The role of liver acyl-CoAcholesterol acyltransferase 2 (ACAT2), earlier shown to be the principal ACAT enzyme within primate hepatocytes, as a regulator of the hypercholesterolemia induced by dietary cholesterol was studied. At the end of low and high cholesterol diet periods, liver biopsies were taken from cynomolgus monkeys, a species highly responsive to dietary cholesterol, and less responsive African green monkeys. Liver cholesterol and cholesteryl ester concentrations were highest in cynomolgus monkeys fed cholesterol, despite the fact that in order to induce equivalent hypercholesterolemia, dietary cholesterol levels were 50% lower than was fed to green monkeys. Hepatic cholesteryl oleate secretion rate, measured during liver perfusion as an indicator of ACAT activity, was significantly higher in cynomolgus monkeys. Liver microsomal ACAT activity was 2–3-fold higher in cynomolgus monkeys than in green monkeys. The responses of ACAT2 were compared with those of ACAT1 that is found primarily in Kupffer cells. ACAT2 protein mass was significantly correlated to microsomal total ACAT activity in both species; ACAT1 mass was less well correlated. Dietary cholesterol induced a significant 3-fold increase of ACAT2 protein mass in cynomolgus monkeys, a much greater increase than was found for mRNA abundance; neither ACAT2 mRNA nor protein was diet-responsive in green monkeys. In cynomolgus monkeys but not in green monkeys, liver free cholesterol concentrations were elevated when cholesterol was fed and were correlated with ACAT2 protein levels. The data suggest a mechanism whereby the elevation of hepatic free cholesterol concentrations by dietary cholesterol, seen only in cynomolgus monkeys, resulted in higher ACAT2 protein levels in hepatocytes, either through increased production or stabilization of the protein. Regulation of ACAT2 gene transcription was not a factor.

Nonhuman primates have long been recognized as good models for the study of coronary artery atherosclerosis (CAA) because many characteristics of this disease are shared with humans. To induce CAA in primate models, cholesterol must be fed to elevate plasma cholesterol concentrations (2). The factors that respond to dietary cholesterol and lead to hypercholesterolemia are not fully understood but may include alterations in cholesterol synthesis, clearance of plasma lipoproteins via receptors, degradation of cholesterol into bile acids, secretion of cholesterol and bile acids into bile, and secretion of lipoprotein cholesterol esters into the plasma compartment (3).

The plasma cholesterol response to atherogenic diets is characteristic among primate species and can be low, moderate, or high (4). The genetic pattern of response typically appears polygenic (5, 6), although specific genetic influences remain mostly unidentified. Studies in mice show that many sterol regulatory element-binding proteins and liver X receptor-sensitive genes can be coordinately regulated at the transcriptional level in response to dietary cholesterol (7, 8). In contrast to what has been described for rodents (7), liver X receptor-mediated regulation of the cholesterol 7a-hydroxylase gene does not explain the down-regulation of this enzyme by dietary cholesterol in primates (9). Instead, cholesterol esterification enzymes are candidate sites for regulation. We have shown that when atherogenic diets are fed to primates, the extent of hepatic cholesteryl ester secretion in response to dietary fatty acids is highly correlated to the extent of CAA (10). Hepatic cholesteryl ester secretion in apoB-containing lipoproteins in primates is facilitated by hepatic acyl-coenzyme Acholesterol acyltransferase (ACAT) (11), and the form of ACAT enzyme present in the hepatocyte is ACAT2 (12, 13). The deletion of the gene for ACAT2 in the mouse led to a very low level of plasma lipoprotein cholesteryl esters upon dietary cholesterol challenge, clearly demonstrating that this enzyme is of key importance to hepatic lipoprotein cholesteryl ester secretion (13). Lecithin:cholesterol acyltransferase activity was intact in these animals. However, because the mouse has no functional cholesteryl ester transfer protein, the deficiency of cholesteryl esters in the apoB-containing lipoproteins appears to have been because of the lack of hepatic ACAT2. Furbee et al. (14) have shown that 70–80% of the cholesteryl esters in lecithin:cholesterol acyltransferase-deficient mice were still present in plasma, indicating that ACAT2 can provide many of the cholesteryl esters in lipoproteins in such animals.

It is not yet possible to generate monkeys with gene deletions, so we are unable to clarify the specific roles of individual enzymes with the same precision in the primate model as has been done in mice. However, we have identified species of primates with low and high plasma cholesterol responses to dietary cholesterol (4), and within any one species, a wide range of dietary cholesterol responsiveness is also apparent (15). By contrasting diet responses between species, we can make inferences about the roles played by individual enzymes. This is the approach taken in the present study, in which characteristic response differences of ACAT1 and ACAT2 to

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The abbreviations used are: CAA, coronary artery atherosclerosis; ACAT, acyl-CoA:cholesterol acyltransferase; Cyno, cynomolgus; ANOVA, analysis of variance; Mops, 4-morpholinopropanesulfonic acid.
dietary cholesterol were identified between two species of non-human primates. Given that the difference in response to dietary cholesterol of ACAT2, the cholesterol esterification enzyme found in primate hepatocytes (12), was identified between the dietary cholesterol-sensitive cynomolgus monkeys and the more diet-resistant African green monkeys, hepatic ACAT2 is implicated as an important factor in atherosclerosis susceptibility.

**MATERIALS AND METHODS**

**Experimental Animals and Procedures**—The monkeys used in these studies were feral animals imported into this country as young adult males. Both cynomolgus monkeys (Macaca fascicularis) from Indonesia and African green monkeys, also called St. Kitts vervet monkeys (Cercopithecus aethiops sabaeus), from St. Kitts island in the Caribbean were used in these studies. As part of a protocol to establish diet groups with equal variability in responsiveness to an atherogenic diet, the monkeys were fed a low cholesterol diet (<0.5 mg/kcal) during an equilibration period for at least 16 weeks. Monkeys then were fed for 10 weeks a challenge diet containing saturated fat as palm oil (35% of energy) supplemented with cholesterol at levels of 0.4 mg/kcal for cynomolgus and 0.6 mg/kcal for green monkeys (16). The monkeys were maintained in individual cages throughout the experiment in an AAALAC-accredited animal facility, and all procedures with monkeys were approved by the Institutional Animal Care and Use Committee.

Near the end of each diet period, animals were fasted overnight and blood was drawn into Vacutainer® tubes containing Na2EDTA while the animals were restrained with ketamine (10 mg/kg intramuscularly). Plasma was separated by centrifugation, and an aliquot was subjected to heparin-manganese precipitation of apoB-containing lipoproteins for analysis of high density lipoprotein cholesterol (17). Liver biopsies were also taken at the end of each of the diet periods from animals after an overnight fast. Biopsies (1–2 g) were taken surgically via laparotomy, and the animals were anesthetized with ketamine and isoflurane. Samples were immediately frozen in liquid nitrogen and stored at −80 °C (21). ACAT assays were done with 50 μg of microsomal protein in a final reaction volume of 300 μl of 0.1 M K2HPO4, pH 7.4, buffer containing 1 mg of bovine serum albumin. The microsomes were preincubated for 30 min at 37 °C with 50 nmol of cholesterol in cholesterol-saturated hydroxypropyl-β-cyclodextrin before the reaction was started by the addition of 25 μM (14C)oleoyl-CoA (500,000 dpm). Reaction time was 10 min, and the reaction was stopped with the addition of chloroform/methanol, 2:1.

**Western Blotting**—An aliquot containing 50 μg of protein in liver microsomes was suspended in an equal volume of protein solubilization buffer (120 mM Tris, pH 6.8, 20% (v/v) glycerol, 4% (v/v) SDS, 0.01% (w/v) bromphenol blue), and dithiothreitol was added to a final concentration of 0.1 M. Samples were incubated at 37 °C for 30 min before electrophoresis on 10% SDS-PAGE gels. Proteins were blotted to nitrocellulose in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol, pH 8.3) at 70 V for 16 h. Nonspecific binding sites on the nitrocellulose were blocked in 5% nonfat dry milk dissolved in a solution containing 100 mM Tris buffer, pH 7.4, 150 mM NaCl, 0.02% (v/v) Tween 20. Monospecific fusion protein antibodies to the N-terminal 100–110 amino acids of ACAT1 and ACAT2 were prepared in rabbits as described by Lee et al. (12) and were immunopurified by using a maltose-binding column. Removal of the maltose-binding protein antibodies was done prior to immunoblotting. The purified antibodies to ACAT were applied to the nitrocellulose at a concentration of 3 μg/ml in the blocking solution. A goat-antirabbit peroxidase-conjugated secondary antibody (Sigma) was used at a 1:15,000 dilution. Peroxidase signal was detected using Pico West Substrate (Pierce) and captured on X-OMat film (Eastman Kodak). ACAT protein mass was then quantified using the ChemiImager 5500 (Alpha Innotech).

**Statistical Analyses**—The data were evaluated for statistically significant differences and diet differences using ANOVA with post hoc analysis for individual group differences by the Fisher’s Protected Least Significant Difference test. Pearson’s correlation and linear regression analyses were also performed. The levels of significance of identified differences were determined according to the formulae provided with the statistical software (Statview, Mountain View, CA). Differences were considered statistically significant at p < 0.05.

**RESULTS**

The animals used in these studies were feral males that had been equilibrated to the laboratory environment for at least 16 weeks before the studies were begun. Liver biopsies were taken after the monkeys had been fed a low cholesterol diet, and a diet with cholesterol was added to elevate plasma cholesterol concentrations into a range in which atherosclerosis develops. The whole plasma and lipoprotein cholesterol concentrations are shown in Table I. Whole plasma cholesterol concentrations rose from 2.9 ± 0.5 mmol/l to 7.9 ± 0.6 mmol/l in the high cholesterol diet in both species. All of the increase was in the very low density lipoprotein + low density lipoprotein cholesterol, and there was no significant difference in these values between species. The very high density lipoprotein cholesterol was significantly higher in the green monkeys on the low cholesterol diets but, during the high cholesterol diet period, de-
creased to about the same concentration in both species.

Data on liver cholesterol concentrations in the biopsies taken from the two primate species for each of the diet periods are shown in Table II. No significant species differences in free cholesterol or cholesteryl ester concentrations were found when the low cholesterol diets were fed. The concentration of hepatic free cholesterol was significantly higher when cynomolgus monkeys were fed the high cholesterol diets, but no difference due to dietary cholesterol was found in green monkeys. When monkeys were fed the high cholesterol diet, the concentration of liver cholesteryl ester increased 30-fold in cynomolgus monkeys and 5-fold in green monkeys. Both of these differences were highly significant. The liver cholesteryl ester concentration from the high cholesterol diet period in cynomolgus monkeys was significantly higher than in green monkeys.

The hepatic ACAT activity levels (Fig. 1) were measured in assays of liver microsomes. By ANOVA, a statistically significant species difference was identified. When the high cholesterol diet was fed to cynomolgus monkeys, activity increased significantly, whereas in green monkeys, no significant change was found. When the high cholesterol diets were fed, the ACAT activity in liver microsomes isolated from cynomolgus monkeys was over 3-fold higher than the value in microsomes from green monkey liver, and this was a highly significant difference. In separate groups of each species, fed the high cholesterol diet, the secretion rates of cholesteryl oleate measured during isolated liver perfusion were obtained as a direct measure of the rate of hepatic ACAT product formation and secretion. The livers of cynomolgus monkeys secreted cholesteryl oleate at a rate of 3.3 ± 0.3 mg/hg (mean ± S.E.), which was over 3 times higher than the rate of secretion by African green monkey livers (0.89 ± 0.2 mg/hg). An even greater species difference was observed in the concentration of hepatic cholesteryl oleate, which was 28.7 ± 6.3 mg/g of liver in the cynomolgus monkeys and only 2.2 ± 0.45 mg/g of liver for green monkeys. Thus, in agreement with in vitro ACAT activity measurements, in vivo measures of both ACAT product secretion and accumulation by the liver indicated a significantly higher hepatic ACAT activity in cynomolgus monkeys than in green monkeys fed dietary cholesterol.

The mRNA abundance for ACAT1 and ACAT2 was measured in the liver biopsies (Fig. 2). Values for ACAT1 mRNA were comparable when the low and high cholesterol diets were fed to either species, and no difference between the two species was found. For ACAT2 mRNA abundance, values were higher in cynomolgus monkeys than in green monkeys fed comparable diets, and the addition of cholesterol to the diet in cynomolgus monkeys resulted in a statistically significant increase of about 30%. The levels of ACAT2 mRNA were comparable between the two diet periods in green monkeys. Values of ACAT2 mRNA abundance were significantly higher in cynomolgus monkeys than in green monkeys fed comparable diets.

Western blotting was used to estimate the levels of the two ACAT proteins in the liver biopsies from these animals. Fig. 3 shows a representative gel with the data for both ACAT1 and ACAT2 in liver biopsies of four different monkeys from each species. Single bands near 50 kDa in size for either enzyme were identified for each liver sample. When the blots for 8—10 animals of each species were quantified, the data in Fig. 4 were obtained. For ACAT1, the protein levels appeared comparable between the two species fed the low cholesterol diets, assuming similar antibody cross-reactivity for both species. However, a 3-fold increase in enzyme protein was identified when cynomolgus monkeys were fed the high cholesterol diet. A small but statistically significant increase in ACAT1 protein was found when green monkeys were fed the high cholesterol diet. The ACAT2 protein mass estimates were similar in cynomolgus and green monkeys fed the low cholesterol diets. A 3-fold increase for ACAT2 protein was found when the high cholesterol diet was fed to cynomolgus monkeys. By contrast, no increase in ACAT2 protein was seen when higher dietary cholesterol levels were fed to green monkeys. Thus, a clear species difference in response of hepatic ACAT1 and ACAT2 protein to dietary cholesterol was apparent, with major dietary cholesterol-induced increases in liver ACAT1 and ACAT2 being found in cynomolgus monkeys, whereas in green monkeys no diet cholesterol-related increase in ACAT2 occurred, and only a small increase in ACAT1 was found.

In liver microsomes, the ACAT activity assay reflects a contribution from both ACAT enzymes, although more of a contribution from ACAT2 was suspected because it is in hepatocytes. Total ACAT activity was compared with the estimates of ACAT1 and ACAT2 protein. A strong positive correlation in

### Table II

| Species/diet | n | Free chol. | Chol. ester |
|--------------|---|------------|-------------|
| Cyno, low    | 10| 2.63 ± 0.06a | 1.40 ± 1.4a |
| Cyno, high   | 11| 5.91 ± 1.02b | 45.61 ± 9.32b |
| AGM, low     | 12| 3.04 ± 0.05a | 1.92 ± 0.07a |
| AGM, high    | 13| 2.86 ± 0.38a | 10.29 ± 2.01c |

**TABLE II**

**Liver free cholesterol (chol.) and cholesteryl ester concentrations**

All values are mean ± S.E. Values within a column are significantly different if their letter is different.

**Fig. 1.** Species and diet comparisons of hepatic ACAT activity. Cyno, cynomolgus monkeys; AG, African green monkeys. The open bars represent the low cholesterol diet data, and the gray shaded bars are the high cholesterol diet data; all values are mean ± S.E., n = 20 for cyno, n = 8 for African green monkeys. ACAT activity was measured in cholesterol pre-loaded liver microsomes using [14C]oleyl-CoA as described under “Materials and Methods.” ANOVA showed a statistically significant species difference; bars with different letters are significantly different by post hoc analysis.

**Fig. 2.** Species and diet comparisons of mRNA abundance for hepatic ACAT1 and ACAT2. Abbreviations and bar shadings are as described for Fig. 1. No statistically significant differences were seen for ACAT1. Different letters indicate significant differences for ACAT2. The radiolabeled probes used in the solution hybridization assays were made based on the African green monkey sequence, but no base sequence dissimilarities were identified between species.
The levels of liver ACAT1 and ACAT2 protein were then compared with free and ester cholesterol concentrations. No significant correlation was found between ACAT concentrations and ester cholesterol concentrations for either species or diet situation (data not shown). On the other hand, a significant correlation was seen in cynomolgus monkeys between unesterified cholesterol concentration and ACAT2 concentration (Fig. 6). A wide range in hepatic cholesterol concentrations was found among cynomolgus monkeys but only when the high cholesterol diet was fed. The unesterified cholesterol concentrations in green monkey liver were the same whether the animals had been fed low or high cholesterol diets, obviating detection of any association with ACAT protein.

DISCUSSION

Cynomolgus monkeys have been found previously to be more responsive to dietary cholesterol than African green monkeys, with plasma total and low density lipoprotein cholesterol concentrations increasing 2–3 times more given the same dietary cholesterol challenge (4). In this study we investigated the likelihood that regulation of liver cholesterol esterification is, at least in part, responsible for the species difference in responsiveness to dietary cholesterol. We studied liver from each species when they were fed low cholesterol diets and were normocholesterolemic. We then fed enough cholesterol so that each species attained an equivalent degree of hypercholesterolemia and analyzed a second liver sample. Even though plasma cholesterol concentration was equivalent, liver free cholesterol and cholesteryl ester concentrations were significantly higher in the hypercholesterolemic cynomolgus monkeys (Table II). Liver cholesterol esterification was catalyzed by two enzymes, ACAT1 and ACAT2, and for comparison, the responses of both were documented. ACAT2 was found primarily in hepatocytes, whereas ACAT1 was most abundant in Kupffer cells (12).

Because hepatocytes are the cells of the liver that accumulate and secrete cholesteryl esters in lipoproteins, we hypothesized that any characteristically different responses to dietary cholesterol would most likely be a result of ACAT2 action. The data showed that hepatic ACAT2 protein concentration was highly correlated to microsomal ACAT activity (Fig. 5) and that species differences in the ACAT2 response to dietary cholesterol were significant, i.e. no change in green monkeys in contrast to a 3-fold increase in the diet-responsive cynomolgus monkeys (Fig. 4). The fact that hepatic free cholesterol concentrations increased in response to dietary cholesterol only in the cynomolgus monkeys and correlated to ACAT2 protein concentration (Fig. 6) suggests that the higher cholesterol concentration may stimulate greater ACAT2 protein levels, either through increased production or decreased catabolism of the enzyme. Regulation does not appear to be primarily transcriptional given that mRNA abundance was less responsive to diet.
than protein mass (Fig. 2). The tendency of cynomolgus monkeys to develop higher hepatic ACAT2 protein concentrations appears closely associated with the increased diet cholesterol responsiveness of this species. To our knowledge, this is the first identification in primates of a specific gene product that responds to dietary cholesterol in a species-specific manner.
The data derived in this study do not make clear if the increase in ACAT2 protein in cynomolgus monkeys is a direct result of the increase in hepatic cholesterol concentration, but because ACAT2 protein mass is correlated to the increase in cholesterol, and more ACAT2 would be expected to decrease available unesterified cholesterol, it seems likely that cholesterol somehow activates or stabilizes the enzyme. The consequence of this increase may well be the significantly higher cholesteryl ester accumulation and secretion. In the same vein, we have obtained preliminary data in cells stably transfected with ACAT2 showing that depletion of cellular cholesterol is associated with a decrease in ACAT2 protein mass. The data are consistent with the possibility that cholesterol somehow regulates enzyme degradation such that enzyme stability is increased when more cholesterol is available and esterification might be preferred. This would be consistent with the allosteric self-association of monomers of ACAT2, as suggested by Chang et al. (22), except that in addition to facilitating enzyme activity, the stability of the enzyme molecule is enhanced, as monitored by an increase in mass. The alternative would be that dietary cholesterol promotes increased enzyme production, although this effect would need to be post-transcriptional because differences in mRNA abundance were not as large as those in protein levels. Whereas a similar correlation between ACAT2 mRNA and protein mass was found in cynomolgus (r = 0.65) and green monkeys (r = 0.63), the apparent mass of ACAT2 protein per amount of mRNA (slopes of 231 versus 81 AU/pg mRNA) was almost 3 times higher in cynomolgus monkeys than in green monkeys. One interpretation for such a difference is that the translational efficiency for the ACAT2 protein could be higher in cynomolgus monkeys, possibly influenced by the increased cholesterol availability. Further experimentation will be needed to assess the likelihood of such a possibility.

The data in Fig. 4 also show an increase in ACAT1 mass in cynomolgus monkeys fed the higher cholesterol diet. This ACAT1 protein increase was also proportional to ACAT activity (Fig. 5), but the degree of association was not as high as for ACAT2. No significant correlation was seen for ACAT1 mass and total liver free cholesterol concentration (Fig. 6). We interpret these data as showing that in the cholesterol-fed cynomolgus monkeys, the Kupffer cell, as the primary site of ACAT1 (12), may be responding to higher dietary cholesterol in a similar manner to ACAT2 in the hepatocyte. However, because the Kupffer cell represents a minority of cells in the liver, the correlations with whole liver end points were not as strong. Although limited data are available, ACAT1 appears to be sensitive to cholesterol in an analogous manner to ACAT2 (22). Thus, it is reasonable to assume that similar mechanisms might signal both enzymes even though they are in different cell types.

The issue of which cell types contain ACAT1 and ACAT2 in the livers of experimentalprimates has not been questioned, and a similar pattern appears to be found in mice as well (23, 24). Controversy exists with regard to whether the cellular distribution pattern in these two animals was also seen in human liver. The Chang group (22) has found that little ACAT2 protein can be demonstrated in isolated human hepatocytes, whereas ACAT1 was more easily detectable. Immunodepletion analyses and immunohistochemical staining confirmed that ACAT2 levels in human livers were low and often below the limits of detection (22). Unfortunately, the cellular location of either enzyme could not be clearly ascertained in immunohistochemical stains given the low magnifications employed (22). We have obtained a limited amount of data in human tissue collected at surgery showing 50-fold lower levels of ACAT2 protein and mRNA than was seen in green monkeys, which appears to correlate with lower total ACAT activity (about 25%) as seen in a microsomal assay of human liver. These data appear to corroborate the lower ACAT2 abundance in human liver reported by Chang et al. (22), although they make no statement about cellular distribution. Our findings in human liver suggest that most of the ACAT1 signal from immunohistochemical staining is in Kupffer cells, as was true for monkey liver (12). Thus, the cellular distribution of ACAT1 and ACAT2 in human liver may still prove to be the same as in mice and monkeys, but more data are needed to define this distribution accurately. An interesting association is in the responsiveness to dietary cholesterol that can be rank-ordered as follows: cynomolgus > green monkeys > humans. This appears to be the same rank order as for hepatic ACAT2 expression. Clearly more work is needed to define the most pertinent relationships. The data in the present work lead us to hypothesize that hepatic ACAT2 is a major player in the responsiveness to dietary cholesterol.

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