Colestervam improves insulin resistance in a diet-induced obesity (F-DIO) rat model by increasing the release of GLP-1

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Colestervam improves insulin resistance in a diet-induced obesity (F-DIO) rat model by increasing the release of GLP-1. Am J Physiol Gastrointest Liver Physiol 298: G419–G424, 2010. First published December 31, 2009; doi:10.1152/ajpgi.00362.2009.—Bile acid sequestrants have been shown to lower glucose levels in patients with type 2 diabetes. To investigate how colestervam (CL) HCl improves hyperglycemia, studies were conducted in diet-induced obesity (F-DIO) rats, which develop insulin resistance when fed a high-energy (high fat/high sucrose) diet (HE). The rats were fed HE; HE + 2% CL; HE + 0.02% SC-435 (SC), an apical sodium-dependent bile acid transporter inhibitor; and regular chow (controls). After 4 wk of treatment, both in the HE group and the SC + HE group, plasma glucose and insulin levels remained elevated compared with baseline values throughout an oral glucose tolerance test (OGTT). In contrast, in the CL + HE group, plasma glucose levels returned to baseline by the end of the test, and insulin peaked in 15–30 min and then returned to baseline. CL induced release of glucagon-like peptide-1 (GLP-1) because the area under the curve of plasma total GLP-1 in the CL-induced release of glucagon-like peptide-1 (GLP-1) because the area under the curve of plasma total GLP-1 in the CL + HE group was significantly greater than in the HE group during the OGTT. Bile acid concentrations in the portal blood did not decrease in the HE group but declined significantly both in the CL + HE and SC + HE groups with reduced farnesoid X receptor activation compared with controls. We concluded that CL reduces plasma glucose levels by improving insulin resistance in this rat model. It is unlikely that the improvement is attributable to decreased bile acid flux to the liver but is likely secondary to induced GLP-1 secretion, which improves insulin release.

EXCESSIVE GLUCONEOGENESIS in the liver contributes greatly to fasting and postprandial hyperglycemia. Recently, it has been shown that both bile acids and the bile acid sensor farnesoid X receptor (FXR), in addition to their well-known roles in bile acid metabolism and enterohepatic homeostasis, are involved in glucose homeostasis. Treatment with bile acids inhibits the expression of genes involved in gluconeogenesis in HepG2 cells as well as in mice (4, 22). The suppressive effects of bile acids and FXR on gluconeogenesis was confirmed by the observation by Ma et al. (17) that activation of FXR by cholic acid (CA) suppressed the expression of genes involved in gluconeogenesis in wild-type but not FXR−/− mice, in which gluconeogenesis in the liver was dysregulated. More importantly, it has been noted that FXR also plays an important role in the regulation of insulin sensitivity because glucose intoler-

MATERIALS AND METHODS

Animal experiments. Experiments were conducted in insulin-resistant F-DIO rats (15), initially generated from F344 rats. To induce insulin resistance in these animals, F-DIO rats must be fed a high-energy diet (HE) containing 15.5% fat and 28% sucrose (42 g butter fat, 113 g corn oil, and 278 g sucrose per kg diet). Our studies were carried out in 7-wk-old male F-DIO rats weighing 250–350 g.

There were four study groups (8 rats/group) treated for 4 or 8 wk as follows: 1) untreated controls, F-DIO rats fed regular Purina rat chow (no. 5001) serving as normal controls; 2) HE group, rats fed the HE diet that serve as the insulin-resistant models; 3) CL + HE group, rats fed HE diet containing 2% CL, serving as the CL-treated, insulin-resistant models; and 4) SC + HE group, rats fed the HE diet containing 0.02% SC, an inhibitor of ASBT to serve as SC-treated, insulin-resistant models. The special diets for the HE, CL, and SC + HE groups were made by Research Diets (New Brunswick, NJ). At the completion of the study (after 4 or 8 wk of treatment), a 2-h oral glucose tolerance test (OGTT) was performed after an overnight fast. Glucose was fed at 200 mg (0.2 ml of 1 g/ml glucose solution)/100 g body wt by gavage. Blood specimens were collected from the tail at baseline (t = 0 min) immediately before and at 15, 30, 60, 90, and 120 min after the initiation of glucose feeding. Plasma levels of glucose, insulin, and glucagon-like peptide-1 (GLP-1) were quantitatively evaluated in these samples. The animals then were euthanized after 2 days of further treatment. Under anesthesia, blood was collected from the portal vein for evaluation of changes in the bile acid flux returning
to the liver. Tissues were collected and frozen immediately in liquid nitrogen for measurement of mRNA expression of FXR target genes in the ileum (short heterodimer partner (SHP), ileal bile acid binding protein (IBABP), and ASBT) and in the liver (cholesterol 7α-hydroxylase, CYP7A1). The animal protocol was approved by the Institutional Animal Care and Use Committee at the Veterans Affairs Medical Center, East Orange, New Jersey.

**Assays for GLP-1, glucose, and insulin.** For GLP-1 levels, blood samples were collected in EDTA-plasma tubes containing 50 μM of the dipeptidyl peptidase IV inhibitor (Millipore, Billerica, MA). The collected samples were immediately placed on ice and then centrifuged at 4°C. Plasma was separated and stored at −70°C until assayed. The concentrations (pmol/l) of total GLP-1 were determined using a GLP-1 total radioimmunoassay kit (no. GLP-IT-36HK, Millipore) according to the manufacturer’s instructions. This kit detects all forms of GLP-1, i.e., GLP-1 (7–36) amide, GLP-1 (7–37), GLP-1 (9–36) amide, GLP-1 (9–37), GLP-1 (1–36) amide, and GLP-1 (1–37). Briefly, rabbit anti-GLP-1 antibody was added to the plasma sample and then kept at 4°C for 20–24 h. On day 2, 125I-labeled GLP-1 (7–36) amide tracer was added and then incubated at 4°C for 22–24 h. On day 3, the precipitating reagent was added, and the reaction was kept at 4°C for 20 min. The sample was centrifuged and the pellet counted by Auto-GAMMA (Cobra).

Blood glucose concentrations (mg/dl) were determined using an automated glucose oxidase method with an Analog glucose analyzer. Insulin levels (ng/ml) were determined by radioimmunoassay (Linco Research, St. Charles, MO) using rat insulin radioimmunoassay kit (no. R1–13K, Millipore).

**Assays for mRNA expression FXR target genes.** mRNA expression was quantitated by comparison to the internal standard. Each gene expression is expressed as a ratio **as the internal standard.** Reactions were performed in triplicate in a 7300 Real Time PCR Systems (Applied Biosystems) with thermal cycling conditions as manufacturer’s instructions. For quantitative RT-PCR analysis, 1 μg mRNA from each sample was reverse transcribed to cDNA using the first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA) for rat *CYP7A1*, *SHP*, *IBABP*, and *ASBT*, respectively. All reactions were performed in triplicate in a 7300 Real Time PCR System (Applied Biosystems) with thermal cycling conditions as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative mRNA levels were calculated by the comparative threshold cycle method using GAPDH as the internal standard. Each gene expression is expressed as a ratio between control (chow-fed) and treated rats.

**Measurements of portal bile acid concentration.** Concentrations returned to baseline levels in the F-DIO rats fed CL (CL + HE) and were significantly higher than in the SC + HE group (0.56 ± 0.19 mM, *P* < 0.05) but not different from the chow group (0.77 ± 0.22 mM) or SC + HE group (1.03 ± 0.50 mM).

**RESULTS**

**CL improved plasma glucose levels.** Figure 1 demonstrates that glucose tolerance was improved in the insulin-resistant rat model (HE-fed F-DIO rats) treated with CL (CL + HE) but not in those treated with SC (SC + HE). To show the difference between curves, the data in Figs. 1 and 2 are presented as **means ± SE.** Data are presented as means ± SE, whereas the data presented in the text and other figures are means ± SD. The AUC in Fig. 1 calculated using the trapezoidal rule is 16,256 ± 1,410 (mean ± SD) for the chow group and 17,559 ± 1,229 for the CL + HE group, the latter being 11.7% less (*P* < 0.05) than the AUC observed in the HE group (19,900 ± 1,688). The AUC of the SC + HE group (19,694 ± 1,930) is almost the same as that of the HE group. Fasting glucose levels in the four groups were measured at **time zero** when the rats had been fasted overnight and before being fed glucose. There was no significant difference among the four groups: chow (111 ± 12 mg/dl), HE (119 ± 13 mg/dl), CL + HE (111 ± 11 mg/dl), and SC + HE (117 ± 12 mg/dl). Similar to chow-fed F-DIO rats (chow), plasma glucose concentrations returned to baseline levels in the F-DIO rats fed CL + HE, whereas HE-fed F-DIO rats (insulin-resistant model) or SC + HE-treated rats did not. After 90 min, plasma glucose levels in CL + HE rats were 20% lower than that in the HE group (140 ± 8 vs. 174 ± 16 mg/dl, *P* < 0.01) or 16% lower than the SC + HE group (166 ± 24 mg/dl, *P* < 0.05); after 120 min, glucose levels in the CL + HE group (127 ± 9 mg/dl) were 23% lower than in the HE group (166 ± 16 mg/dl, *P* < 0.001) and 18% lower than in the SC + HE group (154 ± 21 mg/dl, *P* < 0.01). There were no differences in plasma glucose levels between the HE and SC + HE groups at anytime during the test.

The body weights from the four groups were similar at the beginning/end of the experiment as follows: chow, 333 ± 27 g; HE, 334 ± 28 g; CL + HE, 321 ± 28 g; SC + HE, 325 ± 32 g. Fasting plasma triglyceride concentrations (8) in the HE group (1.50 ± 0.50 mM) tended to be elevated and were significantly higher than in the CL + HE group (0.56 ± 0.19 mM, *P* < 0.05) but not different from the chow group (0.77 ± 0.22 mM) or SC + HE group (1.03 ± 0.50 mM).
Fasting plasma cholesterol levels increased 69% ($P < 0.001$) in the HE group (76 ± 10 mg/dl) compared with the chow-fed rats (45 ± 7 mg/dl). However, cholesterol levels in the CL + HE group (63 ± 8 mg/dl) and SC + HE group (62 ± 10 mg/dl) were lower ($P < 0.05$) than that in the HE group.

**CL improved the insulin resistance.** Plasma insulin levels during the OGTT in rats with different treatments are depicted in Fig. 2. The AUC of insulin during the OGTT in the HE group (420 ± 148) was bigger than in the CL + HE group (220 ± 106, $P < 0.05$). There was no significant difference in the plasma insulin levels between the chow-fed and CL + HE animals during the test. However, fasting basal insulin levels in the HE group were higher than in the CL + HE group (2.28 ± 1.08 vs. 1.25 ± 0.56 ng/ml, $P < 0.05$) but not different from the SC + HE group (2.13 ± 0.68 ng/ml). Insulin levels in rats treated with CL + HE were lower than those with HE alone (1.11 ± 0.72 vs. 3.71 ± 1.73 ng/ml, $P < 0.001$) 90 min after glucose feeding. The insulin levels in CL + HE rats were also lower than levels in HE rats (0.96 ± 0.48 vs. 3.11 ± 1.33 ng/ml, $P < 0.001$) or SC + HE rats (2.75 ± 0.82 ng/ml, $P < 0.01$) 120 min after glucose feeding. It should be noted that there was no reduction in insulin levels in rats treated with HE or SC + HE after 60 min but that plasma glucose levels remained elevated.

Plotting glucose vs. insulin levels, linear regression analysis was performed using GraphPad Prism 4 (GraphPad). The slopes of the regression lines in Fig. 3 represent the ratio of glucose/insulin in the HE, CL + HE, and SC + HE groups during the OGTT. Higher slope indicates less efficiency of insulin. Obviously, the slope of the CL + HE group (10.96 ± 3.33) is significantly lower ($P < 0.05$) than the HE group (27.01 ± 9.00), indicating improvement of insulin resistance.

**Effect of CL and SC on bile acid composition and flux in the portal blood.** Because the portal blood flow is believed to be similar in rats with similar size and body weight, the total concentration of bile acids in the portal blood should be a measure of the bile acid flux returning to the liver. The results shown in Fig. 4A suggest that feeding HE alone does not decrease the portal bile acid flux compared with chow-fed F-DIO rats (162 ± 80 μM vs. 166 ± 41 μM). Adding CL or SC with HE reduced total bile acid concentrations in the portal blood 53% (81 ± 37 μM, $P < 0.05$) and 61% (66 ± 25 μM, $P < 0.05$), respectively, compared with HE alone. In addition, concentrations of the potent FXR ligands chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and CA were measured. As seen in Fig. 4B, after adding CL or SC to the HE diet, the FXR ligand concentration in the portal blood decreased 66% (33 ± 11 μM, $P < 0.01$) and 71% (28 ± 12 μM, $P < 0.001$), respectively, compared with rats treated with HE alone (96 ± 41 μM). The concentrations of individual bile acids in the portal blood are listed in Table 1. The proportion of potent FXR ligands (CDCA + DCA + CA) was similar (48%) in the HE + CL group and HE + SC group, and both tended to decrease compared with those in the HE group (61%). Non-FXR ligands in the portal bile acid flux were mainly the muricholic acids.

**The effect of CL and SC on the expression of the genes reflecting FXR activation.** The expression of IBABP and SHP are positively regulated by FXR. CYP7A1, the rate-limiting
The enzyme of classic bile acid synthesis, and ASBT are negatively regulated by FXR. To study the effects of the different treatments on FXR activation, we measured the expression of these genes in the ileum (IBABP, SHP, and ASBT) and liver (CYP7A1), which serve as surrogate markers of activation status of FXR. The mRNA expression was determined by real-time PCR and presented as relative units.

To further evaluate whether CL treatment enhances release of GLP-1, we extended the treatments to 8 wk in these same animal models and then carried out the OGTT. The AUC of the CL + HE group in Fig. 6A (22,462 ± 4,109) is 56% more (P < 0.01) than that of the HE group (14,407 ± 3,970). The increase of GLP-1 levels in plasma from rats fed CL + HE now became significant at a number of time points compared with the HE group, the untreated insulin-resistant rat. Baseline (fasting) levels of total GLP-1 in rats fed CL + HE (108 ± 40 pmol/l) were elevated twofold (P < 0.05) compared with rats given HE alone (52 ± 12 pmol/l). In addition, plasma GLP-1 levels in the rats fed CL + HE were significantly higher than those fed HE at 15 (P < 0.05), 30 (P < 0.05), and 60 min (P < 0.001) during the OGTT. GLP-1 levels in the CL + HE group also were higher than the levels of the SC + HE group (Fig. 6B). The AUC of the CL + HE group is 73% more (P < 0.001) than that of the SC + HE group (13,010 ± 1,843) in Fig. 6B. The GLP-1 concentration in the CL + HE and SC + HE groups reached its peak level at 60 min, whereas that in the HE group reached its peak level at 90 min after being fed glucose.

**DISCUSSION**

This study demonstrates that CL but not SC reduces hyperglycemia in insulin-resistant rats (HE-fed F-DIO rats) by improving the insulin resistance. In those F-DIO rats fed HE alone (insulin-resistant) or HE with added SC, plasma insulin levels stayed elevated, whereas plasma glucose levels re-

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**Table 1. Bile acid concentrations in the portal blood**

<table>
<thead>
<tr>
<th></th>
<th>CDCA</th>
<th>DCA</th>
<th>CA</th>
<th>UDCA</th>
<th>MCAs</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>4.7 ± 3.2</td>
<td>18.2 ± 5.4</td>
<td>98.6 ± 17.9</td>
<td>2.5 ± 2.2</td>
<td>42.8 ± 19.7</td>
<td>166 ± 41</td>
</tr>
<tr>
<td>HE</td>
<td>9.4 ± 12.8</td>
<td>7.2 ± 2.8</td>
<td>80.0 ± 36.4</td>
<td>2.3 ± 2.2</td>
<td>63.4 ± 42.2</td>
<td>162 ± 80</td>
</tr>
<tr>
<td>CL + HE</td>
<td>4.9 ± 4.7</td>
<td>8.4 ± 4.2</td>
<td>20.7 ± 7.1</td>
<td>0.5 ± 0.9</td>
<td>43.8 ± 27.5</td>
<td>81 ± 37</td>
</tr>
<tr>
<td>SC + HE</td>
<td>6.7 ± 3.2</td>
<td>12.9 ± 8.9</td>
<td>8.9 ± 3.3</td>
<td>0.2 ± 0.3</td>
<td>42.2 ± 26.1</td>
<td>67 ± 25</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD (n = 6) in µM. CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; CA, cholic acid; UDCA, ursodeoxycholic acid; MCAs, muricholic acids; HE, high-energy diet; CL, colesevelam; SC, SC-435.
have been the result of enhanced GLP-1 secretion because GLP-1 is one of the most potent insulinotropic substances (10). Our hypothesis was confirmed by the finding that both baseline and postprandial plasma levels of GLP-1 in the CL + HE group, but not in the SC + HE group, were increased and were significantly higher than that in the HE group. Although both CL and SC inhibit intestinal bile acid absorption and block bile acid flux through the ileal enterocyte-portal blood-liver pathway, there remains a fundamental difference between them. CL sequesters bile acids, sharply decreasing the concentration of micelle-forming bile acids throughout the intestinal lumen. In contrast, SC operates at the intestinal surface, blocking bile acids from entering the ileal enterocytes so that the micelle-forming bile acids are probably not decreased at all. A critical level of bile acids in the intestine is essential to create micelles that enable fatty acids to be solubilized and absorbed. Knoebel (13) reported that, in lymph-fistula and bile-fistula rats, fatty acids were absorbed more distally when the concentration of infused bile salt (taurocholate) was decreased. It has also been reported that fatty acids induce secretion of GLP-1 (6, 18, 19) and that fat-induced GLP-1 secretion is dose dependent (23). Furthermore, it has been proposed that free fatty acids promote the secretion of GLP-1 by stimulating a G protein-coupled receptor, GPR120 (9), and that the fatty acid derivative oleoylethanolamide enhances GLP-1 from L cells through activation of another G protein-coupled receptor, GPR119 (14). We hypothesize that CL sequesters bile acids, thereby suppressing fatty acid absorption in the small intestine so that increased amounts of fatty acids, which should have been absorbed in the proximal portion of the small intestine, reach the ileum and stimulate L cells to release GLP-1. However, SC does not sequester bile acids or interfere with fatty acid absorption in the proximal small intestine, so there is no increase in the quantity of fatty acids that reach the L cells in the ileum. As a result, treatment with SC would not induce release of GLP-1 or improve insulin resistance, as we have observed. It has also been reported that colestidemide, another bile acid sequestant, reduced postprandial plasma glucose concentrations with increased GLP-1 levels in patients with type 2 diabetes (21). The results in humans support the hypothesis proposed above.

Because CL, but not SC, improved glycemic control in the insulin-resistant rat, it is unlikely that reducing the bile acid flux through the ileal enterocyte to the liver, and thereby stimulating bile-acid synthesis, could produce a beneficial effect on glycemic control. As expected, both CL and SC reduced the bile acid flux in the portal blood and FXR activation, and both caused increases in bile acid synthesis as indicated by the induced expression of CYP7A1. However, we do not expect that, in those rats treated with either CL or SC, biliary bile acid outputs would increase significantly. The biliary bile acid output consists of the resecreted bile acid flux that has been returned to the liver plus the newly synthesized bile acid. Increased bile acid synthesis, in general, is only able to compensate for the reduced bile acids returning to the liver via the portal system but does not increase total biliary bile acid output. Our previous work demonstrated that neither bile acid pool size nor biliary bile acid secretion was increased in SC-fed rabbits (16). Thus the bile acid flux in the small intestine would not increase in the rats either fed CL + HE or SC + HE though the hepatic CYP7A1 expression was maintained significantly higher than baseline values throughout the OGTT, a typical pattern associated with insulin resistance. In contrast, in the CL-treated, insulin-resistant rat models (CL + HE), the response of plasma glucose was similar to that in controls. Plasma glucose levels declined to baseline and became significantly lower than those in the HE and SC + HE groups 90 min after the glucose bolus, whereas plasma insulin levels declined to baseline soon after an early and sharp elevation. These results indicate that CL, a bile acid sequestant, but not the ASBT inhibitor SC, improves insulin resistance and reduces postprandial hyperglycemia.

A very important question is how CL accomplishes this. We observed that, in CL-treated, insulin-resistant rats, the improvement in glycemic control was related to a sharp and strong increasing of insulin secretion after glucose feeding. This observation led us to inquire whether this event could...
BILE ACID MALABSORPTION AND GLP-1 RELEASE

upregulated and bile acid synthesis was induced. In vitro studies have demonstrated that bile acids promote GLP-1 secretion through activation of bile acid surface receptor TGR5, a novel G protein-coupled receptor in the intestine (11), and that those bile acids potent for FXR activation are also the potent ligands for TGR5 (12). It has also been found that infusing taurocholate at a concentration of 20 mM, but not 10 mM, into the ileum lumen in rats elevated portal GLP-1 levels when infusing taurocholate at a concentration of 20 mM, but not 10 mM, into the ileum lumen in rats elevated portal GLP-1 levels in rats treated with SC (12). It has also been found that increased amounts of fatty acids reach L cells in the ileum to stimulate GLP-1 secretion. In summary, this study demonstrates that, in HE-fed F-DIO rats (insulin resistant), the bile acid sequestrant CL increases plasma glucose levels and insulin response, whereas SC, which blocks bile acid absorption in the ileum, does not. It is unlikely that the beneficial effect of CL on glycemic control is simply attributable to decreased bile acid absorption or to the resulting stimulation of bile acid synthesis because this beneficial effect was not observed in the rats treated with SC. We suggest, instead, that the induced release of GLP-1 observed in CL-treated rats may play a crucial role in the improvement of insulin resistance. We hypothesize that CL inhibits uptake of fatty acids in the jejunum by sequestering bile acids, which should have been available to form micelles. Consequently, increased amounts of fatty acids reach L cells in the ileum to stimulate GLP-1 secretion.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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