Of the hypolipidemic therapy on cholesterol homeostasis in freshly isolated mononuclear cells from patients with heterozygous familial hypercholesterolemia

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ABSTRACT Freshly isolated mononuclear leukocytes have been reported to show changes in cholesterol synthesis and high-affinity degradation of low-density lipoproteins (LDL) that parallel those that occur in the liver. To examine whether hypolipidemic therapy in patients with heterozygous familial hypercholesterolemia influences cholesterol homeostasis in their mononuclear cells we assessed the effects of colestipol and nicotinic acid (alone and in combination) on the rates of high-affinity 125I-labeled LDL degradation and on the rates of cholesterol and phosphatidylcholine biosynthesis by freshly isolated cells. Rates of 125I-labeled LDL degradation were lower in mononuclear cells from patients with heterozygous familial hypercholesterolemia on no medication (3.1 ng per 4 × 10^6 cells per 5 hr) than in cells from normal control subjects (6.1 ng per 4 × 10^6 cells per 5 hr) and, in the former patients, the values were not significantly affected by therapy with nicotinic acid. In contrast, freshly isolated mononuclear cells from patients receiving colestipol degraded 125I-labeled LDL at near-normal rates (5.0 ng per 4 × 10^6 cells per 5 hr). The rates of cholesterol synthesis were also higher in mononuclear cells isolated from patients treated with colestipol than in cells from untreated patients or from those receiving nicotinic acid; in contrast the rate of synthesis of phosphatidylcholine did not show any consistent changes. Similar results were obtained in a smaller number of patients studied longitudinally, in which colestipol therapy significantly increased rates of cholesterol synthesis and high-affinity degradation of 125I-labeled LDL by freshly isolated mononuclear cells. We conclude that previously observed changes in cholesterol homeostasis in the liver of patients treated with bile acid sequestrants are paralleled by similar changes in freshly isolated mononuclear cells and that these cells offer an accessible model for further studies on how diet and pharmacologic agents influence cellular cholesterol homeostasis in humans.

Optimal treatment of patients with heterozygous familial hypercholesterolemia (FH) necessitates the use of diet and hypolipidemic medication, among which the bile acid-binding resins (colestipol and cholestyramine) and nicotinic acid are the most efficacious (1, 2). Recent studies have clarified the mechanisms by which colestipol and cholestyramine decrease plasma concentrations of low-density lipoproteins (LDL) in both experimental animals and in humans (3, 4). Both drugs bind bile acids in the intestinal lumen, thereby interrupting the enterohpetic circulation and stimulating an increase in the hepatic conversion of cholesterol to bile acids. This in turn depletes the hepatic pool of cholesterol and results in two compensatory changes; an increase in the de novo biosynthesis of cholesterol (5) has been shown in humans and an increase in the number of specific high-affinity LDL receptors on hepatocyte membranes has been shown in rabbits and dogs (4, 6). The latter change also promotes an increase in the rate of catabolism of LDL (3), so that overall the concentrations of this lipoprotein in plasma are decreased. In contrast, the hypolipidemic effect of nicotinic acid is thought to result primarily from a decrease in the rate of LDL production (7) with little change in the fractional rate of catabolism of LDL.

Combined drug therapy with colestipol and nicotinic acid is the most efficacious regimen currently available for maximally decreasing the concentrations of LDL cholesterol in patients with heterozygous FH (8–11). This regimen is presumed to act by a dual mechanism that affects both the synthesis (decreased by nicotinic acid) and catabolism (increased by colestipol) of LDL.

Recent studies have suggested that changes in the rate of synthesis of cholesterol in the liver may be accompanied by parallel changes in nonhepatic tissues such as mononuclear leukocytes (12–14). To date, however, no data are available on whether such changes occur in freshly isolated mononuclear leukocytes from patients with heterozygous FH and whether these changes are paralleled by differences in the rates of uptake and degradation of LDL. In the present study, we have examined the effects of hypolipidemic therapy with diet, colestipol, and nicotinic acid (alone and in combination) on the rates of synthesis of cholesterol and phospholipids as well as the rates of high-affinity degradation of 125I-labeled LDL by freshly isolated mononuclear leukocytes from patients with well-characterized heterozygous FH.

MATERIALS AND METHODS

Patients. Blood samples were obtained after an overnight fast from normolipidemic controls and from adult patients with heterozygous FH who were attending the Lipid Disorders Clinic or Clinical Research Center at the Oregon Health Sciences University. Informed consent was obtained in all cases and the protocol was approved by the Human Research Committee of the Oregon Health Sciences University. The diagnosis of heterozygous FH was based on the presence of at least three of the four following criteria in each patient: (i) primary hypercholesterolemia (>300 mg/dl) in the index patient and at least one other family member, with an absence of multiple phenotypes in other family members, (ii) an inheritance pattern consistent with autosomal dominant, (iii) the presence of xanthomas in the index case or at least one first-degree relative, (iv) other.

Abbreviations: LDL, low-density lipoprotein; FH, familial hypercholesterolemia.
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and (io) the presence of hypercholesterolemia in children within the family.

Patients on estrogens or steroid hormones and patients with disorders (such as diabetes) that are known to affect lipid metabolism were excluded from the study. Blood samples were obtained from patients on one or more of four treatment regimens in a cross-sectional study as well as from a smaller number of patients who were studied longitudinally. The four treatment groups were as follows: diet only, colestipol (20–30 g per day), nicotinic acid (3–8 g per day), or colestipol and nicotinic acid in combined drug therapy. The patients in each group were maintained on a low cholesterol (<300 mg per day) diet, in which fats composed <30% of calories (P:S ratio, 0.8–1.2), or the indicated drug therapy for at least 4 weeks prior to the mononuclear cell studies. Plasma lipid values were stable and all studies were therefore performed under steady-state conditions.

Materials. Penicillin, streptomycin, and RPMI 1640 with glutamine were purchased from Gibco; [2-14C]acetate, [methyl-3H]choline, and 125I were purchased from Amersham. Culture flasks were purchased from Falcon. All other chemicals and reagents were analytical grade.

Laboratory Methods. Blood for plasma lipid and lipoprotein determinations was drawn into tubes containing EDTA (1 mg/ml) as anticoagulant. Plasma was separated by centrifugation and total cholesterol and triglycerides were determined with the Autoanalyzer II (15). Lipoproteins were separated by the methods described (15) using heparin/Mn2+. Where indicated, major lipid classes and individual phospholipids were separated by TLC on silica gel H. Phospholipid phosphorus was assayed in aliquots of the total lipid extract (16) by the method of Bartlett (17). LDL was isolated by ultracentrifugation between the densities of 1.019 and 1.060 g/ml. The LDL thus obtained was washed one time by refloation at a density of 1.060 g/ml and was then exhaustively dialyzed against 5 mM sodium phosphate/0.9% sodium chloride, pH 7.4. Protein was measured by the method of Hartree (18) and free and esterified cholesterol were determined by gas/liquid chromatography. Purified LDL was radiolabeled using the iodine monochloride method of McFarlane (19) as modified by Langer et al. (20). Free 125I was removed by gel filtration on a Sephadex G50 column after which the 125I-labeled LDL was dialyzed exhaustively against 12–15 liters of 5 mM sodium phosphate/0.9% sodium chloride, pH 7.4 and then filtered through a 0.45-μM Millipore filter. The specific activity of the 125I-labeled LDL used in these experiments was between 250 and 500 dpm per ng of protein.

Mononuclear Cell Studies. Mononuclear cells were isolated from 40–50 ml of heparinized whole blood by centrifugation on a Ficoll-Paque density gradient (21). The mononuclear cell layers were washed three times in RPMI 1640 and counted in a Coulter Counter. Viability was assayed by trypan blue exclusion and was consistently >95%. The relative numbers of lymphocytes, monocytes, and granulocytes were determined by differential counts of Wright-stained smears obtained from the mononuclear cell preparation. These consistently disclosed <2% granulocytes and 7–12% monocytes in the cell preparations. This distribution of cells was similar in the normal and FH patients and was not influenced by drug therapy.

Synthesis of cholesterol and phosphatidylcholine was measured by incubating aliquots of 6–12 × 106 cells for 5 hr at 37°C in autologous serum buffered to pH 7.4 and containing 3 mM [methyl-3H]choline and 1.5 mM [2-14C]acetate. Previous experiments have shown that the rates of incorporation of both [2-14C]acetate and [methyl-3H]choline proceeded linearly for incubation periods ranging from 1 to 6 hr and that the chosen substrate concentrations were not rate limiting. At the end of the incubation period, lipids were extracted by the addition of chloroform/methanol, 1:1 (vol/vol). Lipids were partitioned into chloroform after addition of water to the chloroform/methanol extract, and aliquots were taken for sterol and phospholipid assay. For the determination of [2-14C]acetate incorporation into sterols, an aliquot of the chloroform extract was saponified with 1 M KOH in ethanol for 1 hr at 37°C. Sterols were extracted with 3–4 ml of hexane and the nonsaponifiable lipids were further separated by TLC on silica gel G using the solvent system ethyl acetate/hexane/diethyl ether, 10:40:10 (vol/vol). The TLC plate was removed, allowed to dry briefly, and placed in a second solvent system of heptane/diethyl ether, 95:20 (vol/vol). The cholesterol-containing band was scraped from the plate and assayed for radioactivity using a Packard TriCarb liquid scintillation counter. In initial experiments the aqueous extract was acidified, free fatty acids were extracted with hexane, and an aliquot was taken for the determination of radioactivity. No differences were observed in the rates of [2-14C]acetate incorporation into fatty acids by mononuclear cells isolated from patients on different drug regimens, and for this reason this procedure was not routinely undertaken. Phosphatidylcholine was separated by TLC of the chloroform extract on silica gel H plates using the solvent system chloroform/methanol/glacial acetic acid/water, 75:45:12:6 (vol/vol). The degradation of 125I-labeled LDL by freshly isolated mononuclear cells was measured using methods similar to those described by Bilheimer et al. (22). Aliquots of 2–4 × 104 mononuclear cells isolated as described above were incubated for 5 hr in the presence of 125I-labeled LDL (25 μg/ml) in RPMI 1640 medium supplemented with 30% pooled human lipoprotein-deficient serum. Parallel incubations contained 125I-labeled LDL (25 μg/ml) and unlabeled LDL (500 μg/ml). Blank values were obtained by parallel incubations containing 125I-labeled LDL but no cells. Total degradation of 125I-labeled LDL was defined as the amount of trilinchoeratic acid-soluble noniodide radioactivity present in the medium after correction for the blank. Nonspecific degradation of 125I-labeled LDL was measured as the amount of trilinchoeratic acid-soluble noniodide radioactivity present in the incubations containing unlabeled LDL (500 μg/ml) and specific degradation of 125I-labeled LDL as the difference between total and nonspecific degradation. Preliminary experiments indicated that high-affinity degradation of 125I-labeled LDL was linearly related to the number of mononuclear cells in the incubation medium within a cell density of 1–20 × 106 cells per ml and that after a 2-hr lag, high-affinity degradation of 125I-labeled LDL proceeded linearly for at least 6 hr. A concentration curve indicated that degradation of 125I-labeled LDL by freshly isolated mononuclear cells proceeded linearly up to a concentration of 25 μg/ml, above which the rate of degradation proceeded at a decreased but still linear rate. This relationship is indicative of a high-affinity component of LDL degradation with saturation at a concentration of ~25 μg/ml. These results are consistent with those of other investigators using various human cell lines. Because of the low rates of 125I-labeled LDL degradation that occur in freshly isolated mononuclear cells, assays were routinely carried out using an 125I-labeled LDL concentration of 25 μg/ml. Statistical analyses were by two-tailed or paired t tests.

RESULTS

The plasma lipid values for each of the four treatment groups of patients with heterozygous FH as well as those in the normal controls are shown in Table 1. In the FH patients on colestipol, plasma cholesterol values were 18.1% lower than in the patients on no medication. The addition of nicotinic acid increased the magnitude of this difference to 25.6%. In those patients re-
receiving single-drug therapy with nicotinic acid, total cholesterol values were 15.5% lower than in the FH patients on no hypolipidemic drugs. These changes in total cholesterol were accompanied by parallel but greater differences in the concentrations of LDL cholesterol (Table 1).

The rates of high-affinity degradation of \(^{125}\)I-labeled LDL by freshly isolated mononuclear cells from the patients with FH on diet therapy only (Fig. 1) were significantly lower than those of normal subjects (3.1 vs. 6.1 ng per 4 \(\times\) 10\(^6\) cells per 5 hr). This result is in agreement with the earlier studies of Bilheimer et al. (22) and is consistent with the known metabolic abnormality in patients with familial hypercholesterolemia. In making this comparison it should be emphasized that the control subjects were studied on an ad lib diet, whereas the FH patients were habituated to a diet low in cholesterol and saturated fat. However, cholesterol feeding has been shown to decrease rates of \(^{125}\)I-labeled LDL degradation by freshly isolated mononuclear cells (14) from normolipidemic humans, and this suggests that differences in cholesterol intake between the control and FH patients should tend to decrease rather than increase the differences in high-affinity degradation of \(^{125}\)I-labeled LDL observed between them. Freshly isolated mononuclear cells from FH patients treated with colestipol had rates of high-affinity degradation of \(^{125}\)I-labeled LDL that were significantly higher (5.0 ng per 4 \(\times\) 10\(^6\) cells per 5 hr) than those observed in mononuclear cells from FH patients on diet only. Significantly increased rates of high-affinity degradation of \(^{125}\)I-labeled LDL were not, however, seen in freshly isolated mononuclear cells from patients treated with nicotinic acid (3.5 ng per 4 \(\times\) 10\(^6\) cells per 5 hr) despite the fact that the concentrations of LDL cholesterol in these patients were similar to those of the patients treated with colestipol. Rates of specific degradation of \(^{125}\)I-labeled LDL by freshly isolated mononuclear cells from patients on combined therapy with colestipol and nicotinic acid (3.9 ng per 4 \(\times\) 10\(^6\) cells per 5 hr) were intermediate between those in patients on single-drug therapy with nicotinic acid and colestipol but were not statistically different from patients on diet therapy alone.

The studies of LDL degradation in freshly isolated mononuclear cells were complemented by parallel studies in which the rates of synthesis of cholesterol and phosphatidylcholine were examined (Fig. 2 and Table 2). The rates of cholesterol synthesis from \([2-\text{C}]\)acetate were significantly \((P < 0.05)\) higher in freshly isolated mononuclear cells from FH patients treated with colestipol than in patients on diet only. In contrast, the

Table 1. Plasma lipids in the study population

<table>
<thead>
<tr>
<th>Plasma Chol</th>
<th>LDL Chol</th>
<th>HDL Chol</th>
<th>Plasma triglyceride</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet only</td>
<td>381 ± 6</td>
<td>308 ± 13</td>
<td>47 ± 2</td>
<td>138 ± 11</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>322* ± 33</td>
<td>226* ± 25</td>
<td>65 ± 4</td>
<td>117 ± 38</td>
</tr>
<tr>
<td>Colestipol</td>
<td>312* ± 12</td>
<td>230* ± 11</td>
<td>50 ± 2</td>
<td>108 ± 9</td>
</tr>
<tr>
<td>Colestipol/ nicotinic acid</td>
<td>272** ± 13</td>
<td>192* ± 12</td>
<td>61 ± 5</td>
<td>99 ± 13</td>
</tr>
<tr>
<td>Normal controls</td>
<td>184 ± 4</td>
<td>113 ± 7</td>
<td>57 ± 2</td>
<td>69 ± 8</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (mg/dl) for the indicated number of patients \((n)\) in each group. Chol, cholesterol.

\(*P < 0.001\) vs. diet only treatment group.

\(1P < 0.05\) vs. colestipol treatment.

Fig. 1. Effect of hypolipidemic therapy on high-affinity degradation of \(^{125}\)I-labeled LDL by freshly isolated mononuclear cells from patients with heterozygous FH. Freshly isolated mononuclear cells (4 \(\times\) 10\(^6\) cells) were incubated with \(^{125}\)I-labeled LDL (25 mg/ml) in the presence and absence of unlabeled LDL (500 mg/ml) and high-affinity degradation was determined. Triplicate incubations were carried out for each assay. n, Number of patients in each group; *, \(P < 0.05\) vs. patients with FH on diet treatment only; **, familial hypercholesterolemia; _, normal control subjects.

Fig. 2. Effect of hypolipidemic therapy on cholesterol synthesis from \([2-\text{C}]\)acetate by freshly isolated mononuclear cells from patients with heterozygous FH. n, Number of patients in each group; *, \(P < 0.05\) vs. FH patients on diet treatment only; **, familial hypercholesterolemia; _, normal control subjects.

Table 2. Effect of hypolipidemic therapy on cholesterol and phosphatidylcholine synthesis by freshly isolated mononuclear cells

<table>
<thead>
<tr>
<th>Cholesterol synthesis</th>
<th>Ac -&gt; Chol, pmol per 10(^7) cells per 5 hr</th>
<th>Cho -&gt; PtdCho, pmol per 10(^7) cells per 5 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>FH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet only</td>
<td>77.0 ± 8.3 (27)</td>
<td>6.0 ± 2.3 (4)</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>56.6 ± 14.4 (6)</td>
<td></td>
</tr>
<tr>
<td>Colestipol</td>
<td>108.9 ± 13.2* (12)</td>
<td>4.6 ± 1.1 (7)</td>
</tr>
<tr>
<td>Colestipol/ nicotinic acid</td>
<td>80.0 ± 8.8 (13)</td>
<td>3.7 ± 0.6 (6)</td>
</tr>
<tr>
<td>Normal controls</td>
<td>82.8 ± 6.8 (26)</td>
<td>2.9 ± 0.9 (7)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of duplicate determinations from patients on the indicated drug regimen. Values in parentheses indicate the number of patients in each group. Ac, acetate; Chol, cholesterol; Cho, choline; PtdCho, phosphatidylcholine.

\(*P < 0.05\) vs. diet only treatment group.
rates of cholesterol synthesis by freshly isolated mononuclear cells from FH patients receiving niacinic acid as single-drug therapy were similar to those of patients on diet therapy alone (Fig. 2). The rates of incorporation of [methyl-3H]choline into phosphatidylcholine by freshly isolated mononuclear cells from FH patients on various treatment regimens were not different (Table 2) and suggest that the stimulatory effects of colestipol on LDL degradation and cholesterol synthesis were not mirrored by parallel increases in phosphatidylcholine biosynthesis. Longitudinal studies in a smaller number of patients with heterozygous FH have confirmed the stimulatory effects of colestipol therapy on both cholesterol synthesis and rates of high-affinity degradation of 125I-labeled LDL by freshly isolated mononuclear cells (Table 3). As in the cross-sectional study, significant differences were found in the rates of cholesterol synthesis (46 ± 11 pmol per 107 cells per 5 hr on diet only vs. 85 ± 8 pmol per 107 cells per 5 hr on diet and colestipol) and high-affinity degradation of 125I-labeled LDL (2.3 ± 0.4 ng per 4 × 107 cells per 5 hr vs. 3.8 ± 0.4 ng per 4 × 107 cells per 5 hr) in the same patients studied sequentially under steady-state conditions on diet treatment only as compared to diet and colestipol.

**DISCUSSION**

Recent studies have suggested that freshly isolated mononuclear leukocytes respond to many of the factors known to affect cholesterol biosynthesis in the liver in vitro or in cultured cell lines in vitro. Young and Rodwell (12) documented parallel changes in the activity of hydroxymethylglutaryl-CoA reductase from freshly isolated leukocytes and hepatic tissue from rats fed cholesterol (a decrease in hydroxymethylglutaryl-CoA reductase) or cholestyramine (an increase in enzyme activity). McNamara et al. (13) have shown that this may also be true for freshly isolated human mononuclear cells. Rates of cholesterol biosynthesis in mononuclear cells from a heterogeneous group of patients with hyperlipidemia were found to be 2 times higher when obtained from subjects receiving cholestyramine than in cells from control subjects or other hyperlipidemic patients treated with clofibrate. These changes parallel the increase in cholesterol synthesis previously observed in the liver of patients treated with cholestyramine (23). The feeding of cholesterol to human volunteers has been reported to result in parallel decreases in the activity of hydroxymethylglutaryl-CoA reductase in and LDL receptor activity in freshly isolated mononuclear cells (14). Similarly, treatment of diabetics with insulin leads to an increase in 125I-labeled LDL degradation by their freshly isolated mononuclear cells (24). Overall, these studies suggest that comparative changes in cholesterol synthesis and in the rates of high-affinity degradation of LDL by freshly isolated mononuclear leukocytes from patients on different diet and drug regimens may parallel those that occur in the liver. The results of the present study provide evidence that hypolipidemic therapy with colestipol but not with nicotinic acid leads to an increase in the rates of cholesterol synthesis and high-affinity LDL degradation by freshly isolated mononuclear cells from patients with heterozygous FH.

The mechanism(s) by which bile acid sequestrants, which are not absorbed, may influence the rates of cholesterol synthesis and high-affinity LDL degradation in mononuclear cells is unknown. In the present study and in that of McNamara et al. (13) no correlation was found between the rates of sterol synthesis in freshly isolated mononuclear cells and the concentrations of LDL in plasma. Studies in other model systems, however, suggest that decreases in the cellular concentration of cholesterol (25, 26), or possibly an increase in the sphingomyelin content (27), result in compensatory increases in cholesterol synthesis and usually, but not invariably (27), the number of LDL receptors. Cholesterol homeostasis in freshly isolated mononuclear cells may potentially be influenced by several factors including variations in the composition of LDL (28) or changes in the distribution of LDL within different subfractions (29, 30), both of which may be influenced by the residence time of LDL in plasma as well as by variations in the concentrations of several hormones known to influence lipid metabolism.

Several investigators have reported differences in the composition of LDL isolated from the plasma of patients with familial hypercholesterolemia as compared with those of normolipidemic control subjects (31–33). These differences include an increase in the cholesterol/phospholipid ratio, an increase in the proportion of cholesterol esters, and an abnormally low lecithin/sphingomyelin ratio. These acquired changes, which are believed to reflect decreases in the fractional rate of catabolism of LDL, are more pronounced in patients with homozygous FH and can be partially ameliorated by plasma exchange therapy or by treatment with colestipol or cholestyramine (31). Although increases in the cholesterol ester and sphingomyelin content of LDL have been reported to decrease its binding affinity to the high-affinity LDL receptors on cultured human fibroblasts (34), an earlier study (35) showed no difference in the rates of cellular metabolism of LDL isolated from the plasma of normal subjects as compared with that from patients with homozygous FH. Thus, it is unclear whether the higher rates of high-affinity LDL degradation and the increases in cholesterol biosynthesis by freshly isolated mononuclear cells from patients treated with colestipol, which were observed in the present study, are related to changes in LDL composition that result from an increased fractional catabolic rate of this lipoprotein.

In summary, our results provide evidence that the rates of cholesterol biosynthesis and high-affinity degradation of LDL by freshly isolated mononuclear cells from patients with heterozygous FH can be stimulated by treatment with colestipol and, therefore, show changes that parallel those known to occur in the liver. These observations also suggest that freshly isolated mononuclear cells are not an appropriate tool with which to biochemically diagnose heterozygous FH if the cells are taken from a patient on certain hypolipidemic medication, specifically the bile acid sequestrants. Because of their accessibility, freshly isolated mononuclear cells provide a useful model system in which the effects of hypolipidemic medications or different dietary constituents on cellular cholesterol homeostasis can be evaluated in humans.
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