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Human noninsulin-dependent diabetes: Identification of a defect in plasma cholesterol transport normalized *in vivo* by insulin and *in vitro* by selective immunoadsorption of apolipoprotein E

(lecithin:cholesterol acyltransferase/cholesteryl ester transfer)

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ABSTRACT **Plasma cholesterol metabolism in patients with poorly controlled noninsulin-dependent diabetes was characterized by inhibition of cholesterol net transport between cultured cells (fibroblasts) and plasma, inhibition of cholesterol esterification, and inhibition of cholesteryl ester transfer to low and very low density lipoproteins, relative to a normal control group. Plasma from these patients also contained a 2-fold higher level of apolipoprotein E (apo E). Effective control of hyperglycemia with insulin normalized both the parameters of plasma cholesterol metabolism and plasma levels of apo E. Removal of apo E by immunoaffinity chromatography normalized cell-to-plasma cholesterol transport but was without effect on the rate of cholesterol esterification or of cholesteryl ester transfer. These findings suggest that an inhibition in the chain of reactions by which cellular cholesterol is transferred in esterified form to low and very low density lipoproteins is associated with the appearance of an apo E-dependent "shunt" pathway, returning cholesterol from plasma back to the cells and so nullifying the normal cell-to-plasma transport pathway.**

The excess of atherosclerotic vascular disease associated with human diabetes often has been attributed to abnormalities of plasma lipoprotein composition (1). Increased levels of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) and a decreased concentration of high density lipoprotein (HDL) have most frequently been reported (2–6). However, diabetic subjects are not necessarily hypercholesterolemic, and there has been little obvious connection in metabolic terms between the lipoprotein abnormalities in diabetes and in the genetic hypercholesterolemic syndromes (such as familial hypercholesterolemia and dysbetalipoproteinemia), which are also associated with increased incidence of atherosclerosis (7, 8).

Although abnormalities of lipoprotein composition and concentration in all these syndromes have been analyzed extensively, there has been little investigation of the activity of the enzymes and lipid transport factors of cholesterol metabolism that, in considerable part, determine lipoprotein cholesterol composition by their effects on the direction and magnitude of free and ester cholesterol fluxes between plasma lipoproteins and peripheral cells. The most important of these parameters appear to be those determining the transport of cholesterol from cells to plasma, the esterification of cholesterol, and the transfer of cholesteryl ester between the major plasma lipoprotein classes. When fibroblasts are incubated in normal plasma, cholesterol leaving cell membranes becomes complexed with a specific cholesterol carrier lipoprotein (9). This binds to lecithin: cholesterol acyltransferase (LCATase) and apolipoprotein (apo) D (10), and this complex catalyzes both the esterification of the cholesterol and the transfer of the esters formed to acceptor lipoproteins, mainly the LDL and VLDL fractions (11).

In this research the activities of these reactions have been determined in the plasma of patients with noninsulin-dependent diabetes mellitus who were untreated or poorly controlled with either insulin or oral hypoglycemic agents. The results indicate that a characteristic syndrome of abnormalities of cholesterol transport and cholesteryl ester metabolism exists in patients with poorly controlled noninsulin-dependent diabetes, which can be rapidly normalized when hyperglycemia is well controlled with insulin.

MATERIALS AND METHODS

Patients selected for this study had fasting plasma glucose concentrations of >140 mg/dl on repeated testing and satisfied recently developed criteria for diagnosis of noninsulin-dependent diabetes (12). The study population consisted of six patients with untreated noninsulin-dependent diabetes, six patients who remained hyperglycemic (>200 mg/dl) on maximal sulfonylurea (glipizide) treatment, six insulin-treated patients whose diabetes was poorly controlled, and eight nondiabetic normal control subjects. The clinical characteristics of these groups (Table 1) demonstrate that the age and degree of obesity were comparable in all groups. The untreated patients were admitted to the Stanford Clinical Research Center and their hyperglycemia was controlled by intravenous insulin administration. These patients were restudied after 1 wk in the Center and again after 10 wk of intensive diabetic control as outpatients with multiple daily injections of insulin. Sulfonylurea-treated patients were also admitted to the Clinical Research Center for 1 wk. They were maintained on their usual treatment program and studied at the beginning and end of this period. Their plasma glucose levels were stable during this interval. All subjects were fasted overnight before blood donation. Blood was drawn into 1/20 vol of 0.2 M sodium citrate (pH 7.0) and plasma was recovered by centrifugation (30 min, 1,000 × *g*, 4°C). Samples of plasma were taken for analysis of glucose, total triglyceride, and total cholesterol by automated analyzer.

Determination of Cell-to-Plasma Cholesterol Net Transport. Normal human skin fibroblasts were cultured and prelabeled with [1,2-^{3}H]cholesterol as described (9). The 6-cm dishes contained 8–10 μg of cell cholesterol when used in the experiments. Plasma freed of fibrinogen by affinity chromatography

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Abbreviations: LCATase, lecithin:cholesterol acyltransferase; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); apo, apolipoprotein(s).

Table 1. Clinical characteristics of the study population

Group	No.	Age, yr	BMI*	Glucose, mg/dl	Triglyceride, mg/dl	Cholesterol, mg/dl
Untreated diabetics	6	52 ± 5	25.3 ± 1.1	277 ± 31	150 ± 55	200 ± 33
Uncontrolled diabetics						
Treated with oral agents	6	61 ± 3	26.7 ± 0.9	260 ± 14	189 ± 125	163 ± 27
Treated with insulin	6	51 ± 7	25.9 ± 1.2	271 ± 13	171 ± 51	188 ± 41
Normal controls	8	47 ± 11	24.5 ± 2.6	78 ± 4	54 ± 25	182 ± 16

* BMI (body mass index) = weight/(height)^2 as (kg × 10^4)/cm^2. In the previously untreated diabetic group, fasting plasma glucose levels were 105 ± 15 mg/dl after 1 wk and 103 ± 13 mg/dl after 10 wk of rigorous control. Values are means ± SD.

(9) (which retained the whole of plasma cholesterol and apoproteins) was diluted with phosphate-buffered saline to 1.2% (vol/vol). Under these conditions about one-half of free cholesterol demand for the LCATase reaction in normal plasma is met by cholesterol transport from the cells. Pentuplicate empty dishes, and the same number of dishes containing cells, were used for each determination of cholesterol transport. Three milliliters of medium was added to each dish; 1 ml was removed for analysis of free and esterified cholesterol (13) and the remainder was incubated for 60 min at 37°C, when a further 1-ml sample was taken for analysis. The initial and final cholesterol radioactivity of medium in the dishes was determined by liquid scintillation spectrometry. Cell cholesterol specific activity was calculated from radioactivity and cholesterol mass. Because the specific activity of cholesterol efflux did not differ significantly from that of the cells (9), efflux was calculated directly from the rate of appearance of radioactivity in the medium. Because cholesterol esterification was identical in the presence or absence of fibroblasts (9), cholesterol net mass transport from cells to medium could be determined as the difference between the decrease in free cholesterol mass during incubation in the presence and absence of cells. When net transport is from cells to medium, this function has a positive sign; if there is net uptake of cholesterol from medium to cells, it has a negative sign. Cholesteryl ester net transport was determined analogously, from cholesteryl ester medium increments under these conditions.

Determination of Cholesterol Esterification Rate in Plasma. LCATase activity was determined in terms of the rate of decrease in plasma free cholesterol mass in the course of incubation at 37°C. The plasma was diluted 5-fold into 0.15 M NaCl containing 1 mM Na_2EDTA and 10 mM Tris·HCl (pH 7.4). Pentuplicate initial 100-μl samples were taken for the enzymatic determination of free cholesterol (13). The diluted plasma was incubated for 60–120 min and pentuplicate samples were taken as previously for analysis. Decrease in free cholesterol was linear with time over the incubation period. This decrease was inhibited >95% by 1.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Nbs_2), an inhibitor of LCATase (14).

Determination of Cholesteryl Ester Transfer Rate in Plasma. When LCATase was inhibited with Nbs_2, cholesteryl ester transfer to LDL and VLDL was not decreased (11). Accordingly, transfer activity was determined as the rate of decrease of HDL lipoprotein cholesteryl ester during incubation at 37°C. Plasma containing Na_2EDTA and Tris·HCl, as described, was brought to 1.5 mM with Nbs_2. VLDL and LDL were precipitated from an aliquot of the plasma with heparin and $MnCl_2$ (11) at final concentrations of heparin at 100 units/ml and 0.1 M $MnCl_2$. The remaining plasma was then incubated for 60–120 min and portions were taken for precipitation with heparin/$MnCl_2$. The mass of ester cholesterol in the supernatant solution was determined enzymatically and the rate of cholesteryl ester transfer was determined as a function of time.

Determination of Plasma Lipoprotein apo. The levels of apo A-I, A-II, B, D, and E in diabetic and control plasma were determined by specific radial immunodiffusion assay, as described (9, 10, 15). Antibodies were obtained by injection of the corresponding pure antigens into New Zealand White rabbits, and the antibodies raised were purified from antibody-containing plasma by chromatography on the corresponding antigen immobilized on Sepharose-CNBr (16). The specific antibodies were released with 3 M NaSCN (pH 7.0) and then dialyzed against 0.15 M NaCl. For immunoaffinity chromatography, purified antibodies to individual apo were coupled to Sepharose-CNBr. Plasma was depleted of the corresponding antigen by passing it through columns (1 × 10 cm) of immobilized antibodies equilibrated with 0.15 M NaCl/1 mM Na_2EDTA, pH 7.0. Fractions containing detectable protein were pooled and utilized in the cell incubation experiments described above. The complete removal of the antigen was confirmed by radial immunodiffusion assay of the eluate pool under denaturing conditions where total plasma antigen was expressed (10).

RESULTS

Cholesterol Net Transport Rates in Diabetic and Normal Plasma. In healthy subjects free cholesterol net transport is in the direction of cells to medium, as cholesterol efflux is coupled to esterification by LCATase and to cholesteryl ester transfer (9, 10). As shown in Table 2, the rate of cholesterol net transport was decreased and, on average, reversed in the plasma from untreated diabetics. Consequently, the level of cholesterol in the medium decreased upon incubation to a greater extent in the presence than in the absence of fibroblasts. The rate of efflux was determined in the same experiments from the rate of appearance of [^{3}H]cholesterol radioactivity in the medium from prelabeled cells. Efflux (mean ± SD) was 0.60 ± 0.05 μg per dish per hr from cells to control plasma and 0.58 ± 0.05 μg per dish per hr from cells to diabetic plasma in these experiments. Because the rates of net transport in the control and untreated diabetic groups (Table 2) were 0.45 and −0.11 μg per dish per hr under the same conditions, cholesterol influx from medium to cells (which is equivalent to efflux − net transport) (9) was therefore 0.15 and 0.69 μg per dish per hr in the control and untreated diabetic groups. Values comparable to those found in untreated diabetics were also obtained with the diabetic group treated only with sulfonylureas. Free cholesterol transport rates were somewhat higher than this in the plasma of patients whose diabetes was poorly controlled by insulin, but these rates were still significantly ($P < 0.01$) below those of the healthy control group. There was no significant difference between control and diabetic groups in the small net uptake of esterified cholesterol from the medium into the cells.

The apo-dependence of efflux was determined as described for total efflux, by using plasma from which individual apo had been removed by immunoaffinity chromatography. The proportion of efflux dependent upon each apo was determined from

Table 2. Cholesterol net transport, esterification, and transfer rates in diabetic and normal plasma

Group	Cholesterol net transport, μg/dish/hr*		Cholesterol esterification rate, μg/ml of plasma/hr†	Cholesteryl ester transfer rate, μg/ml of plasma/hr‡
	FC	CE		
Untreated diabetics	−0.11 ± 0.24	−0.17 ± 0.16	23.9 ± 5.1	−0.3 ± 6.4
Uncontrolled diabetics				
Treated with oral agents	−0.11 ± 0.18	−0.02 ± 0.10	24.4 ± 6.2	2.8 ± 1.9
Treated with insulin	+0.09 ± 0.11	−0.08 ± 0.10	27.2 ± 6.9	2.3 ± 4.1
Normal controls	+0.45 ± 0.13	−0.08 ± 0.06	31.2 ± 6.6	20.3 ± 5.9

Positive transfer rates represent mass transfer of cholesteryl ester to VLDL and LDL; negative transfer rates represent net mass transfer of cholesteryl ester from VLDL and LDL to HDL. Positive cholesterol transport rates represent net transport from cells to plasma medium; negative cholesterol transport rates represent net transport from plasma medium to cells. FC, cholesterol as free sterol; CE, cholesterol as ester. Values are means ± SD.

* At a plasma concentration of 1.2% (vol/vol).

† As cholesterol mass esterified.

‡ As cholesterol mass transferred.

the extent to which efflux was lower in such incubations than in whole plasma medium. In normal plasma, removal of apo A-I decreased efflux (mean ± SD) by 56 ± 5% and removal of albumin by 43 ± 5%. Removal of apo E was without effect. However, in diabetic plasma, removal of apo A-I, apo E, or albumin decreased efflux by 26 ± 9%, 20 ± 11%, and 55 ± 8%, respectively.

Cholesterol Esterification Rates in Diabetic and Normal Plasma. As shown in Table 2, untreated diabetic subjects, and those treated only with oral hypoglycemic agents, had significantly decreased rates of cholesterol esterification in plasma, relative to the control group ($P < 0.05$). A smaller decrease, which did not reach significance, was observed in the plasma of the insulin-treated group with poor control.

Cholesteryl Ester Transfer Rates in Diabetic and Normal Plasma. In normal plasma the major part of cholesteryl esters derived from the LCATase reaction is transferred to VLDL and LDL (11). In contrast, in the plasma of diabetic subjects there was little or no transfer of cholesteryl ester to these lipoproteins. Although transfer was slightly higher in the patients receiving traditional insulin therapy or oral hypoglycemic agents and whose hyperglycemia was poorly controlled, the mass of esters transferred to VLDL and LDL in all the groups was decreased by at least a factor of 8. Because the reduction of LCATase activity was relatively slight, this difference cannot be ascribed to the reduction in the rate of cholesteryl ester synthesis.

Lipoprotein apo Levels in Diabetic and Normal Plasma. As shown in Table 3, the plasma of patients with diabetes had lower concentrations of apo A-I than did that of healthy control subjects, in agreement with several earlier reports (2–4). There was no significant difference in the level of apo A-II (the other major apo of HDL) or of apo D (complexed with LCATase in plasma) (9, 10). apo B levels were slightly raised, as has been reported previously (17), but the difference did not reach significance in this study. However, there was a significant difference between the apo E concentrations in the plasma of the control and untreated diabetic groups. apo E was shown earlier to be functional in free cholesterol transport in LCATase deficiency (15). The plasma of untreated diabetics and those treated only with oral hypoglycemic agents showed levels of apo E that were significantly greater than those of the control group ($P < 0.01$). Insulin-treated diabetics had levels of apo E that were close to those of the control group (Table 3).

Effects of Normalization of Plasma Glucose Levels on Plasma Cholesterol Metabolism. When plasma glucose levels of untreated diabetics were rigorously controlled with insulin, there were rapid changes in each of the parameters of cholesterol metabolism (Fig. 1). While cholesterol efflux rates were unchanged, cholesterol net transport rates, which had been reversed relative to those of the healthy control group, normalized and rose on average by 0.56 μg/hr (Fig. 1). Consequently, calculated cholesterol influx rates from diabetic plasma into the cells decreased during the same treatment from 0.69 to 0.13 μg/hr, the same rate as in the nondiabetic control group. A major portion of the change was evident within 1 wk of rigorous diabetic control. At the same time, LCATase rates rose significantly ($P < 0.01$) (Fig. 1). Rates of cholesteryl ester transfer to VLDL and LDL also rose significantly ($P < 0.01$), although these had not reached control levels after 10 wk of insulin therapy. The level of apo E in diabetic plasma fell towards normal levels (Fig. 1), but there was no significant change in plasma levels of apo A-I, A-II, B, or D. Finally, plasma triglyceride also fell (from 150 ± 55 to 90 ± 27 mg/dl). However, cholesterol levels in plasma were only slightly altered (from 200 ± 33 to 183 ± 19 mg/dl).

There was no detectable change in the initial and final cholesterol metabolic rates in the plasma of the patients treated only with oral hypoglycemic agents during 1 wk in the Clinical Research Center.

Effects of Immunoaffinity Chromatography on Diabetic Plasma. The findings in the plasma of untreated diabetics indicated a specific syndrome of abnormalities of cholesterol metabolism associated with increased levels of apo E. All these abnormalities, including apo E levels, returned to or towards

Table 3. Plasma lipoprotein apo levels in diabetic and normal plasma

Group	Concentration, mg/ml				
	apo A-I	apo A-II	apo B	apo D	apo E
Untreated diabetics	0.92 ± 0.16	0.29 ± 0.09	1.38 ± 0.34	0.05 ± 0.01	0.14 ± 0.02
Uncontrolled diabetics					
Treated with oral agents	0.87 ± 0.21	0.27 ± 0.02	1.32 ± 0.20	0.05 ± 0.01	0.11 ± 0.02
Treated with insulin	0.90 ± 0.09	0.25 ± 0.05	1.40 ± 0.27	0.07 ± 0.01	0.08 ± 0.01
Normal controls	1.20 ± 0.19	0.28 ± 0.06	1.12 ± 0.23	0.05 ± 0.01	0.07 ± 0.01

Values are means ± SD.

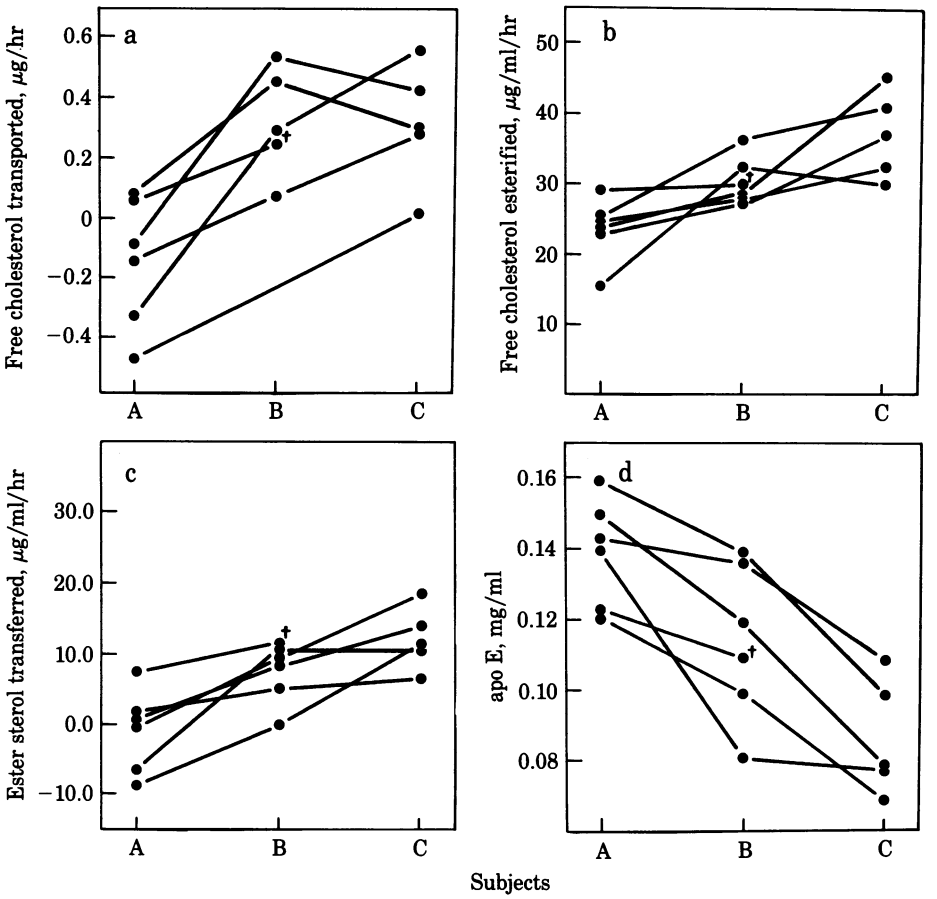

FIG. 1. Effects of diabetic control on plasma rates of cholesterol esterification, cholesteryl ester transfer, cholesterol net transport, and plasma apo E levels. Plasma samples were obtained from fasting untreated subjects (A), after 1 wk of diabetic control with insulin (B), and after 10 wk of diabetic control with insulin (C). One patient (†) was lost from the study after the second sampling. (*a*) Cholesterol net transport between cultured fibroblasts and plasma. Transport was measured in terms of the difference in free cholesterol consumption by LCATase in the presence and absence of cells. (*b*) Rates of cholesterol esterification in plasma measured in terms of the decrease of plasma free cholesterol during incubation at 37°C. (*c*) Cholesteryl ester transfer to VLDL and LDL determined with heparin/$MnCl_2$ (11). Negative rates indicate net loss of cholesteryl ester from VLDL and LDL to HDL. (*d*) Plasma apo E concentrations, determined by radial immunodiffusion assay.

normal during metabolic control with insulin. To determine whether lipoproteins containing apo E played a mediating role in abnormal cholesterol metabolism in diabetes, plasma from untreated diabetics was passed through columns of immobilized antibody to apo E bound to Sepharose. Cholesterol net transport, cholesterol esterification, and cholesteryl ester transfer were then determined and their rates were compared to those in the same plasma retaining its apo E content. Chromatographic removal of apo E resulted in an immediate normalization of cholesterol net transport between cultured fibroblasts and diabetic plasma (Fig. 2), with a mean increase in net transport rates of 0.31 ± 0.05 μg/hr ($n = 4$). Accordingly, influx was decreased in these subjects to 0.21 μg/hr, compared with 0.15 μg/hr in the healthy control group and 0.13 μg/hr in the diabetic groups whose hyperglycemia had been normalized by intravenous insulin.

Removal of apo E also resulted in a slight reduction (7 ± 5%) (not significant) in the rate of cholesterol esterification in plasma, and it was without effect on cholesteryl ester transfer in the same plasma.

Removal of apo E was without significant effect on cholesterol metabolism in the plasma of diabetics whose cholesterol transport had been normalized with insulin. A small positive effect was found in the plasma of the group whose plasma glucose levels were only poorly controlled by traditional insulin therapy. In this group, cholesterol net transport rates rose from 0.09 ± 0.11 μg/hr to 0.25 ± 0.05 μg/hr upon removal of apo E ($n = 4$). However, the apo E levels in plasma in this group were initially only slightly above control values (Table 2).

DISCUSSION

There is considerable evidence that in normal plasma cholesterol net transport, cholesterol esterification by LCATase, and transfer of LCATase-derived cholesteryl esters to VLDL and LDL form a coupled series of reactions by means of which the gradient for cholesterol transport from peripheral cells is established and maintained (9–11). The results of this study indicate that this series of reactions is grossly disturbed in patients with noninsulin-dependent diabetes and documents the existence of a highly abnormal pattern of cholesterol metabolism in the plasma of these subjects. A reduction, or even reversal, of cho-

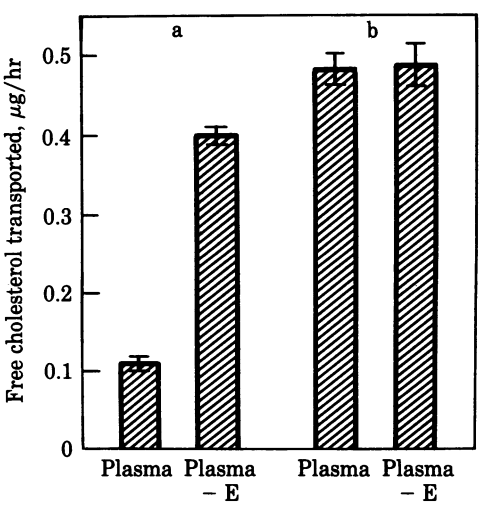

FIG. 2. Cholesterol net transport from cultured fibroblasts to medium containing plasma or plasma from which lipoprotein species containing apo E had been removed by immunoaffinity chromatography. (*a*) Fasting plasma from an untreated diabetic patient. (*b*) Plasma from the same patient after 1 wk of diabetic control with insulin. Cholesterol transport was determined as described in the legend to Fig. 1.

lesterol transport was associated with a significant decrease in the rate of cholesterol esterification. Furthermore, there was an inhibition, essentially complete, of the transfer of cholesteryl ester to VLDL and LDL. Together these findings indicate an interruption of the cell-to-plasma cholesterol gradient in plasma from patients with noninsulin-dependent diabetes.

Other studies have shown that although traditional insulin therapy has little effect on the abnormal levels of lipoproteins in patients with noninsulin-dependent diabetes, continuous insulin infusion rapidly normalizes total plasma triglyceride and cholesterol levels and the distribution of cholesterol within the major plasma lipoprotein classes (18, 19). The present studies show that similar therapy also rapidly normalizes cholesterol metabolic parameters in the plasma of noninsulin-dependent diabetics and, in particular, the direction and activity of the cell-to-plasma cholesterol gradient.

Although apo E levels are triglyceride dependent (20), the increased level of apo E in the untreated diabetics in this study was greater than would be predicted on the basis of their mildly elevated triglyceride levels, just as the decrease in apo E with control of blood glucose was greater than predicted from the fall of plasma triglyceride levels alone. These findings suggested that apo E was a factor in the abnormal cholesterol metabolism of diabetes. Removal of apo E *in vitro* selectively normalized transport without effect on esterification or transfer. However, removal of apo E had no effect on transport in normal plasma or in the plasma of diabetics whose cholesterol metabolism had been normalized by continuous glycemic control. These findings, taken together, suggest that an abnormal lipoprotein species present in the plasma of the untreated diabetic subjects mediates the excessive influx of cholesterol from diabetic plasma to cells, which is the driving force for the reversal of cholesterol net transport. Further research will be required to identify the chemical characteristics of this fraction.

However, several pieces of evidence from the present study suggest that it may act prior to the esterification of cell-derived cholesterol. (*i*) Removal of apo E did not normalize cholesteryl ester transfer, ruling out a direct inhibitory effect of this apo. Other studies (21) confirm that apo E is unlikely to be directly active in transfer, and the presence of significant reversed transfer (from VLDL and LDL to HDL) in several untreated or unresponsive diabetics suggests that the defect of transfer lies in an impaired ability of VLDL and LDL in diabetics to accept cholesteryl ester by transfer. (*ii*) LCATase activity was only partially inhibited in untreated diabetes, and activity was not increased by removal of apo E. (*iii*) In the plasma of untreated diabetics, efflux was partially apo E dependent, suggesting that this apo here played a role in cholesterol transport; the influx of cholesterol to the cells was as free cholesterol rather than as esterified sterol, and this influx was decreased to normal rates by removal of apo E. Because normal plasma contains significant levels of apo E, clearly neither the whole nor possibly a major part of total apo E in diabetic plasma functions to promote cholesterol influx. However, it appears most likely that as a secondary response to the block to transfer of LCATase-derived cholesteryl esters (22), free cholesterol bypasses the LCATase reaction and is diverted to the cells, via an apo E-containing lipoprotein carrier.

It is clear that diabetes mellitus is a heterogeneous syndrome (12). The conclusions of the present study may therefore not apply to other groups of diabetics, such as those with insulin-dependent diabetes or impaired glucose tolerance. The patients in this study were also only mildly hypertriglyceridemic and their cholesterol levels were not increased above that of the normal group. However, it is of interest that although several other groups of subjects at risk (like diabetics) for atherosclerotic vascular disease (such as those with hypertriglyceridemia, hypercholesterolemia, and dysbetalipoproteinemia) show no common abnormality in the level of the major lipoprotein classes, each is characterized by an increased level of apo E in plasma (20, 23), as were the untreated diabetics in this study. Investigation of plasma cholesterol metabolism in these other groups may also indicate a block to cholesteryl ester transfer coupled with reversed cholesterol net transport that could indicate a common biochemical defect in the plasma of the different patient groups.

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