Studies on the Expression of Genes Encoding Apolipoproteins B100 and B48 and the Low Density Lipoprotein Receptor in Nonhuman Primates

**COMPARISON OF DIETARY FAT AND CHOLESTEROL***

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African green monkeys were fed diets containing low and moderate cholesterol concentrations with either polyunsaturated or saturated fat as 40% of calories. Plasma total cholesterol, low density lipoprotein (LDL) cholesterol, and apoB concentrations generally were higher in animals fed (a) the higher dietary cholesterol concentration and (b) saturated fat. At necropsy, liver and intestine were removed, and measurement of mRNAs for LDL receptors (liver) and for apolipoprotein B (liver and intestine) was done. Monkey small intestine mucosa made exclusively apoB48 while the liver made only apoB100, although apoB mRNA in both tissues was the same size (14 kilobases). No dietary cholesterol or fat effects were found for apoB mRNA abundance in the liver, while the animals fed the higher dietary cholesterol level had 50% lower levels of hepatic LDL receptor mRNA. In a separate group of animals, livers were perfused and the rate of apoB secretion was measured. No dietary fat effect on apoB secretion rate was found, and no relationship between plasma LDL cholesterol concentration and the rate of hepatic apoB production existed. These findings support the idea that dietary factors that increase LDL concentrations act by reducing clearance of apolipoprotein-containing particles rather than by increasing production of these lipoproteins. Hepatic LDL receptor mRNA was similar in abundance in polyunsaturated fat and saturated fat-fed animals, suggesting that the difference in plasma cholesterol concentration between these groups is not mediated via effects on LDL receptor mRNA abundance. The level of intestinal apoB mRNA was about 30% higher in animals fed the moderate dietary cholesterol concentration. Earlier studies have shown that more cholesterol is transported in chylomicrons from the intestine when dietary cholesterol levels are higher, and the increased intestinal apoB mRNA abundance may reflect increased intestinal cholesteryl transport and chylomicron apoB48 production.

Plasma low density lipoproteins (LDL) are the primary transport form of cholesterol in the circulation, and elevated LDL cholesterol concentrations have been positively correlated with an increased risk of coronary heart disease (1, 2). Increased LDL cholesterol concentrations occur when cholesterol and saturated fat are consumed in the diet of human beings (3, 4) and when fed to induce atherosclerosis in nonhuman primates (5, 6). However, isocaloric substitution of polyunsaturated fat for saturated fat in the diet has been associated with reduced LDL cholesterol concentrations (3, 7) and decreased severity of atherosclerosis (8, 9).

When elevated LDL cholesterol concentrations are found, increased production and/or decreased catabolism of the LDL particles must occur, although the specific effects of dietary cholesterol and saturated fat remain to be demonstrated. Turnover studies have suggested that in some individuals, elevation of plasma LDL concentrations may occur as a result of increased production of apoB100-containing LDL (10–13). However, in most of these cases, reduced clearance by hepatic LDL receptors of LDL precursors could also occur yielding a more efficient conversion of apoB100 particles into LDL through the delipidation cascade. In this case, the increased production of LDL would not require enhanced hepatic production of apoB100.

The major pathway of LDL clearance is through the LDL receptor which is a highly regulated pathway that has been shown to be down-regulated by dietary cholesterol in experimental animals (14, 15) and in humans (4, 16). The principal tissue for clearance of LDL by LDL receptors is the liver. It has been demonstrated in experimental animals that as much as 75% of LDL catabolism occurs in the liver (17, 18). In the most dramatic example of the role of the liver in LDL clearance, transplant of a normal liver into a familial hypercholesterolemic patient markedly reduced plasma LDL concentrations (19). In hamsters, the role of diet in LDL receptor regulation has been studied in vivo (20). Dietary cholesterol-induced down-regulation of LDL cholesterol concentrations occurred when coconut oil, a saturated fat, was fed; down-regulation of LDL receptor activity was less efficient in animals fed polyunsaturated fat and LDL concentrations were lower (20). Together, the data above strongly support a major role for LDL catabolism via the LDL receptor as a primary regulator of plasma LDL concentrations. In some cases higher LDL concentrations may result from increased LDL produc-

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1 The abbreviations used are: LDL, low density lipoprotein; SDS, sodium dodecyl sulfate.
tion as well, although this would not necessarily be due to increased apoB production by the liver.

The African green monkey is a valuable model for study of the mechanisms of dietary cholesterol-induced atherosclerosis because the modest dietary responsiveness of this species approximates that of human beings at increased risk of coronary heart disease. Dietary cholesterol-induced elevations in LDL cholesterol concentrations have been documented, and this increase in LDL is moderated by polyunsaturated fat (8, 9). Results from liver perfusion studies have indicated that alterations in dietary cholesterol and fat do not alter hepatic production of apoB100-containing particles (21-23), suggesting that dietary effects on LDL concentration are not mediated through alterations in the production of apoB100. The present studies were designed to assess whether nutritional effects of apoB mRNA abundance could be detected that parallel the liver perfusion data on apoB-containing particle accumulation and to determine concomitant effects on intestinal apoB mRNA abundance. Hepatic LDL receptor mRNA levels were measured to learn if dietary cholesterol and fat would exert effects at this level. No dietary effects on hepatic apoB mRNA were found while hepatic LDL receptor mRNA was reduced in concentration in animals fed the higher dietary cholesterol concentration. These findings support the idea that diet-induced increases in LDL concentration are more likely a result of decreased LDL catabolism than of increased production of apoB100 particles. Hepatic LDL receptor mRNA was similar in abundance in polyunsaturated fat and saturated-fat-fed animals, suggesting that the difference in plasma LDL concentration between these groups is not mediated via effects on LDL receptor mRNA abundance. The level of intestinal apoB mRNA was elevated in the animals fed the higher dietary cholesterol concentration, perhaps resulting from the increased demand to transport absorbed cholesterol in these animals.

**Materials and Methods**

**Animals and Diets—**Adult male African monkeys (Cercopithecus aethiops) were fed for 5 years diets containing 40% of calories as either saturated fat (P/S = 0.3) or polyunsaturated fat (P/S = 2.2) and cholesterol at either 0.03 mg kcal or 0.8 mg kcal (for diet compositions, see Ref. 24). Tissues were collected at necropsy, frozen in liquid N2, and stored at -70°C.

A separate group of male and female animals was used for studies of apoB production during liver perfusion. These animals were fed at least 2 years the same high cholesterol diets as those used for mRNA measurements. At the time of necropsy, the livers were isolated and perfused with a chemically defined medium as described previously (21). A 90-min washout period to flush the liver of plasma lipoproteins was followed by a 2-h perfusion period during which aliquots of perfusate were collected for measurements of apoB concentration.

**Tissue Labeling and Immunoprecipitation of ApoB—**Metabolic labeling of liver and small intestinal mucosal cell scrapings with [3H]leucine in short-term (1 h) culture was as described (35). Muscular cells were scraped from the luminal surface of the upper ⅓ of the small intestine (duodenum and jejunum). Immunoprecipitation of radiolabeled tissue extracts was with goat anti-monkey LDL apoB100 as primary antibody and sheep anti-goat γ-globulin as second antibody. Immunoprecipitates were analyzed by SDS, 5% polyacrylamide slab gel electrophoresis followed by scintillation counting of solubilized gel slices (25). Monkey apoB100 from plasma LDL and apoB48 from lymph chylomicrons were run in adjacent lanes as standards.

**Isolation and Characterization of Cellular RNA—**Total RNA from monkey tissue or from HepG2 cells was prepared by the method of Cox (26) with modifications (27). Integrity of apoB mRNA was determined by electrophoresis of formaldehyde-treated RNA on 0.7% agarose gels (28) followed by transfer to nitrocellulose and hybridization (29) with the uniformly labeled apoB cDNA probe described below. Degradation of apoB100 mRNA was noted when RNA was isolated by centrifugation through CsCl but not when isolated by ethanol precipitation from guanidine isothiocyanate-solubilized tissue (30).

**Probes and Solution Hybridization Assay—**The human apoB100 5′ cDNA probe corresponding to nucleotides +713 to +879 (+1 being the A of the translation start codon of the human apoB100 cDNA) was prepared from a fragment of pBS 9 (31) (nucleotides +88 to +879) by HincII and Mlu13mp10. After DNA synthesis and digestion with BstEII, single-stranded probe was isolated as described (32). The apoB100 3′ cDNA probe corresponding to nucleotides +12,898 to +13,106 was prepared from PsIl-HindIII fragment of pBS 9 (33) (nucleotides +12,324 to +13,106) subcloned in M13mp8 and isolated by digestion with AvaII after DNA synthesis. Human LDL receptor cDNA corresponding to nucleotides +1,372 to +1,534 was prepared from HindII-BamHI fragment of pLDR2 (34) inserted into HindII-BamHI cut M13mp8. Single-stranded probe was prepared by digestion with BamHI after DNA synthesis. Each of the human cDNA probes was examined for homology with monkey mRNA by S1 nuclease protection analysis (35).

**Total Plasma Cholesterol, LDL Cholesterol, and apoB Determinations—**Total plasma cholesterol and high density lipoprotein cholesterol concentrations were determined by standardized, automated procedure (36). LDL was chromatographically separated and quantitated as described (37). Plasma and perfusate apoB concentration was determined with a two-site immunoenzymetric assay with enzyme-linked immunosorbent assay, performed essentially as described by us for apoA-I (38).

Briefly, apoB isolated from plasma LDL of cynomolagus monkeys (39) was used to immunize goats. IgG were isolated from the antisera by ammonium sulfate precipitation, and apoB antibodies were isolated by affinity chromatography on columns of apoB immobilized on agarose beads. An aliquot of the apoB antibodies was conjugated with horseradish peroxidase (40), and other aliquots were diluted for coating the microtiter plates with 500 ng/well.

**Results**

Table I shows the changes in total plasma cholesterol, LDL cholesterol, and apoB concentrations in response to the cholesterol level, and the type of dietary fat in male African green monkeys. Each was significantly higher in the high cholesterol diet groups compared with the low cholesterol diet groups. The means for total plasma cholesterol and LDL cholesterol concentrations were increased approximately 75 and 125%, respectively, while that for plasma apoB concentration was increased 45%. The type of dietary fat also influenced these parameters with the values being 10-30% higher with saturated fat as compared with polyunsaturated fat. The effect of the type of fat was statistically significant for total plasma cholesterol but just missed significance at the 5% confidence interval for apoB concentrations (p = 0.07) and (p = 0.09) for LDL cholesterol (Table I). A similar effect of dietary fat was observed at both dietary cholesterol levels. Animals fed polyunsaturated fat had lower high density lipoprotein cholesterol concentrations, and animals fed the higher cholesterol level had higher HDL cholesterol concentrations.

**Tissue Specificity of ApoB100 and ApoB48 Production—**The form of apoB synthesized by monkey liver and small intestine was determined by SDS-polyacrylamide gel electro-
ApoB mRNA in these tissues was characterized by Northern blot analysis using cDNA probes derived from either the 3' or 5' end of human apoB100 cDNA. As shown in Fig. 2, a single 14-kilobase mRNA band was detected with each probe. The amounts of apoB mRNA corresponding to the 5' and 3' ends of apoB100 mRNA were measured in each tissue by DNA-excess solution hybridization assays with the 3' end and 5' end probes. With RNA samples from seven animals, the molar ratio of 5' ends to 3' ends found in liver RNA was 0.90 ± 0.07 while the ratio in intestinal RNA was 1.08 ± 0.08 (mean ± S.E.).

ApoB mRNA Abundance and Secretion—ApoB mRNA concentrations in liver and small intestine were measured by DNA excess solution hybridization with the 3' end cDNA probe. S1 nuclease protection analysis was used to test the homology between the human 3' end apoB cDNA probe and African green monkey mRNA. As shown in Fig. 3A, the 3' end cDNA probe yielded the expected nuclease-resistant fragment after hybridization with human HepG2 RNA (lane 2). Hybridization with RNA from monkey liver (lane 3) and small intestine (lane 4) yielded fragments of the same size as seen with HepG2 RNA. The cDNA probe was not protected from nuclease digestion by yeast tRNA (lane 1). Similar results were obtained with 5' end apoB100 cDNA probe (Fig. 3B). The human LDL receptor cDNA probe (Fig. 3C) also showed the same nuclelease-resistant fragments after hybridization with human HepG2 RNA (lane 2), monkey liver RNA (lane 3), and monkey adrenal RNA (lane 4) but not hybridization with yeast tRNA (lane 1). These data show that the
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**Fig. 3.** Characterization of apoB and LDL receptor mRNA by S1 nuclease protection. A, ApoB100 3' cDNA probe was hybridized to 100 µg of RNA from yeast (lane 1), HepG2 cells (lane 2), African green monkey liver (lane 3), and African green monkey small intestine (lane 4). These samples were digested with 300 units of S1 nuclease, and analyzed by electrophoresis through a 7 M urea, 6% polyacrylamide gel. Probe incubated in the absence of S1 nuclease is shown in lane P. Lane M shows labeled MspI restriction fragments of pBR322 as size markers. B, shows the same series of RNAs studied as in A except that the apoB100 5' end probe was used. C, LDL receptor cDNA probe was hybridized to 100 µg of yeast tRNA (lane 1), HepG2 cells (lane 2), African green monkey liver (lane 3), and African green monkey adrenal (lane 4). S1 nuclease-resistant probe fragments were not present in the low molecular weight region of the gels which has not been included in this figure.

**TABLE II**
Liver and small intestine apoB mRNA abundance

| Diet                    | No. | Fat source | ApoB mRNA (pg/µg RNA) | Significance determined by two-way analysis of variance |
|-------------------------|-----|------------|-----------------------|--------------------------------------------------------|
|                         |     |            |                       |                                                        |
| Low cholesterol         | 4   | Saturated  | 168 ± 45, 75 ± 24     | Dietary fat: NS, Dietary cholesterol: NS, Interaction: NS |
|                         | 5   | Polyunsaturated | 185 ± 17, 81 ± 11 |                                                        |
| High cholesterol        | 5   | Saturated  | 181 ± 29, 119 ± 3    | Dietary fat: NS, NS, Dietary cholesterol: p < 0.03, Interaction: NS |
|                         | 5   | Polyunsaturated | 192 ± 16, 100 ± 13 |                                                        |

*NS, not significant.

human apoB and LDL receptor cDNA probes are suitable for the measurement of the corresponding monkey mRNAs by solution hybridization.

ApoB mRNA concentrations in liver and small intestine were determined for animals in each diet group. The results in Table II show that apoB100 mRNA abundance in liver was not significantly altered by dietary fat or cholesterol. In comparison, apoB48 mRNA abundance in the small intestine was increased in both high cholesterol diet groups. ApoB48 mRNA abundance in the small intestine was unaffected by the type of dietary fat.

Livers from a separate group of animals were used for perfusion to quantitate apoB secretion. The data in Fig. 4 show that the appearance of apoB in perfusate from both saturated and polyunsaturated fat-fed groups was linear throughout the 2-h perfusion and was the same for both groups. The data in Table III show the plasma LDL cholesterol concentrations and hepatic apoB secretion rates in the 17 animals used in these studies. Although all animals in each dietary fat group were fed cholesterol-enriched diets, in each case there was a range of plasma LDL cholesterol responses. The data for each dietary fat group is shown separately, and no correlation (r = -0.15) between the plasma LDL cholesterol concentration and the hepatic apoB secretion rate was apparent. By contrast, a positive correlation between the rate of hepatic cholesterol secretion and plasma LDL cholesterol concentration was found for these animals, r = 0.7, p < 0.005.

Taken together, these data suggest that dietary effects on
apoB production, as estimated by hepatic secretion and apoB mRNA abundance, are not the major determinants of plasma apoB and LDL cholesterol concentrations.

**LDL Receptor mRNA Abundance**—To estimate the effects of dietary cholesterol and fat on LDL catabolism, LDL receptor mRNA abundance in liver was measured, as shown in Table IV. The concentration of liver LDL receptor mRNA was lower by approximately 50% in the high cholesterol compared to low cholesterol diet groups. No significant difference in liver LDL receptor mRNA abundance was noted between the polyunsaturated and saturated diet groups. In contrast to these data for liver, the concentration of adrenal LDL receptor mRNA (Table IV) was as much as 10-fold higher than found in the liver and was not significantly altered by the cholesterol content or the type of fat in the diet.

Correlation analysis was performed to determine if a relationship between liver LDL receptor mRNA abundance and various plasma parameters occurs in vivo. Since individual groups were small, data from all groups were pooled. Significant inverse correlations were found between liver LDL receptor mRNA and plasma apoB100 concentration ($r = -0.52$, $p < 0.02$, $n = 19$), and with total plasma cholesterol ($r = -0.94$, $p < 0.01$, $n = 19$) as shown in Fig. 5. An inverse correlation was also noted between liver LDL receptor mRNA and plasma LDL cholesterol concentration (data not shown) ($r = -0.44$) although this correlation did not reach statistical significance. No correlation existed between LDL receptor mRNA abundance and plasma HDL cholesterol or apoA-I concentrations which were also measured in these animals.

**DISCUSSION**

In the African green monkeys of this study, as has been observed in humans, dietary cholesterol and saturated fat increased plasma total and LDL cholesterol concentrations, while dietary polyunsaturated fat moderated this cholesterol-induced increase. We were able to investigate mechanisms responsible for these dietary effects by studying the liver and intestinal concentrations of mRNA for apoB and for liver LDL receptor mRNA since these tissues were available at necropsy. For comparison, we measured the rates of apoB appearance during liver perfusion. The measurements of liver RNA (Table II) showed that apoB mRNA abundance was the same in each of the dietary groups. No effect of the type of dietary fat was found on the rate of hepatic apoB secretion (Fig. 4) and no correlation between plasma LDL cholesterol.
concentration and hepatic apoB secretion rate was found (Table III), although a good correlation between LDL cholesterol concentration and hepatic cholesterol secretion was seen. Based on the outcome of these measurements, we estimate that the diet-induced changes in plasma cholesterol concentrations are primarily due to effects on catabolism of apoB-containing lipoproteins rather than on synthesis and secretion of these lipoproteins from liver. This conclusion is the same as reached previously with liver perfusion studies where no clear dietary effects were seen on the rate of secretion of apoB-containing lipoprotein particles as measured by protein and apoB accumulation in recirculating liver perfusion (21-23). Since the apoB of plasma LDL is all apoB100 which is derived exclusively from the liver in African green monkeys (Fig. 1 and Ref. 21), and since this apoprotein is not exchangeable but remains on the particle of its secretion, these data also support the idea that diet-induced increases in the concentrations of LDL in plasma primarily result from modifications in the removal mechanisms of apoB100-containing lipoproteins.

Previous studies have shown that differences in LDL receptor mRNA abundance in rabbits can be correlated with differences in plasma total and LDL cholesterol concentrations (43, 44). In addition, in vivo studies have shown that, in parallel with plasma LDL cholesterol concentrations, LDL receptor function is decreased much more significantly in hamsters fed saturated fat and cholesterol than in those fed polyunsaturated fat and cholesterol (20). Since we have found similar effects on plasma total and LDL cholesterol concentrations in the animals of this study, it was of interest to determine whether we could demonstrate diet-induced differences in hepatic LDL receptor mRNA abundance. We found higher levels of LDL receptor mRNA in the groups fed low cholesterol concentrations (Table III). This diet-related difference is consistent with the idea that the dietary cholesterol-induced increase in plasma total and LDL cholesterol concentrations is due, at least in part, to a decrease in LDL clearance from plasma. It should be noted that the "high" dietary cholesterol concentration of our study, 0.8 mg of cholesterol/kcal or 0.4% by weight, is really moderate for experimental animal studies, e.g. Ref. 45.

On the other hand, we did not find a difference in LDL receptor mRNA abundance between the groups of animals fed polyunsaturated and saturated fat, even though plasma total and LDL cholesterol concentrations were lower in the former case. Since diet-induced apoB production differences have not been found, and in keeping with the ideas of Spady an Dietzchay (20), it is possible that the difference in LDL receptor activity in polyunsaturated fat versus saturated fat-fed animals is more related to an effect on the function of the LDL receptor protein. For example, an effect of polyunsaturated fat to increase membrane fluidity might increase the efficiency of receptor recycling and/or clustering in the coated pits. Alternatively, dietary-saturated fat could decrease receptor number by modifying translational or post-translational processing events.

We are aware of one report in baboons that has concluded that there is a dietary fat effect on LDL receptor mRNA levels (45). However, no dietary fat effect on plasma total or LDL cholesterol concentration was present in that study. LDL receptor mRNA was correlated to plasma HDL but not LDL cholesterol concentration, and the difference in LDL receptor mRNA was confined to a small subset of the animals studied. Since numerous experimental details of the baboon study, including diet compositions, were different from the present study, the reason(s) for the contrasting outcomes is not clear.

A surprising finding was that intestinal apoB mRNA abundance was increased in the animals fed the higher cholesterol concentration. Since we found that all of the apoB synthesized in intestinal cells and detected in lymph chylomicrons is apoB48 (Fig. 1 and Ref. 46), it is not likely that plasma apoB100-containing lipoprotein concentrations are increased directly because of increased intestinal apoB secretion. We have shown previously that increased dietary cholesterol results in increased chylomycin cholesterol content (47), and it is possible that more chylomycin apoB48 is secreted as well. We speculate that increased cholesterol delivery to the liver via chylomycin remnants contributes to the higher liver cholesterol concentration (48) and to the decrease in LDL receptor mRNA observed in animals fed the high cholesterol diet.

An interesting finding in these studies was in the characterization of apoB mRNA in the liver and intestine. We found (Fig. 3) that the apoB mRNA of the liver and intestine is all the same size (14 kilobases). Therefore, the apoB48 that is made in the intestine (Fig. 1) is all derived from a full length mRNA, and no evidence for a shorter mRNA was found. This is in contrast to the data of Chen et al. (49) who have shown that apoB mRNA from human intestine is 85% of a small size (7 kilobases) and only about 15% of the full length for apoB100. The difference may be related to a difference among primate species or to some other unknown factor. We have not sequenced the cDNA from the monkey to know that the signal for the stop codon for apoB48 (49) is present in the apoB mRNA of monkey intestine, but our apoB production data suggest that it is almost certainly present.

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