triglyceride transfer protein, acyl-coenzyme A:cholesterol acyltransferase 2, ApoA2 and ApoC2 messenger ribonucleic acid expressions were observed in the mice treated with repeated doses of anagliptin.

Conclusions: These findings suggest that anagliptin might exert a cholesterol-lowering action through DPP-4-dependent and glucagon-like peptide 1-independent suppression of intestinal cholesterol transport.

INTRODUCTION

In patients with type 2 diabetes mellitus, dyslipidemia is an important modifiable cardiovascular risk factor, and it is often

necessary to improve the lipid profile by drug intervention[1–3]. Dipeptidyl peptidase 4 (DPP-4) inhibitors, which are widely used for the treatment of type 2 diabetes mellitus, suppress the degradation of incretins, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), by inhibiting DPP-4 enzyme activity[4]. GLP-1 and GIP act on the

pancreatic islets, and exert glucose-dependent insulinotropic actions in patients with type 2 diabetes mellitus[5,6]. In addition, GLP-1 is known to reduce the secretion of glucagon, a hormone that promotes gluconeogenesis[6].

In addition to their effect of improving glycemic control, DPP-4 inhibitors have recently also been reported to exert extrapancreatic actions, including ameliorating the risk of diabetic complications, such as nephropathy, retinopathy and neuropathy, and also the risk of cardiovascular disease[7–12]. Several previously published studies, including a meta-analysis, have also reported the lipid-lowering effect of DPP-4 inhibitors[13–16]. Two meta-analyses have shown that DPP-4 inhibitors, such as sitagliptin, vildagliptin, alogliptin, saxagliptin and linagliptin, reduce both the serum cholesterol and serum triglyceride levels[13,14]. Sitagliptin has been reported to significantly decrease the serum total cholesterol and non-high-density lipoprotein cholesterol (non-HDL-C) levels, with a reduction also of the serum low-density lipoprotein cholesterol (LDL-C) levels observed only in patients with elevated baseline triglyceride levels[15]. Similarly, alogliptin has been shown to significantly reduce the serum total cholesterol, non-HDL-C and LDL-C levels in newly diagnosed drug-naive type 2 diabetes mellitus patients[16]. Anagliptin has been reported to reduce the serum total cholesterol and LDL-C levels in Japanese type 2 diabetes mellitus patients with hyperlipidemia, suggesting that the drug also exerts a lipid-lowering action, in addition to its antidiabetic effect[17–19]. Recently, DPP-4 inhibition with anagliptin was shown to suppress serum lathosterol, a clinical marker of cholesterol synthesis[20]. Although clinical studies suggest that anagliptin suppresses the hepatic cholesterol synthesis, cholesterol metabolism is regulated in the small intestine, as well as in the liver, and the involvement of cholesterol transport from the small intestine in the cholesterol-lowering action of DPP-4 inhibitors has not yet been fully investigated.

In the present study, we investigated the effect of anagliptin on the cholesterol metabolism and transport of the small intestine in non-diabetic hypercholesterolemic animals to clarify the mechanism(s) underlying the cholesterol-lowering action of DPP-4 inhibitors.

METHODS
Animals
Male 6-week-old spontaneously apolipoprotein E (ApoE)-deficient hypercholesterolemic mice (C57BL/6.KOR/StmSlc-*Apoe*[shl]; B6.SHL) were purchased from Japan SLC Inc. (Hamamatsu, Japan). The mice were housed under a 12-h light/dark cycle (07.00/19.00 h), with normal chow (CRF-1; Oriental Yeast, Tokyo, Japan) provided *ad libitum*. For the experiment, the mice were divided into two groups at 8–9 weeks-of-age, with one group given 0.3% anagliptin mixed in the diet for 8 weeks. The animal care and experimental procedures were approved by the Animal Care Committee of Sanwa Kagaku Kenkyusho Co., Ltd.

Measurement of the plasma DPP-4 activity, serum glucose and serum lipid parameters
Blood samples were collected for measurement of the plasma DPP-4 activity, serum glucose and serum lipid levels. Plasma DPP-4 activity was measured using the fluorescent substrate, Gly-Pro-MCA (Peptide Institute, Osaka, Japan), as reported previously[21]. Serum glucose and serum total cholesterol and HDL-C levels were measured using commercially available assay reagents (Wako Pure Chemical, Osaka, Japan), according to the manufacturers' instructions. Non-HDL-C was calculated as the serum total cholesterol minus HDL-C. The lipid profile was examined by a high-performance liquid chromatography method, as previously described[22].

Measurement of ^{14}C-labeled cholesterol uptake in mice
To clarify the cholesterol transport from the small intestine, cholesterol uptake from the intestine after oral radiolabeled cholesterol loading was investigated in the B6.SHL mice. After 4-weeks' treatment with anagliptin, the mice were orally loaded with corn oil-diluted ^{14}C-labeled cholesterol (^{14}C-Chol; 4 mg/1.85 MBq/kg; American Radiolabeled Chemicals, St. Louis, MO, USA). Then, 2 and 72 h after the ^{14}C-Chol loading, the radioactivity levels in the plasma, liver and small intestine (upper, middle, lower) were measured with a liquid scintillation system (AccuFLEX LSC-7400; Hitachi Aloka Medical, Tokyo, Japan). Fecal cholesterol excretion was also assessed as follows[23]. Mice were orally loaded with ^{14}C-Chol and ^{3}H-labeled sitosterol (4 mg/9.25 MBq/kg and 1.6 µg/7.25 MBq/kg, respectively; American Radiolabeled Chemicals) after 4 weeks of anagliptin treatment, and feces were collected over 72 h after the loading. Fecal excretion of ^{14}C-Chol was calculated by determining the percent excretion relative to the total administered radioactivity. The absorption ratio of ^{14}C-Chol was estimated as follows: absorption ratio of ^{14}C-Chol (%) = (1 − fecal excretion ratio of ^{14}C-Chol/fecal excretion ratio of ^{3}H-sitosterol) × 100.

Effect of exendin-4
A total of 27 male B6.SHL mice were divided into three groups at 8 weeks-of-age, and two groups were administered the GLP-1 receptor agonist, exenatide (Byetta; AstraZeneca, London, UK) by subcutaneous injection at the dose of 8 or 40 µg/kg twice daily for 4 weeks. After 4 weeks' treatment with exenatide, the mice received oral ^{14}C-Chol loading at 4 mg/1.85 MBq/kg, followed by blood collection at 2 h after the loading for plasma radioactivity measurements.

Real-time quantitative polymerase chain reaction for measurement of the target messenger ribonucleic acid expression levels in the mouse tissues
Expressions of the target genes related to lipid metabolism and transport in the small intestine were quantified by real-time quantitative polymerase chain reaction. Isolated tissues were homogenized in TRIZOL Reagent (Invitrogen, Yokohama,

Japan). Total ribonucleic acid (RNA) was purified using TRI-ZOL Reagent and the RNeasy mini Kit (QIAGEN, Tokyo, Japan). Complementary deoxyribonucleic acid was synthesized from 0.1 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Yokohama, Japan). Real-time polymerase chain reaction was carried out using the LightCycler 480 system (Roche Diagnostics, Tokyo, Japan). All target-specific hydrolysis primers and probe sets were purchased from Applied Biosystems.

Measurement of ^{14}C-Chol uptake in DPP-4-deficient rats

Six-week-old male F344/DuCrlCrlj (DPP4-deficient) rats were purchased from Charles River Japan (Osaka, Japan). The rats were housed under a 12-h light/dark cycle (07.00/19.00 h) with normal chow (CRF-1; Oriental Yeast) provided *ad libitum*. At 11 weeks-of-age, the rats were randomly divided into two groups, as follows: the control group ($n = 8$) and the anagliptin (anagliptin 0.3% mixed in the diet)-treated group ($n = 8$); in the latter group, the drug was administered mixed in the diet for 4 weeks. The rats then received oral loading of ^{14}C-Chol (4 mg/0.95 MBq/kg) diluted with corn oil. Then, 4 and 72 h after the loading, the plasma radioactivity of ^{14}C-Chol was measured with a liquid scintillation system.

Statistical analysis

All the values are expressed as mean ± standard error of the mean. The data between two groups were compared using the unpaired Student's *t*-test, and Dunnett's test was used among three groups, using EXSUS version 8.0.0 (CAC EXICARE Corporation, Tokyo, Japan). $P < 0.05$ was considered as indicative of statistical significance.

RESULTS

Effect of anagliptin on the hyperlipidemia in ApoE-deficient mice

Table 1 shows the baseline data before and after treatment with anagliptin in the ApoE-deficient spontaneously hyperlipidemic (SHL) mice. No significant changes in the bodyweight or serum

glucose concentrations were observed in the anagliptin-treated mice as compared with the control SHL mice. Significant suppression of the plasma DPP-4 activity was noted at 4 and 8 weeks after the start of treatment. The serum total cholesterol and non-HDL-C concentrations in the anagliptin-treated SHL mice were significantly lower than those in the control SHL animals at 8 weeks after treatment. Lipoprotein analysis showed that the cholesterol-lowering effect was predominantly observed in the chylomicron fraction, with a tendency towards decrease also in the very low-density lipoprotein (VLDL) fraction at 8 weeks of treatment (Figure 1). Additionally, data of triglyceride concentration of each fraction are shown in Figure S1.

Effect of anagliptin on the cholesterol transport after oral ^{14}C-Chol loading in SHL mice

After 4 weeks of anagliptin treatment, the SHL mice received oral ^{14}C-Chol loading. Figure 2 shows the plasma, hepatic and intestinal radioactivities of ^{14}C-Chol at 2 h after the loading.
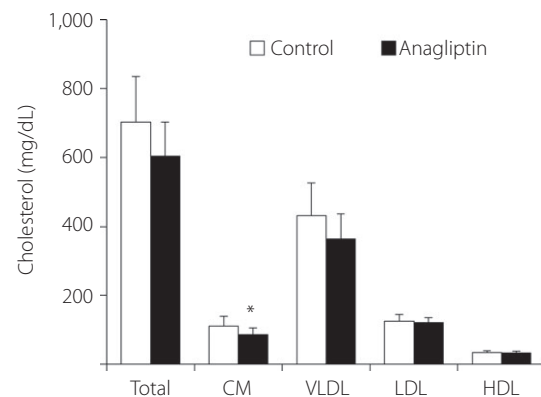

Figure 1 | Serum lipoprotein profiling by high-performance liquid chromatography in apolipoprotein E-deficient mice treated with anagliptin for 8 weeks. Data represent the mean ± standard error of the mean for 12 animals. *$P < 0.05$, significant differences as compared with the control. CM, chylomicron; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VLDL, very low-density lipoprotein.

Table 1 | Effect of anagliptin on bodyweight, serum glucose, plasma dipeptidyl peptidase 4 activity and serum cholesterol levels in spontaneously hypercholesterolemic mice

	0 weeks		4 weeks		8 weeks	
	Control	Anagliptin	Control	Anagliptin	Control	Anagliptin
Bodyweight (g)	23.5 ± 0.3	23.6 ± 0.4	29.3 ± 0.5	28.7 ± 0.4	32.5 ± 0.7	31.2 ± 0.3
Serum glucose (mg/dL)	167 ± 4	177 ± 5	167 ± 8	149 ± 6	146 ± 5	140 ± 6
Plasma DPP-4 activity (nmol/min/mL)	8.06 ± 0.34	8.07 ± 0.54	5.06 ± 0.40	1.21 ± 0.05***	8.17 ± 0.50	1.24 ± 0.07***
Serum cholesterol (mg/dL)						
Total-C	650 ± 37	628 ± 42	597 ± 24	515 ± 36	811 ± 58	659 ± 42*
Non-HDL-C	631 ± 38	608 ± 42	572 ± 24	492 ± 37	781 ± 56	634 ± 41*
HDL-C	19.4 ± 0.8	19.6 ± 1.2	24.6 ± 1.3	22.8 ± 1.4	29.7 ± 2.4	25.0 ± 1.8

Each value is expressed as the mean ± standard error of the mean of 12 individuals. *$P < 0.05$, ***$P < 0.001$ vs control, respectively. DPP-4, dipeptidyl peptidase 4; HDL-C, high-density lipoprotein cholesterol; Total-C, total cholesterol.
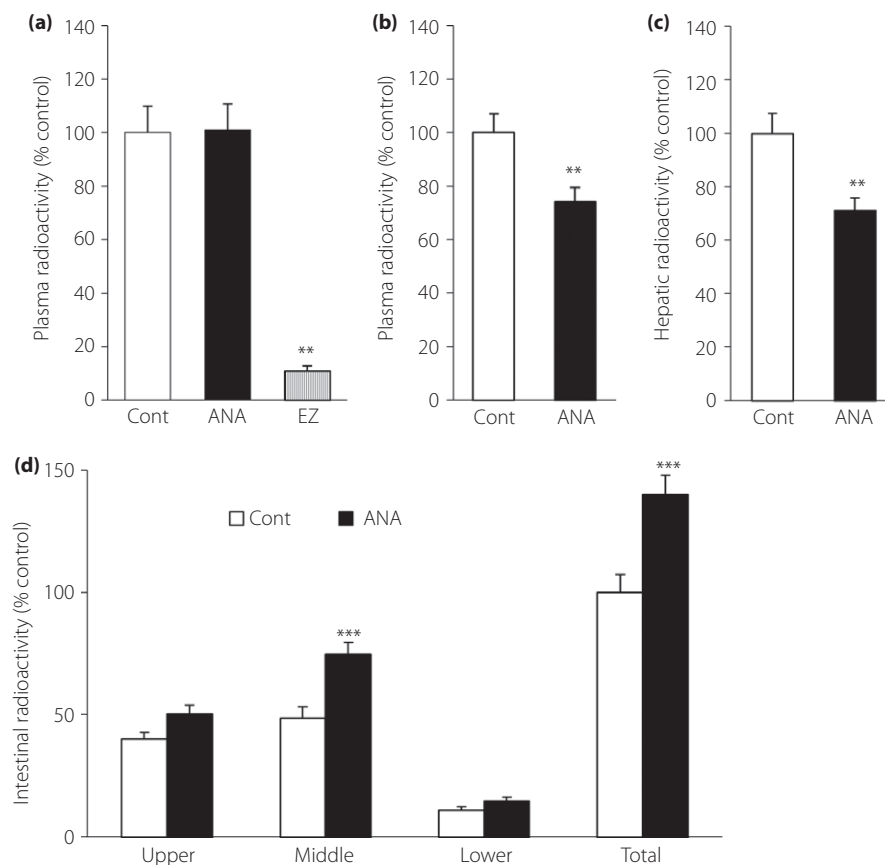
Figure 2 | Effect of anagliptin (ANA) treatment on cholesterol uptake at 2 h after oral ^{14}C-labeled cholesterol loading in apolipoprotein E-deficient mice. Plasma radioactivity of ^{14}C-labeled cholesterol (a) after single-dose administration of ANA or ezetimibe (EZ) and (b) in mice treated daily with ANA for 4 weeks. (c) Hepatic and (d) intestinal radioactivity during repeated-dose ANA treatment for 4 weeks. Data represent the mean ± standard error of the mean for 16 animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significant differences compared with the control (Cont).

Although single-dose administration of anagliptin had no effect on the ^{14}C-Chol radioactivity level in the plasma (Figure 2a), significant decreases of the plasma and hepatic radioactivity levels by 26 and 29%, respectively, were observed in the animals that had received repeated daily doses of anagliptin for 4 weeks (Figure 2b,c). In contrast, an increase of the radioactivity was observed in the small intestine (by 26, 53 and 40% in upper, middle and total, respectively) at 2 h after the radiolabeled cholesterol loading (Figure 2d).

Interestingly, in the animals that had received anagliptin treatment for 4 weeks, while the plasma radioactivity of ^{14}C-Chol was suppressed even at 72 h after the oral loading, the intestinal radioactivity remained unchanged (Figure 3a,b).

Figure 3c shows the fecal radioactivity of ^{14}C-Chol at 24, 48 and 72 h after radiolabeled cholesterol loading in the SHL mice. Excretion of ^{14}C-Chol in feces was completed by 24 h after the oral radiolabeled cholesterol loading, and a single-dose administration of ezetimibe increased the cholesterol excretion by approximately 2.5-fold in comparison with that in the untreated control. Fecal cholesterol excretion was significantly increased by 38% up to 72 h after ^{14}C-Chol loading with repeated doses of anagliptin. The estimated absorption, calculated from the excreted fecal cholesterol, was completely inhibited by ezetimibe, and anagliptin also reduced the estimated absorption of cholesterol by 19% (Figure 3d).

Effects of a GLP-1 receptor agonist on the cholesterol transport

Exendin-4 was administered by subcutaneous injection at the dose of 8 or 40 μg/kg (twice daily) to the SHL mice for 4 weeks. Neither low-dose nor high-dose administration of exendin-4 had any effect on the bodyweight changes in the SHL mice (data not shown). Exendin-4 treatment even at the dose of 40 μg/kg had no effect on the plasma radioactivity of ^{14}C-Chol measured at 2 h after the cholesterol loading (Figure 4a). Thus, exendin-4 administration had no effect on the plasma total cholesterol concentration in the SHL mice (Figure 4b).

Effect of anagliptin on the target messenger ribonucleic acid expressions in the intestine in normolipidemic mice

Figure 5 shows the changes in the target messenger ribonucleic acid (mRNA) expressions in the small intestine of
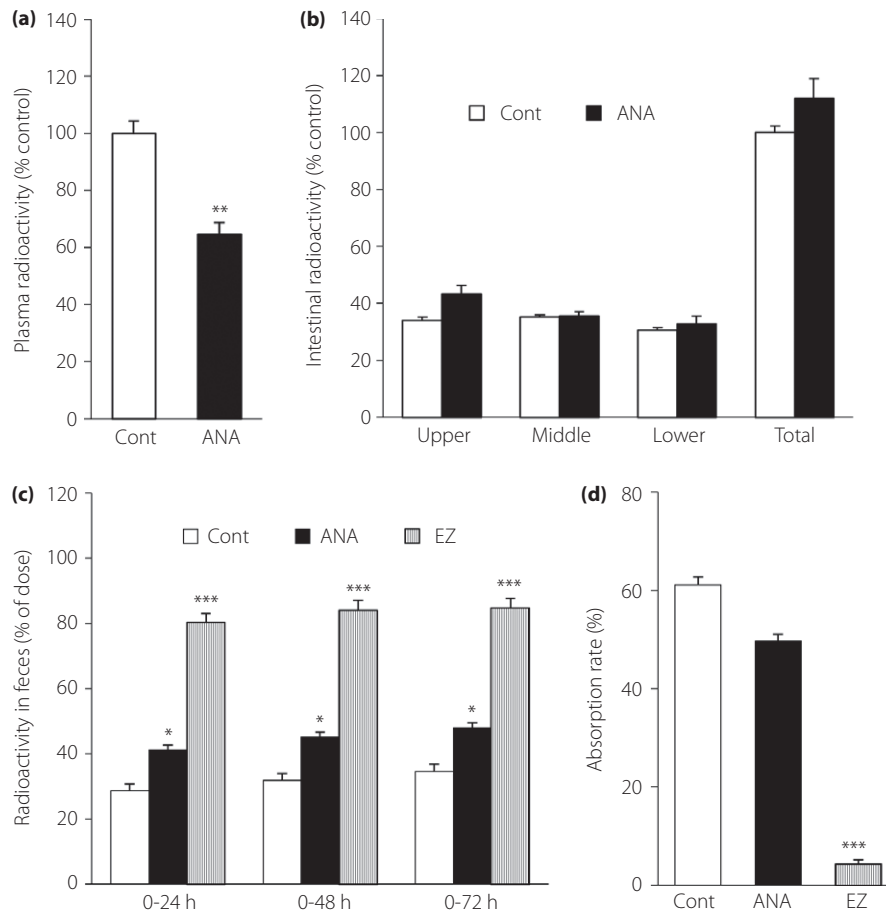
Figure 3 | Effect of anagliptin (ANA) treatment on the cholesterol uptake at 72 h after oral ^{14}C-labeled cholesterol loading in apolipoprotein E-deficient mice. (a) Plasma and (b) intestinal radioactivity of ^{14}C-labeled cholesterol at 72 h after oral radiolabeled cholesterol loading in mice treated daily with ANA for 4 weeks. (c) Fecal excretion and (d) estimated absorption rate of radiolabeled cholesterol after repeated-dose treatment with ANA for 4 weeks. Data represent the mean ± standard error of the mean for five animals. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significant differences as compared with the control (Cont). EZ, ezetimibe.

normolipidemic C57BL/6 mice treated with anagliptin mixed in the diet for 5 weeks. Treatment with anagliptin was associated with decreased mRNA expressions of microsomal triglyceride transfer protein (MTTP), acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2), ApoA2 and ApoC2, which are known to be involved in cholesterol transport, especially lipoprotein assembly and secretion, from the intestinal mucosa to lymph. In contrast, anagliptin treatment had no effect on the gene expressions of transporters, such as Niemann-Pick C1-like 1, scavenger receptor class B type 1, adenosine triphosphate-binding cassettes and ApoB. We also confirmed the inhibitory effect of anagliptin on the hepatic target gene expression related to cholesterol synthesis and on the serum total cholesterol concentration (Figures S2 and S3).

Effects of DPP-4 deficiency on the effect of anagliptin on cholesterol transport

Using F344/DuCrlCrlj rats, which are spontaneously DPP-4-deficient, we investigated the effect of anagliptin on

cholesterol transport, in order to clarify the involvement of the DPP-4 enzyme activity. As shown in Figure 6, anagliptin did not significantly reduce the plasma radioactivity of ^{14}C-Chol at either 4 or 72 h after oral loading of radiolabeled cholesterol.

DISCUSSION

In the present study, we investigated the effects of anagliptin on the intestinal cholesterol metabolism and transport in normoglycemic and spontaneously hypercholesterolemic ApoE-deficient mice. Normoglycemic mice have been used in this study for the reason that we would like to investigate the lipid-lowering effect of a DPP-4 inhibitor independent of glucose-lowering effect. Because lipid metabolism could be closely related to glucose metabolism, and DPP-4 inhibitors could affect glucose metabolism in diabetic animals, these studies using diabetic animals were considered to be difficult to confirm the direct effect of DPP-4 inhibitors on lipid metabolism. First of all, we confirmed the lipid-lowering effect of anagliptin in the mice, just as in a previous clinical study[17–20], with
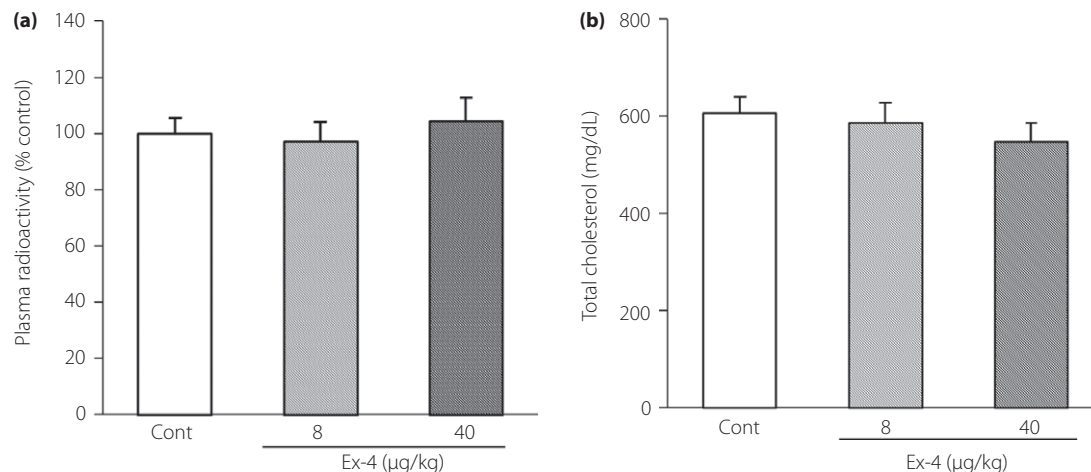
Figure 4 | Effect of exendin-4 on the cholesterol uptake after oral ^{14}C-labeled cholesterol loading in apolipoprotein E-deficient mice. (a) Plasma radioactivity of ^{14}C-labeled cholesterol at 2 h after oral radiolabeled cholesterol loading, and (b) plasma total cholesterol concentration in mice treated twice daily with exendin-4 for 4 weeks. Data represent the mean ± standard error of the mean for nine animals. Cont, control; EX-4, exendin-4.
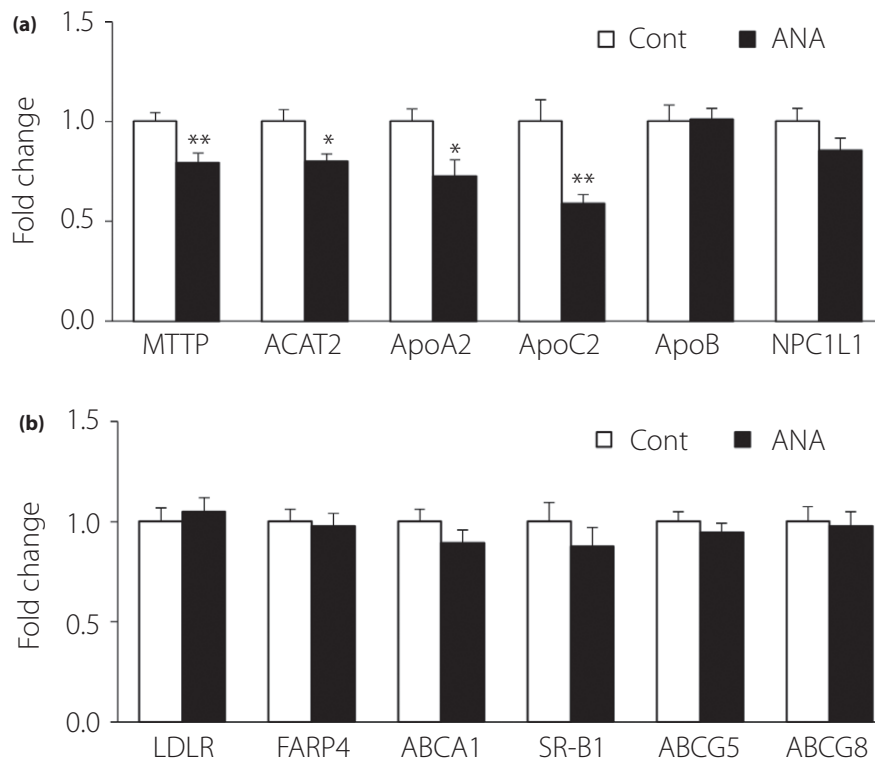

Figure 5 | Target gene expressions in the small intestines of mice after repeated-dose administration of anagliptin (ANA). Expressions of (a) microsomal triglyceride transfer protein (MTTP), acyl-coenzyme A:cholesterol acyltransferase 2 (ACAT2), apolipoprotein (Apo)A2, C2 and B, and Niemann-Pick C1-like 1 protein (NPC1L1), (b) low-density lipoprotein receptor (LDLR), fatty acid transport protein (FATP)-4, adenosine triphosphate-binding cassette transporter 1 (ABCA1), scavenger receptor class B member 1 (SR-B1), and adenosine triphosphate-binding cassette subfamily G member 5 (ABCG5) and 8 (ABCG8) were examined. Data represent the mean ± standard error of the mean for eight animals. *$P < 0.05$, **$P < 0.01$ significant differences as compared with the control (Cont).
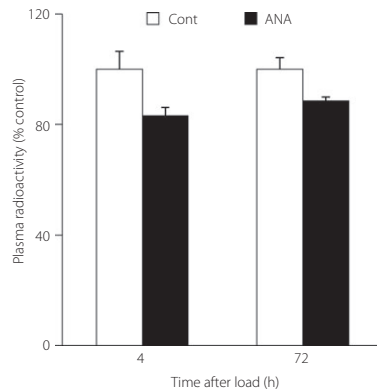
Figure 6 | Effect of dipeptidyl peptidase 4 deficiency on the effect of anagliptin (ANA) on cholesterol uptake after oral ^{14}C-labeled cholesterol loading in rats. Plasma radioactivity of ^{14}C-labeled cholesterol at 4 and 72 h after oral radiolabeled cholesterol loading in F344/DuCrlCrlj rats treated daily with ANA for 4 weeks. Data represent the mean ± standard error of the mean for eight animals. Cont, control.

anagliptin treatment reducing the serum total cholesterol and non-HDL-C concentrations. Similar effects of anagliptin have been shown in ApoE-deficient mice, LDL receptor knockout mice and cholesterol-fed rabbits[24–26]. Yano et al.[25] suggested that downregulation of hepatic lipid synthesis was involved in the cholesterol-lowering effect of anagliptin, because DPP-4 inhibition by anagliptin was associated with downregulation of the gene expression of sterol regulatory element-binding protein 2, as well as reduction of the serum levels of total cholesterol, VLDL-C and LDL-C. Actually, in the present data, the hepatic target gene expression related to cholesterol synthesis was decreased in mice with repeated treatment with anagliptin (Figure S2), which reproduced the results in the previous reports. In contrast, anagliptin also tended to decrease the serum cholesterol level in dietary cholesterol-fed rabbits with hypercholesterolemia[26]. Although rabbits cannot synthesize cholesterol endogenously, the effect of anagliptin appears to be attributable to reduction in the absorption of dietary cholesterol from the small intestine. In fact, the serum concentrations of sitosterol and campesterol, both markers of cholesterol absorption, in these rabbits also showed a tendency to decrease with anagliptin treatment, and were closely correlated with the serum cholesterol concentrations. In the present study, this effect was predominantly observed in the chylomicron and VLDL fractions, which is consistent with previous reports[26].

Next, we investigated the effect of anagliptin on cholesterol transport after exogenous ^{14}C-Chol loading. In the present study, repeated daily administration of anagliptin, but not single-dose administration, delayed and reduced cholesterol transport from the intestine to the blood stream in the ApoE-deficient mice; based on the results of the experiments carried out using DPP-4-deficient rats and the GLP-1 receptor agonist, this effect of anagliptin appears to be DPP-4-dependent

and incretin-independent. Some studies have shown the effects of GLP-1 and other related peptides on lipid transport, including reduction of the lymph flow and chylomicron production[27,28]. In intestinal lymph duct-cannulated rats, infusion of recombinant GLP-1 has been shown to decrease the intestinal lymph flow, triglyceride absorption and apoprotein (ApoB and ApoA4) production, while having no effect on the cholesterol absorption[27]. The GLP-1 receptor agonist, exendin-4, and DPP-4 inhibitor, sitagliptin, also suppressed postprandial hypertriglyceridemia by reducing intestinal production/secretion of triglyceride-rich lipoprotein, and blockade of endogenous GLP-1 receptor (GLP-1R) signaling by the antagonist, exendin (9-39), or genetic elimination of GLP-1R signaling in GLP-1R knockout mice was shown to enhance intestinal ApoB-48 secretion[28]. Therefore, DPP-4 inhibitors might reduce postprandial triglyceride absorption, at least in part, through GLP-1R signaling. Cholesterol transport does not run in parallel with triglyceride transport. It was shown that GLP-1 does not affect lymphatic cholesterol absorption after intraduodenal infusion of radiolabeled cholesterol and triolein, although triolein absorption was dramatically reduced by GLP-1 treatment[27]. In fact, in the present study, while anagliptin also reduced postprandial serum triglyceride level after oral oil loading (Figure S1), 4 weeks of treatment with the GLP-1R agonist exendin-4 was found to have no effect on the cholesterol transport after oral radiolabeled cholesterol loading in mice with hypercholesterolemia. Furthermore, DPP-4-deficiency abolished the effect of anagliptin on cholesterol transport, and sitagliptin showed a similar effect on ^{14}C-Chol transport in mice (data not shown). Taken together, DPP-4 inhibition by DPP-4 inhibitors might negatively regulate the cholesterol transport in a GLP-1-independent manner, which might be a common effect of DPP-4 inhibitors.

We also carried out quantitative polymerase chain reaction analysis of target gene expressions in the small intestine of mice to clarify the mechanisms underlying the suppression of cholesterol transport. The mRNA expression of Niemann-Pick C1-like 1 (NPC1L1) remained unchanged after anagliptin treatment. The transporter, NPC1L1, a target of ezetimibe, is mainly expressed on the gastrointestinal tract epithelial cells and regulates the absorption of dietary cholesterol from the intestinal lumen into the intestinal epithelial cells[29,30]. In the present study, as the cholesterol radioactivity in the intestinal tissue was not decreased, but actually increased (or remained unchanged) after radiolabeled cholesterol loading, it would seem that anagliptin has no effect on the cholesterol absorption per se, which is consistent with the results of the quantitative polymerase chain reaction analysis. In contrast, the mRNA expressions of MTTP, ACAT2, ApoA2 and ApoC2 decreased after repeated-dose treatment with anagliptin. These proteins are known to play roles in lipoprotein assembly and secretion from the intestinal mucosa to the lymph in the course of cholesterol transport and metabolism. As MTTP catalyzes the transport of triglyceride, cholesteryl ester and phosphatidylcholine between membranes, MTTP might have a role in lipoprotein

assembly[31]. ACAT2 is a member of the small family of acyl-CoA:cholesterol acyltransferases that produces intracellular cholesterol esters from long-chain fatty acyl CoA and cholesterol[32]. ACAT2 is known to be responsible for cholesterol ester formation and secretion of lipoproteins[33]. The ApoC2 genes encode ApoC-II, and ApoC-II activates the enzyme, lipoprotein lipase, which hydrolyzes triglycerides[34]. One possible explanation for the mechanisms underlying the suppression of cholesterol transport is that these decreased proteins might inhibit the lipoprotein assembly and secretion, and lipoprotein lipase activity in the small intestine, resulting in the suppression of lipoprotein metabolism and delayed cholesterol transport into the intestinal lymph. However, further studies are required to precisely clarify these mechanisms, because it remains unclear how anagliptin treatment changes the target gene expressions in the intestine.

Regarding the differences of efficacy among DPP-4 inhibitors, meta-analysis suggests that the cholesterol-lowering effect seems to be a 'class effect', and the present study shows the suppression of intestinal cholesterol transport would be DPP-4-dependent. However, Kurozumi *et al.*[19] showed that in a comparison between alogliptin and anagliptin, only anagliptin exhibited a significant reduction of serum LDL-C levels when a DPP-4 inhibitor was changed from another DPP-4 inhibitor in type 2 diabetes mellitus patients. Furthermore, anagliptin, but not alogliptin, significantly reduced serum ApoB-100 levels after 24 weeks of treatment, suggesting that hepatic cholesterol synthesis might be suppressed by anagliptin[19]. Similar effects of anagliptin on cholesterol synthesis in the liver were reported by others[20,25], and we also confirmed them, as mentioned above. At present, whether the effect on cholesterol synthesis in the liver is a common effect of DPP-4 inhibitors is not clarified; therefore, further studies are required to elucidate the details of how anagliptin can regulate the cholesterol metabolism in the liver, as well as in the small intestine.

In conclusion, these data suggest that in addition to reducing the blood glucose levels in type 2 diabetes mellitus patients, the DPP-4 inhibitor, anagliptin, might also exert a beneficial effect on the lipid metabolism, at least in part, through DPP-4-dependent and GLP-1-independent inhibition of intestinal cholesterol transport.

DISCLOSURE
MG, SF, SY, HH and NK are employees of Sanwa Kagaku Kenkyusho Co., Ltd. WY and NI are employees of Kowa Company, Ltd. KK is an advisor to, received honoraria for lectures from and received scholarship grants from Novo Nordisk Pharma, Sanwa Kagaku Kenkyusho, Takeda Pharmaceutical, Taisho Pharmaceutical, MSD, Kowa Company, Sumitomo Dainippon Pharma, Novartis Pharma, Mitsubishi Tanabe Pharma, AstraZeneca, Nippon Boehringer Ingelheim, Chugai Pharmaceutical, Daiichi Sankyo and Sanofi.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 | Postprandial plasma triglyceride profiling in lipoproteins by high-performance liquid chromatography in apolipoprotein E-deficient mice treated with anagliptin.

Figure S2 | Target gene expressions in the liver of mice after repeated-dose administration of anagliptin.

Figure S3 | Serum total and non-high-density lipoprotein cholesterol concentrations in C57BL/6 mice with 5 weeks of treatment with anagliptin.