While bile acids (BAs) have long been known to be essential in dietary lipid absorption and cholesterol catabolism, in recent years an important role for BAs as signalling molecules has emerged. BAs activate mitogen-activated protein kinase pathways [1, 2], are ligands for the G-protein-coupled receptor (GPCR) TGR5 [3, 4] and activate nuclear hormone receptors such as farnesoid X receptor α (FXR-α; NR1H4) [5-7]. Recently, it has been reported that bile acids, cholic acid, induce energy expenditure by promoting intracellular thyroid hormone activation [8] indicating that BAs might be able to function beyond the control of BA homeostasis as general metabolic integrators. Despite these advances, further study is needed to determine the precise mechanism underlying the effect of bile acid on glucose metabolism. In addition, bile acid itself is not applicable for clinical use because of toxic effects.

Colestimide is a new anion exchange resin which has shown strong hypolipidemic effects in rabbits [9]. Colestimide is reported to increase cholic acid content among bile acids [10], thus, colestimide may have hypoglycemic effect in diabetes. In practice, there is increasing evidence that this effect of bile acid sequestrants may be mediated through the farnesoid X receptor (FXR/bile acid receptor), liver X receptor, and the influence of fibroblast growth factor-19 and TGR5 on intestinal glucose metabolism.

Colestimide improves glycemic control via hepatic glucose production in db/db mice

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Abstract. The objective of this study was to assess the chronic effects of a bile acid sequestrant, colestimide, on glucose metabolism. After db/db mice were fed a diet containing colestimide or cholic acid (CA) for 12 weeks, we investigated the impact of these agents on glucose and lipid metabolism. Colestimide significantly reduced the elevated fasting blood glucose level (p<0.01), and CA even more markedly reduced fasting blood glucose. The blood glucose level after an oral glucose load was significantly lower in the CA group than in the control group, but the colestimide group showed no significant difference. The insulin response to a glucose load was abolished in the control and colestimide groups. A hyperinsulinemic-euglycemic clamp study revealed that colestimide significantly improved the GIR (p=0.013). Hepatic EGP and Rd were also improved by colestimide, suggesting that it alleviated insulin resistance by suppressing hepatic glucose production and increasing peripheral glucose usage. CA significantly increased both the weight and cholesterol content of the liver, while colestimide reduced these parameters. Colestimide suppressed hepatic gene expression of SHP, but enhanced SREBP2 expression. On the other hand, CA increased the expression of SHP and lipogenic enzymes such as ACC and SCD-1, but had no effect on SREBP2. The present study demonstrated that colestimide improves hyperglycemia and hyperlipidemia, as well as reducing the hepatic lipid content. In contrast, CA exacerbates hyperlipidemia and increases the hepatic lipid content, although it improves glycemic control. Thus, colestimide is a well-balanced drug for the treatment of diabetes mellitus.

Key words: Colestimide, Bile acid, Diabetes
absorption and/or hepatic glucose metabolism, in addition to influences on peripheral insulin sensitivity, an incretin effect, an influence on energy homeostasis [11, 12].

We previously reported that colestimide improved glycemic control in patients with type 2 diabetes. However, the mechanism responsible for the hypoglycemic effect of colestimide has remained unclear [13, 14]. In addition, there are no direct comparison among cholic acid and colestimide for glycemic control in diabetes. These circumstances prompt us to investigate the effects of cholic acid and colestimide on insulin sensitivity and glucose metabolism in db/db mice to pursue the possibility of new mechanisms explaining these beneficial effects.

### Materials and Methods

#### Mice

C57BL/KsJ-db/db mice (6-week-old) were obtained from a commercial breeder (Japan CREA, Tokyo, Japan). Animals were housed in a room with a 12-h light/dark cycle and were allowed free access to food and water. The animal care and procedures of the experiments were approved by the animal care committee of the Yokohama City University.

#### Study design

On day 0, animals were divided to 3 groups (10-12 animal/group) in a way that average random glucose of one group was not significantly different from that of the other. One group (control) was maintained on the basal diet, 2nd group received colestimide added to the diet (1.5% w/w), and 3rd group received cholic acid added to the diet (0.5% w/w). Colestimide and cholic acid were mixed into the pelletized diet respectively by Oriental Co., Ltd. and the treatment period was 12 weeks. Body weight, food consumption, and plasma glucose were measured every 2 weeks.

At the end of the study, animals were anesthetized by inhalation of ether after a 16-h fast, and blood was collected from the retro-orbital plexus for further analysis.

#### Oral glucose tolerance test

The db/db mice, which were on a 2-week dosing regimen, were fasted overnight on day 13. On day 14, an oral dose of vehicle alone or compound was given in the morning, and blood samples were collected from the tail vein for determination of baseline values (0 min). The mice were then gavaged with an oral bolus of glucose (2 g/kg), and additional blood samples were collected at regular intervals (15, 30, 60, and 90 min) for glucose and insulin measurement. Insulin levels were measured by using an enzyme linked immunosorbent assay kit (Morinaga, Kanagawa, Japan).

#### Insulin tolerance test

Each group of mice was given an insulin tolerance test. Mice were given free access to food and were then intraperitoneally injected with 0.75 mU of insulin per gram of body weight. The glucose levels were then measured at 0, 15, 30, 60, and 120 minutes using whole blood obtained from the tail vein and a portable blood glucose analyzer (Glutest Neo).

#### Hyperinsulinemic-euglycemic clamp study

Clamp studies were performed as described previously [15-18]. Briefly, 2 to 3 days before the study, an infusion catheter was inserted into the right jugular vein under general anesthesia with sodium pentobarbital. Studies were performed on mice under conscious and unstressed conditions after a 6-hour fast. A primed continuous infusion of insulin (Humulin R; Eli Lilly and Company, Indianapolis, IN, USA) was given (5.0 mU/[kg min]), and the blood glucose concentration, monitored every 5 minutes, was maintained at 120 mg/dL through the administration of glucose (5 g of glucose per 10 mL enriched to approximately 20% with [6,6-2H2] glucose [Sigma, Tokyo, Japan]) for 120 minutes. Blood was sampled via tail tip bleeds at 90, 105, and 120 minutes to determine the rate of glucose disappearance (Rd). Rd was calculated using non–steady-state equations, and endogenous glucose production (EGP) was calculated as the difference between Rd and the exogenous glucose infusion rate (GIR).

#### Measurement of Lipoproteins

If HPLC is performed by an on-line single detection technique combined with a selective enzymatic reaction, this allows the lipid constituents of lipoproteins in the column effluent to be detected and monitored without the need for subsequent analysis of column fractions. The on-line detection technique eliminated laborious and time-consuming procedures associated with fraction collection and achieved a high throughput of samples, while improving analytical precision and detection sensitivity. However, this method required the separate injection of each enzyme reagent, which is both inefficient and wasteful of samples. Multiple injections
may be impossible for small samples, e.g., those from individual mice. Usui et al. [19] developed a new dual detection HPLC system for lipoprotein analysis that made it possible to simultaneously measure cholesterol and triglycerides in a single sample, thus reducing the number of analytical runs and tests needed. Therefore, plasma lipoproteins at the age of 18 weeks were analyzed by an on-line dual enzymatic method that allowed simultaneous quantification of cholesterol and triglycerides by HPLC at Skylight Biotech Inc. (Akita, Japan) according to the procedure described by Usui et al. [19]. In brief, 200 µL of serum diluted to 1:20 with saline was injected into two TSK gel LipropakXL columns (300 × 7.8-mm; Tosoh) connected in tandem, and the levels of cholesterol and triglycerides in lipoproteins separated by size were determined by using enzymatic reagents prepared by Kyowa Medex (Tokyo, Japan). Total cholesterol and triglyceride concentrations (mg/dL) were calculated by comparison with the total area under the chromatography curves for calibration standards of known concentrations [20].

**Histology**

The $db/db$ mice of the 3 groups and their lean littermates were used for histological studies. Liver were removed, and then fixed with 10% formalin, embedded in paraffin, and sectioned. Samples were processed and embedded in paraffin, sectioned at 5 µm using a Zeiss microtome and stained for connective tissue using the Masson Trichrome method.

Cryostat sections were cut at 5 µm from the liver samples and stained for haematoxylin and eosin.

**Isolation of RNA and Real-Time PCR Analysis**

When aged 18 weeks, mice were sacrificed for tissue collection. Tissues were homogenized and total RNA was extracted with Trizol reagent (Gibco-BRL Life Technologies). The quantity and quality of each RNA sample were evaluated by spectrophotometry (Beckman Coulter, DU640B) at 260 and 280 nm. RNA quality was also examined by electrophoresis on 1% formaldehyde agarose RNA gel. The messenger RNA levels in the liver were quantitatively analyzed using fluorescence-based reverse transcriptase polymerase chain reaction (PCR). The reverse transcription mixture was amplified using specific primers and an ABI Prism 7500 sequence detector equipped with a thermocycler. The primers were purchased from Applied Biosystems (Foster City, CA). The relative expression levels were compared after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GAPDH TaqMan Control Reagent Kit; ABI Prism 7700, Perkin-Elmer Inc.).

**Statistical Analysis**

Data are presented as the mean±SD. The unpaired Student’s t-test was used to assess the significance of differences between 2 groups. For analysis of lesion size, comparisons between groups were performed by the Mann-Whitney U test. In these analyses, $p<0.05$ was considered to be statistically significant.

**Results**

**Effect of colestimide on body weight and food consumption**

To determine whether colestimide or cholic acid affected body weight or food consumption, either agent was added to the basal diet of $db/db$ mice. Both colestimide and cholic acid had no effect on the weight or food consumption of $db/db$ mice (Fig. 1).

**Effect of chronic administration of colestimide on glycemic parameters (FPG, OGTT, and ITT)**

In $db/db$ mice, the fasting plasma glucose level increased along with age. Colestimide significantly decreased the fasting plasma glucose level compared with that of control mice ($p<0.01$) at 6, 8, 10 weeks, and cholic acid caused an even more marked decrease of fasting plasma glucose (Fig. 2A) at 4, 6, 8, 10, 12 weeks. To further clarify the effects of cholic acid and colestimide on glucose metabolism and insulin secretion, an oral glucose tolerance test was performed after 12 weeks of treatment. After an oral glucose load, the blood glucose level was significantly lower in the cholic acid group than in the control group, while the colestimide group showed no significant difference from the control group (Fig. 2B). The insulin response to glucose was completely abolished in the control group and the colestimide group, indicating the marked impairment of insulin secretion in both groups (Fig. 2C). To more directly evaluate whether insulin resistance was decreased by treatment with colestimide or cholic acid, an intraperitoneal ITT was performed. Colestimide tended to promote the hypoglycemic effect of insulin in $db/db$ mice, but there were no significant differences among any of the groups (Fig. 2D).

**Hyperinsulinemic-euglycemic clamp study**

We determined the metabolic response to insulin
Fig. 1 The changes in (A) body weight and (B) food intake in db/db mice treated with normal chow control (closed square), cholic acid (open circle), and colestimide (closed triangle). The values are the means ± SE.

Fig. 2 Impact of cholic acid and colestimide on glucose tolerance and insulin sensitivity in db/db mice. A, Blood glucose levels for 12 weeks of treatment with control (closed square), cholic acid (open circle), or colestimide (closed triangle). B, Blood glucose levels during an oral glucose tolerance test conducted after 12 weeks of treatment in db/db mice. D, plasma glucose levels during insulin tolerance test after 12 weeks of treatment. Statistical significance Values are the means ± SE.
by performing a hyperinsulinemic-euglycemic clamp study, which revealed that colestimide significantly improved the GIR ($p=0.013$). The hepatic EGP and Rd also tended to improve with colestimide treatment, suggesting that this agent improves insulin resistance by suppressing hepatic glucose production and increasing peripheral glucose usage. In contrast, cholic acid had no effect on insulin sensitivity (Fig. 3).

**Effect of chronic administration of cholic acid or colestimide on lipid metabolism**

Cholic acid significantly increased the total cholesterol level, while colestimide had no effect on serum lipid levels (Fig. 4A). The cholesterol content of the very low density lipoprotein (VLDL) and LDL fractions was significantly higher in the cholic acid group than in the control group, but the TG content was not significantly different. In the colestimide group, the cholesterol content of the VLDL fraction and chylomicrons was significantly lower than in the cholic acid group or the control group. In contrast, the cholesterol content of the high density lipoprotein (HDL) fraction was significantly higher in the colestimide group than in the control group (Fig. 4B). The increase of the cholesterol content in the cholic acid group was more prominent for the larger particles, while the decrease of the cholesterol content in the colestimide group was greater for the larger particles (Fig. 4D). The TG content was significantly reduced in both groups, especially that of the larger particles (Fig. 4C, E).

**Effect of chronic administration of cholic acid and colestimide on liver weight and lipid content**

Cholic acid significantly increased the liver weight/body weight compared with that of the control group, but colestimide had no effect on liver weight/body weight (Fig. 5A). The hepatic cholesterol content was also significantly increased by cholic acid treatment, but not by colestimide, while the TG content did not differ significantly between these two groups (Fig. 5B, C). Histological examination revealed that administration of cholic acid increased the accumulation of lipids in the liver. In contrast, colestimide decreased the hepatic lipid content (Fig. 5E).

**Adiponectin**

The plasma adiponectin concentration is decreased in patients with obesity and type 2 diabetes who have insulin resistance, and lack of adiponectin has been implicated in the mechanism of insulin resistance. To investigate the mechanisms by which treatment with cholic acid or colestimide reduced the elevated blood glucose level, we measured the plasma adiponectin concentration. However, there were no significant differences of the adiponectin concentration between the groups (data not shown).

**Effect of colestimide on expression of genes related to lipid and glucose metabolism**

To identify genes that might be related to the effects of cholic acid and colestimide on glucose and lipid metabolism, we examined the hepatic expression profile of genes related to glucose/lipid metabolism (Fig. 6).
Fig. 4  Impact of cholic acid and colestimide on lipid metabolism in db/db mice.
A, Lipid profile after 8 weeks on either diet. Mice were fed control (open bar), cholic acid (black bar), or colestimide (striped bar). B, Cholesterol content in each lipoprotein in each groups (n = 6). The cholesterol contents in chylomicron, VLDL, LDL, and high-density lipoprotein were determined. C, Triglyceride content in each lipoprotein in each groups (n = 6). The TG contents in chylomicron, VLDL, LDL, and high-density lipoprotein were determined. D, Cholesterol content in LDL fraction further analyzed in 6 subfractions according to particle size (n = 6). E, Triglyceride content in VLDL further analyzed in 6 subfractions according to particle size (n = 6). Values are the means ± SE. *p <0.05. CM indicates chylomicron; HDL, high-density lipoprotein.
Fig. 5 Effect of cholic acid and colestimide on liver steatosis in db/db mouse. Mice were fed control (open bar), cholic acid (black bar), or colestimide (striped bar) for 8 weeks and liver weights/body weights (A), TG content in the liver (B), TC content in the liver (C), and serum ALT levels (D) were measured. E. Histological analysis of liver samples with hematoxylin and eosin. Values are the means ± SE. *p<0.05 vs control,
Fig. 6 Effect of cholic acid and colestimide on hepatic gene expression in db/db mouse. Mice were fed control (open bar), cholic acid (black bar), or colestimide (striped bar) for 8 weeks and hepatic gene expression of G6Pase, FAS, SREBP1c, SREBP2 mRNA in the groups of mice indicated normalized to the β-actin mRNA (n=5). Hepatic expression of FXR, SHP, ACC, SCD-1, PEPCK mRNA in the groups of mice indicated normalized to the GAPDH mRNA (n=5).
While the expression of farnesoid X receptor (FXR) mRNA was not significantly different among the three groups, expression of small heterodimer partner (SHP) was significantly decreased in the colestimide group, but was significantly increased in the cholic acid group. In contrast, expression of sterol regulatory element–binding protein 1c (SREBP1c) was decreased in both the cholic acid and colestimide groups compared with the control group. The expression of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), key enzymes for gluconeogenesis, was slightly elevated in the cholic acid group. In addition, cholic acid treatment significantly increased the expression of lipogenic enzymes, including fatty acid synthase (FAS), ACC, and SCD-1. Colestimide also increased the expression of FAS, but did not significantly elevate either ACC or SCD-1 expression. SREBP2 expression was significantly increased in the colestimide group.

Discussion

A previous study suggested a potential effect of colestimide on glycemic control in type 2 diabetes, but the mechanism responsible for this hypoglycemic effect of colestimide has remained unclear. In the present study, we examined the differences between the responses of glucose and lipid metabolism to treatment with colestimide or cholic acid. The first finding was that colestimide treatment significantly reduced the elevated fasting blood glucose level ($p<0.01$) from 2 to 10 weeks in $db/db$ mice compared with that of the control group, while cholic acid more markedly reduced fasting blood glucose. Second, a euglycemic-hyperinsulinemic clamp study revealed that colestimide improved the GIR and hepatic EGP, and also slightly improved peripheral insulin sensitivity. In contrast, cholic acid enhanced insulin secretion. Third, treatment with cholic acid increased the TC level and induced severe fatty liver via an increase of the hepatic cholesterol content, while colestimide conversely improved fatty liver. Fourth, cholic acid and colestimide had different effects on the expression of lipogenic and gluconeogenic enzymes.

The mechanism(s) by which colestimide lowers blood glucose in diabetes is not yet clearly understood. In the present study, the OGTT showed that insulin secretion was enhanced by cholic acid, but not colestimide. Cholic acid-induced insulin secretion might be mediated through the TGR5 receptor [21]. In contrast, insulin sensitivity was slightly enhanced by colestimide, but not by cholic acid. Colestimide tends to promote insulin sensitivity, but there were no significant differences. On the other hand, the euglycemic clamp study showed significant improvement of insulin sensitivity (Fig. 3). The sensitivity from clamp study evaluate the insulin sensitivity is higher as compared with ITT (Fig. 2D). Therefore, only the euglycemic clamp study was able to show the differences between colestimide and cholic acid.

The euglycemic clamp study showed that administration of colestimide significantly improved the GIR ($p=0.013$). The hepatic EGP and Rd also tended to be improved by colestimide, suggesting that it increased insulin sensitivity by suppressing hepatic glucose production and increasing peripheral glucose usage, which would be compatible with recent reports [22]. In the present study, we focused on the hepatic effects of colestimide.

There is increasing evidence that the hepatic effects of BA sequestrants may be mediated through the farnesoid X receptor (FXR/BA receptor), in addition to the influence of fibroblast growth factor-19 and TGR5 on intestinal glucose absorption and/or hepatic glucose metabolism, as well as an action on peripheral insulin sensitivity, an incretin effect, and an influence on energy homeostasis [11, 23]. The FXR-α regulates the enterohepatic circulation and biosynthesis of BAs by altering the expression of genes such as SHP [24, 25], which inhibits the activity of other nuclear receptors. FXR activation by BAs has been shown to reduce the expression of genes involved in gluconeogenesis, including PEPCK and G6Pase. In addition, the FXR may modulate hepatic glucose production during the fasting period and may also regulate postprandial hepatic glucose utilization [26-28]. In this study, both colestimide and cholic acid slightly increased FXR expression, but their effects on SHP downstream of FXR were different. While cholic acid induced up-regulation, colestimide treatment led to downregulation of SHP, a finding that was compatible with previous reports [29, 30].

There are two possible explanations for these discrepancies as described below. First, the upstream gene expression pattern may affect that downstream. Transiently enhanced SHP, an upstream regulator of SREBP-1c, PEPCK, and CYP7A1, is reported to downregulate expression of SREBP-1c and PEPCK.
However, constitutive overexpression of SHP in the liver altered the expression of the above-mentioned genes in the opposite direction to that observed in the present study, resulting in the development of fatty liver [31]. Recently, targeted disruption of SHP was reported to decrease hepatic lipid content and increase hepatic insulin sensitivity, in association with a decrease in PEPCK expression [32]. The finding that SHP expression was decreased by colestimide suggests that the changes in lipid and glucose metabolism (in addition to cholesterol metabolism) in colestimide-treated mice were mediated by constitutive downregulation of SHP. Second, cholic acid and colestimide may regulate SREBP1c and PEPCK via FXR independent and SHP independent pathways. As described previously, BA sequestrants may also influence other pathways such as, fibroblast growth factor-19, TGR5 and unknowns [23]. Therefore, cholic acid and colestimide may regulate SREBP1c expression not mediated by FSR and SHP. Further investigation is needed to determine the precise mechanism underlying the influence of colestimide on glucose metabolism.

Recently, a BA-binding resin was reported to improve metabolic control through the induction of energy expenditure [30]. Administration of the resin increased energy expenditure, resulting in significant weight reduction and improvement of insulin sensitivity, unlike our present findings since there was no body weight difference among the three groups. This discrepancy might be due to the use of different experimental models, since the other study employed a model of obesity induced by a high-fat diet, while we used db/db mice with the spontaneous onset of obesity and diabetes.

SHP was reported to suppress CYP7A transcription [33]. In the present study, colestimide decreased SHP expression, suggesting that it could increase CYP7A expression via suppression of SHP. An increase of CYP7A1 activity may lead to the depletion of cholesterol in the liver and a subsequent reduction of the hepatic cholesterol content. Taken together, it seems that colestimide increases BA synthesis from cholesterol in the liver and reduces the hepatic sterol content. On the other hand, cholic acid increased the hepatic cholesterol content. Administration BAs reduces their biosynthesis from cholesterol, as reflected by the downregulation of CYP7A1, and thus increases the hepatic cholesterol content. In addition, resins can promote the elimination of circulating LDL particles and subsequently reduce the plasma LDL cholesterol level [34, 35] through an increase of LDL receptor expression and expression of proprotein convertase subtilisin kexin 9 (PCSK9). Induction of these mRNAs was reported to be mediated by an increase of SREBP2 expression induced by administration of the resin [36]. In the present study, colestimide treatment increased SREBP2 expression, suggesting that the mechanism(s) of its action may be compatible with previous findings.

Our study had several strengths, including the performance of a direct comparison between colestimide and cholic acid, and the use of genetically diabetic obese mice, which are a known model of obese type 2 diabetes mellitus. We also identified differences in the mechanism of hypoglycemic activity between colestimide and cholic acid. However, several limitations of this study need to be considered when interpreting the results. First, the hypoglycemic effect of colestimide was found to be weaker than that of cholic acid. Second, the mechanisms underlying the hypoglycemic effect of colestimide have not been fully identified. Third, we only investigated the molecular mechanisms affecting the liver, so further investigation of mechanisms acting on the peripheral tissues is needed.

Despite the beneficial effect of cholic acid on glucose metabolism, the clinical use of this agent is limited by the induction of severe fatty liver and an increase of LDL-cholesterol. On the other hand, we found that colestimide had both hypoglycemic and lipid-lowering effects, as well as reducing the hepatic cholesterol content, without causing significant adverse reactions. Thus, colestimide might be useful for the treatment of type 2 diabetes. Although many agents are already available to improve glycemic control, some may not be tolerated by a substantial number of patients or may have undesirable effects such as weight gain, excessive hypoglycemia, and edema that interfere with achieving good glycemic control and reduce compliance [37-40]. For example, in patients with end-stage renal disease, prolonged hypoglycemia can be induced by sulfonlurea therapy [41], and metformin is contraindicated. On the other hand, colestimide is not absorbed from the gastrointestinal tract and is completely excreted in the feces, so it is safe for such patients. Since the LDL-cholesterol and TG levels are leading predictors of coronary heart disease [42] in patients with diabetes mellitus, it is important to treat dyslipidemia in diabetic patients. Treatment with cholestyramine has
been reported to decrease the incidence of coronary heart disease [43], so colestimide has the potential to improve the outcome of both diabetes and hyperlipidemia, which are two major risk factors for atherosclerosis.

In conclusion, both cholic acid and colestimide improved glucose metabolism in db/db mice, although the hypoglycemic effect of cholic acid was stronger than that of colestimide. However, some significant differences were observed between cholic acid and colestimide. First, the mechanisms of their hypoglycemic effect were different. In addition, colestimide reduced the hepatic cholesterol content, while cholic acid caused accumulation of cholesterol in the liver. These findings suggest that colestimide may potentially be a promising new agent for the treatment of diabetes mellitus.

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Disclosure

None of the authors have any potential conflicts of interest associated with this research.

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