Acyl-Coenzyme A:Cholesterol Acyltransferase-2 (ACAT-2) Is Responsible for Elevated Intestinal ACAT Activity in Diabetic Rats

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Objective—Diabetes-induced dyslipidemia is seen in streptozotocin-induced diabetic rats. This is caused, in part, by elevated intestinal acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity. Because two ACAT isozymes (ACAT-1 and ACAT-2) were identified, in the present study we determined which ACAT isozyme was involved in the elevated intestinal ACAT activity in diabetic rats.

Methods and Results—We cloned a full-length cDNA of rat ACAT-2. Its overexpression in ACAT-deficient AC29 cells demonstrated that the ACAT activity is derived from the cloned cDNA, and a 45-kDa protein of rat ACAT-2 cross-reacts with an anti-human ACAT-2 antibody. The tissue distribution of rat ACAT-2 mRNA revealed its restricted expression to liver and small intestine. Immunohistochemical analyses using an anti-human ACAT-2 antibody demonstrated that ACAT-2 is localized in villus–crypt axis of rat small intestine. The intestinal ACAT activity in diabetic rats was significantly immunodepleted by an anti–ACAT-2 antibody but not by an anti–ACAT-1 antibody. Finally, intestinal ACAT-2 in diabetic rats significantly increased at both protein and mRNA levels as compared with that in control rats.

Conclusions—Our data demonstrate that ACAT-2 isozyme is responsible for the increased intestinal ACAT activity of diabetic rats, suggesting an important role of ACAT-2 for dyslipidemia in diabetic patients. (Arterioscler Thromb Vasc Biol. 2004;24:1689-1695.)

Key Words: acyl-coenzyme A:cholesterol acyltransferase-2; diabetic rats; diabetes-induced dyslipidemia; cholesterol absorption; atherosclerosis

Acyl-coenzyme A (CoA):cholesterol acyltransferase (ACAT) plays an important role in regulation of plasma cholesterol. ACAT, an intracellular enzyme located in the rough endoplasmic reticulum, catalyzes cholesteryl esters from cholesterol and fatty acyl-CoA.1 Physiological roles of ACAT so far clarified are intestinal cholesterol absorption, hepatic assembly of very-low-density lipoprotein (VLDL), and cholesterol storage in steroidogenic tissues for steroid hormone biosynthesis, whereas it plays a pathological role in the formation of macrophages-derived foam cells in atherosclerotic lesions.3 Diabetes-related vascular disorders such as coronary heart disease (CHD) are serious diabetic complications and the major cause of death in diabetic patients.4–5 One of the major causes of these diseases is diabetes-induced dyslipidemia,6–8 in which intestinal cholesterol absorption plays a significant role.9–11 Recently, 3 studies with streptozotocin (STZ)-induced diabetic rats have clarified that an elevated intestinal ACAT activity is responsible for diabetes-induced dyslipidemia.12–14 Jiao et al12 first showed that the intestinal ACAT activity was significantly elevated, which was normalized by insulin treatment. Maechler et al13 made a similar observation and showed that the elevated plasma cholesterol level is reduced by administration of an ACAT inhibitor (CL-277082). Kusunoki et al14 further clarified that the increased plasma triglyceride level as well as lymphatic absorption of total cholesterol (TC) and triglyceride were reduced by an ACAT inhibitor (F-1394). Two ACAT isozymes (ACAT-1 and ACAT-2) were identified in mammals.15 However, it remains unclear which ACAT isozyme is responsible for the elevated intestinal ACAT activity in diabetic rats. This issue was solved in the present study. Our results indicate an important role of ACAT-2 in diabetes-induced dyslipidemia.
Methods

Expression of Rat ACAT-2 in ACAT-Deficient Chinese Hamster Ovary Cells

To express rat ACAT-2 cDNA (online Figure IA, please see http://atvb.ahajournals.org) in cells, rat ACAT-2 cDNA was inserted into a pcDNA3.1(+) vector (Invitrogen). The 5'-RACE polymerase chain reaction (PCR) product inserted into pGEM-T was subcloned into a SacI/Smal site of pSP72 (Promega) to introduce an EcoRI site. Both the 5'-RACE-PCR product in pSP72 and the 3'-RACE-PCR product in pGEM-T were subcloned into the EcoRI site of the pcDNA3.1(+). The plasmid containing full open reading frame of rat ACAT-2 in a proper sequence (pcDNA3.1/HCAT-2) was confirmed by sequencing on both strands and used in the following cellular studies. ACAT-deficient Chinese hamster ovary cells (AC29)14 were transfected with 4 μg/mL pcDNA3.1/HCAT-2 and 20 μL/mL LIPOFECTAMINE 2000 reagent (Gibco-BRL) in OPTI-MEM (Gibco-BRL). Stable transfectants were selected during 2 weeks of incubation with medium containing 800 μg/mL of G418. Unless otherwise specified, cells were incubated at 37°C in 5% CO2.

Animals

Male Wistar rats (6 weeks old, weighing 200 to 220 g) purchased from Charles River Japan were fasted overnight before induction of diabetes. Diabetes was induced by a single intravenous injection of 60 mg/kg of STZ (Sigma) freshly dissolved in 50 mmol/L sodium citrate (pH 4.5) under light ether anesthesia. Age-matched rats were injected with an equal volume of citrate buffer to serve as controls. Two weeks after injection of STZ, animals were starved for 18 hours and euthanized under the ether anesthesia. Blood was collected to measure glucose, TC, and triglyceride using enzymatic kits (Wako). Animals were immediately submerged in RNAlater RNA stabilization reagent (Ambion) after harvesting. Total RNA was extracted from these tissues using RNeasy Mini kit (Qiagen) after harvesting. Total RNA was extracted from these samples using RNeasy Mini kit (Qiagen) and subjected to electrophoresis on 1% agarose gel containing formaldehyde, followed by blotting to a Hybond N+ nylon membrane (Amersham). The membrane was hybridized with the heat-denatured RNA probe (500 ng/mL) and further incubated for 30 minutes with anti-DIG antibody conjugated with alkaline phosphatase. ACAT-2 mRNA was visualized using CDP-Star (Roche) chemiluminescence substrate as described previously.8

Northern Blot Analyses

A fragment of rat ACAT-2 cDNA inserted into pGEM-T was amplified and used for preparation of digoxigenin (DIG)-labeled antisense RNA probes according to the manufacture’s instructions (DIG Northern starter kit from Roche). For RNA protection, samples using RNAeasy Mini kit (Qiagen) and subjected to electrophoresis on 1.0% agarose gel containing formaldehyde, followed by blotting to a Hybond N+ nylon membrane (Amersham). The membrane was hybridized with the heat-denatured RNA probe (500 ng/mL) and further incubated for 30 minutes with anti-DIG antibody conjugated with alkaline phosphatase. ACAT-2 mRNA was visualized using CDP-Star (Roche) chemiluminescence substrate as described previously.8

Immunohistochemical Analysis of ACAT-2 in Rat Small Intestine

Small intestines from normal rats were removed and fixed in 4% paraformaldehyde at 4°C overnight, dehydrated through a graded series of ethanol and xylene, and embedded in paraffin. The paraffin-embedded tissue blocks were cut sequentially into 3-μm-thick sections and mounted on poly-l-lysine-coated slides. Deparaffinized sections were boiled for 10 minutes in 10 mmol/L sodium citrate buffer (pH 6.0) for antigen retrieval. The sample was then incubated for 20 minutes with 5% normal donkey serum and sequentially reacted at 4°C overnight with 2 μg/mL of an anti-human ACAT-2 antibody as a primary antibody followed by an indirect immunoperoxidase method described previously.19

Assay for ACAT Activity

The cultured cells were homogenized with buffer A (50 mmol/L Tris-HCl and 1 mmol/L EDTA at pH 7.8 with protease inhibitors). The tissues were soaked in ice-cold buffer A and homogenized according to method described previously.14 The enzyme activity was determined by the reconstituted assay as described previously.20 Briefly, homogenates obtained from cultured cells and tissues were mixed with 4 mol/L KCl and 2% 3-(3-cholamidopropyl)-dimethyl-ammonium)-1-propanesulfonate (CHAPS) in buffer A to obtain the final concentration of 1 mol/L and 2%, respectively. These samples (80 μg in 20 μL) were reconstituted with 140 μL of sodium taurocholate–cholesterol-phosphatidylcholine (PC) mixed micelles (0.2 as cholesterol/PC molar ratio). The enzyme reaction was initiated by adding 20 μL of 250 μmol/L of [14C]oleoyl-CoA (20 pdim/pmol), followed by incubation for 15 minutes at 37°C. Lipids were then extracted and the radioactive cholesteryl [14C]oleate was determined by thin-layer chromatography.

Antibodies

The rabbit polyclonal antibodies against human ACAT-1 (DM10)21 and human ACAT-2 (DM54)22 were generous gifts from Dr T.Y. Chang (Dartmouth Medical School, Hanover, NH). DM10 cross-reacts with rat ACAT-1 protein.23

Immunodepletion Analyses

An immunodepletion analysis was performed as described previously.22 Briefly, homogenates obtained from cultured cells and tissues were solubilized with 1 mol/L KCl and 2% CHAPS. Each sample (0.9 mg in 450 μL) was incubated with 50 μL of buffer A containing 12 μg of an anti-human ACAT-1 antibody, an anti-human ACAT-2 antibody or nonimmune IgG, followed by constant shaking for 30 minutes at 4°C. After addition of 100 μL of protein A-Sepharose, each reaction mixture was incubated at 4°C overnight, followed by separation of supernatant and immunoprecipitate by centrifugation at 14,000g for 1 minute. Each supernatant was measured for ACAT activity by the reconstituted assay.20

Immunoblot Analyses

Homogenates obtained from cultured cells and tissues were solubilized with 10% sodium dodecyl sulfate (SDS). Each sample (200 μg) was electrophoresed on 10% SDS-polyacrylamide gel (SDS-PAGE), transferred to a polyvinylidene difluoride transfer membrane, and subjected to immunoblot analyses with an anti-human ACAT-1 antibody and an anti-human ACAT-2 antibody as described previously.22 Density of bands was measured with an Image Gauge software in LAS-1000plus (Fuji Film).

Statistical Analysis

Statistical differences were measured by Student t test. Pearson correlation coefficient (r) was used to estimate linear relationships between variables. Simple linear regression analysis was performed to determine the relationship of intestinal ACAT-2 and plasma lipids levels. P<0.05 was regarded as statistically significant.
Cloning and Sequence of Rat ACAT-2 cDNA and Its Expression in AC29 Cells

We cloned a full-length cDNA of rat ACAT-2 (GeneBank Accession No. AB101480), which contained an open reading frame of 1572 bp (Figure 1A). Its deduced amino acid sequence predicts a 524-residue protein with a calculated molecular weight of 64 kDa, showing 83%, 85%, and 84% identity with human, mouse, and monkey ACAT-2, respectively (Figure 1B). Next, rat ACAT-2 cDNA was transfected into AC29 cells to determine whether the cloned rat ACAT-2 cDNA coded for an enzymatically active protein. The ACAT activity of 5 stable transfectants (rat ACAT-2 cells) was 5- to 25-fold higher than that of 2 mock transfectants (Figure 1A). Immunoblot analyses with an anti-human ACAT-2 antibody demonstrated a single 45-kDa band in rat ACAT-2 cells but no band in mock transfectants (Figure 1B). In addition, the level of ACAT-2 mRNA was positively detected in all tissues examined, the kidney, and spleen. Whereas the expression of ACAT-1 mRNA were examined by RT-PCR in small intestine, liver, adrenal gland, testis, brain, stomach, heart, and pancreas. Data represent the mean of 3 separate experiments with triplicate assays. Error bars represent SD. B, These cells were solubilized and electrophoresed on 10% SDS-PAGE, followed by subjection to immunoblot analyses with an anti-human ACAT-2 antibody as described in Methods. Data are representative of 3 independent experiments.

Results

Tissue Distribution of Rat ACAT-2 mRNA and Intestinal Immunohistochemical Localization

The expression of ACAT-2 mRNA were examined by RT-PCR in small intestine, liver, adrenal gland, testis, brain, kidney, and spleen. Whereas the expression of ACAT-1 mRNA was positively detected in all tissues examined, the expression of ACAT-2 mRNA was limited to intestine and liver (Figure 2A). Furthermore, Northern blot analyses showed that ACAT-2 mRNA was detected in rat ACAT-2 cells, McA-RH7777 cells (rat hepatoma-derived cell line), and rat ACAT-2 protein is also recognized by an anti-human ACAT-2 antibody. These results indicate that the 45-kDa protein is derived from rat ACAT-2 cDNA expressed in AC29 cells, suggesting that rat ACAT-2 mRNA was also recognized by an anti-human ACAT-2 antibody.

Effect of Diabetic Conditions on Expression of Rat Intestinal ACAT-2

Fasting blood glucose levels in diabetic rats were ≈22.0 mmol/L at day 4 (4 days after STZ injection), whereas those in control rats remained <6.0 mmol/L. Plasma TC and triglyceride levels in diabetic rats at day 14 were increased 1.5-fold and 2.9-fold, respectively (Table). The intestinal ACAT activity of diabetic rats at day 14 was 2.2-fold higher than that of control rats (Figure 3A). In contrast, the hepatic ACAT activity was not changed in control and diabetic rats (35.1±2.3 versus 41.8±6.6 pmol/mg protein/minute, respectively; NS). Therefore, it is likely that hepatic ACAT does not contribute to diabetes-induced dyslipidemia. Immunodepletion of ACAT-2 in intestinal enterocytes contributed to diabetes-induced dyslipidemia. Immunodepletion of ACAT-2 in intestinal enterocytes contributed to diabetes-induced dyslipidemia. Immunodepletion of ACAT-2 in intestinal enterocytes contributed to diabetes-induced dyslipidemia.

Glucose, Total Cholesterol, and Triglyceride in Normal and Diabetic Rats

<table>
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<tr>
<th>Parameters</th>
<th>Control (n=8)</th>
<th>DM (n=16)</th>
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<tbody>
<tr>
<td>Glucose (mmol/L)</td>
<td>5.36±0.73</td>
<td>22.78±4.95†</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>72.0±10.5</td>
<td>106.6±38.2*</td>
</tr>
<tr>
<td>Triglyceride (mg/dL)</td>
<td>62.3±43.9</td>
<td>170.3±94.4*</td>
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Male Wistar rats were intravenously injected with either STZ (DM) or its vehicle (control). Each value represents mean±SD.

†P<0.05 and †P<0.001 compared with control.
tion analyses revealed that an anti–ACATn-1 antibody significantly reduced the activity of rat ACAT-1 transfectants (rat ACAT-1 cells), but had no effect on the enzyme activity of rat ACAT-2 cells. Similarly, an anti–ACAT-2 antibody completely reduced the enzyme activity of rat ACAT-2 cells but had no effect on rat ACAT-1 cells (Figure 3B). Under these conditions, an anti–ACATn-1 antibody reduced the ACAT activity by >70% of the total intestinal activity of control and diabetic rats, whereas an anti–ACATn-1 antibody did not affect it at all (Figure 3B). Furthermore, we made immunoblot analyses to examine whether the diabetes-induced increase in small intestinal ACAT activity was caused by an increase in ACAT-2 but not ACAT-1. The immunoblot analyses using an anti–ACAT-2 antibody revealed at least 2 bands in rat small intestine: the upper band and the slightly lower band. The amount of intestinal ACAT-2 at protein levels in diabetic rats at day 14 was 2-fold higher than that in the intestine of control rats (Figure 4B). Under these conditions, parallel immunoblot analyses with an anti–ACATn-1 antibody failed to reveal a distinct band (data not shown), indicating that the major intestinal ACAT isozyme both in normal and diabetic rats was ACAT-2. In addition, the expression of intestinal ACAT-2 was significantly correlated with the level of plasma TC ($r=0.56$, $P<0.01$) and triglyceride ($r=0.57$, $P<0.01$) (Figure 5).

Finally, we made Northern blot analyses to demonstrate whether the increased intestinal ACAT-2 protein in diabetic rat was caused by increased ACAT-2 mRNA level. Northern blot analyses could detect a single distinct transcript in small intestine and liver (Figures 2B and 4C). On quantitative analyses based on densitometry of this transcript, ACAT-2 mRNA level were increased by 1.8-fold in diabetic rats (Figure 4C and 4D).

**Discussion**

ACAT plays an important role in cholesterol absorption from intestines and recent reports using ACAT inhibitors demonstrated that the intestinal ACAT activity is responsible for dyslipidemia in diabetic rats. Two ACAT isozymes (ACAT-1 and ACAT-2) were identified in mammals, and it is important to determine which ACAT isozyme is responsible for the increased intestinal ACAT activity in diabetic rats.

**Figure 5.** Correlation between intestinal ACAT-2 and plasma lipids. A, Plasma TC levels. B, Plasma triglyceride levels. Plasma levels of TC and triglyceride were determined in 16 diabetic rats (black circles) and 8 control rats (white circles). The regression equations in (A) and (B) are $r=0.56$ ($P<0.01$) and $r=0.57$ ($P<0.01$), respectively.
The results of the present study have clarified an important role of intestinal ACAT-2 for diabetes-induced dyslipidemia at a transcriptional level.

In parallel with an elevated intestinal ACAT activity, both plasma TC and triglyceride levels were increased in the diabetic rats (Table). The increased TC levels seem to result from an increase in intestinal absorption of dietary cholesterol caused by elevated intestinal ACAT activity, whereas the mechanism of the increased plasma triglyceride levels is not clear. The effect of intestinal ACAT on intestinal triglyceride absorption is not known. Microsomal triglyceride transfer protein (MTP) was reported to play a crucial role in the intestinal chylomicron formation as well as hepatic VLDL assembly. Triglyceride as well as cholesterol esters are essential substrates for MTP. The increased cholesterol absorption in the diabetic rats induced mainly by upregulation of intestinal ACAT activity might also increase the intestinal MTP activity and then enhance intestinal chylomicron synthesis, thus inducing hypertriglyceridemia in the diabetic rats. In fact, recent reports showed that intestinal MTP expression in diabetes was higher than controls in both rabbits and rats.

Recent studies revealed new molecules involved in the intestinal cholesterol absorption; ATP-binding cassette half-transporters ABCG5/ABCG8 and Niemann-Pick C1–like 1 protein (NPC1L1). ABCG5/ABCG8 are highly expressed in the liver and intestine. Mutations in the human genes encoding ABCG5/ABCG8 resulted in sitosterolemia with a reduced biliary secretion as well as an enhanced intestinal absorption of plant sterols (sitosterol, campesterol), suggesting an important role of ABCG5/ABCG8 in hepatobiliary cholesterol secretion and intestinal cholesterol absorption. In STZ-induced diabetic rats, both intestinal and hepatic ABCG5/ABCG8 expressions were reduced at a mRNA level that contributed to a reduction of hepatobiliary cholesterol secretion and an increased plant sterols concentrations. Therefore, it is possible that both the decreased intestinal ABCG5/ABCG8 and the increased intestinal ACAT-2 contribute to diabetes-induced dyslipidemia. NPC1L1 localized in jejunal enterocytes was demonstrated to be critical for intestinal cholesterol absorption. Ezetimibe, a selective inhibitor of intestinal cholesterol absorption, had no effect in NPC1L1-deficient mice, suggesting that NPC1L1 serves as an ezetimibe-dependent pathway for intestinal cholesterol absorption. NPC1L1-deficient mice had reduced intestinal uptake of plant sterols but did not change triglyceride absorption. It does not seem likely that NPC1L1 may contribute to hypertriglyceridemia induced under diabetic conditions.

Previous studies showed that intestinal ACAT activity was increased in diabetic rats, which was restored by insulin treatment, suggesting insulin deficiency might lead to the elevated intestinal ACAT activity under the diabetic condition. Furthermore, no significant differences in the intestinal ACAT activity could be detected between diabetic animals fed control chow and those fed the atherogenic diet. In vitro study with Caco-2 cells, human intestinal cell-derived cell line, showed that incubation of these cells with insulin significantly reduced the ACAT activity of these cells, suggesting an inhibitory effect of insulin on the intestinal ACAT activity. Our in vitro experiment with Caco-2 cells also resulted in reduction of ACAT activity by insulin but not high glucose (unpublished data). It is likely from these data that the intestinal ACAT activity is regulated by insulin but not by high glucose or diet.

Recent studies clarified the ACAT-2 promoter. Song et al identified the ACAT-2 promoter region whose function was increased during the differentiation of Caco-2 cells. In this connection, the expression of Cdx-2, a transcriptional regulator localized in nucleus, increased greatly during the differentiation of Caco-2 cells but was not detected in hepatoblastoma cell line HepG2 cells. Sequence analyses by computer showed that human, mouse, and rat ACAT-2 promoter region contained 4, 3, and 4 Cdx-2 elements, respectively. Electrophoretic mobility shift assay showed that Cdx-2 was able to efficiently bind to mouse and human ACAT-2 promoter regions, suggesting that Cdx-2 is a tissue-specific ACAT-2 regulator in intestinal cells. This could be the case under diabetic conditions because ACAT activity in intestine, but not in liver, was increased in diabetic rats (Figure 3A).

ACAT inhibition is emphasized as a potential antiatherogenic strategy. However, results obtained from ACAT-1-deficient mice have not supported this notion unanimously. Animal experiments by Accad et al using ACAT-1-deficient mice bred either on apoE-deficient mice or LDL receptor-deficient mice revealed that deletion of ACAT-1 did not prevent these animals from development of atherosclerotic lesions. Yagyu et al using similar animal model systems reached the different conclusion that ACAT-1 deficiency reduces atherosclerotic lesions. However, Fazio et al showed that reconstitution of LDL receptor-deficient mice with ACAT-1-deficient macrophage resulted in a more significant increase in atherosclerotic lesions than LDL receptor-deficient mice reconstituted with wild-type macrophage. With these reports taken together, effectiveness of ACAT-1 inhibitors for anti-atherogenic therapy in humans remains controversial.

Although the expression of ACAT-1 is ubiquitous, the expression of ACAT-2 is restricted. Human ACAT-2 was expressed in intestine and mice ACAT-2 in intestine and liver, and rat ACAT-2 was shown to localize in intestine and liver in the present study. ACAT-2-deficient mice were completely resistant to diet-induced hypercholesterolemia. On further gene knockout of apoE, atherogenic lesions of these mice were significantly reduced when compared with those of apoE-deficient mice. These animal studies revealed that complete depletion of ACAT-2 prevents mice from atherosclerosis-prone hyperlipidemia, suggesting an importance of ACAT-2-derived intestinal and hepatic ACAT activity in diet-induced hypercholesterolemia.

Development of ACAT inhibitors has faced difficulty, caused mainly by adrenotoxicity, which might be induced by ACAT-1 suppression and isozyme-specific ACAT inhibitors are expected to be a potential target. In this connection, results of the present study and previous ones using ACAT-2-deficient mice suggest the possibility that ACAT-2-specific inhibitors are beneficial as an anti-atherosclerotic drug by normalization of diabetes-induced dyslipidemia.
In conclusion, the cloning of rat ACAT-2 cDNA with its characterization have provided convincing evidence that the increased intestinal ACAT activity in diabetic rats is caused largely by a significant upregulation of ACAT-2 at both protein and mRNA levels but not ACAT-1, suggesting a major catalytic role of ACAT-2 in diabetes-induced dyslipidemia. Although results from animal studies with mice or rats may not be directly applicable to human, selective inhibition for ACAT-2 might ameliorate diabetes-induced dyslipidemia in human and even cardiovascular disease, because intestinal cholesterol absorption is reported to be significantly higher in diabetic patients with CHD than those without CHD.10

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