Human plasma phospholipid transfer protein accelerates exchange/transfer of α-tocopherol between lipoproteins and cells

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α-Tocopherol (α-T), an important anti-oxidant of plasma lipoproteins and cell membranes, is secreted from liver together with very-low-density lipoproteins into the blood stream. Other serum lipoprotein classes gain α-T by exchange and transfer processes. We show here that the lipoprotein-free $d > 1.22$ g/ml fraction of human or pig serum increases the exchange rate of α-T by a factor of 2–4 as compared with spontaneous exchange/transfer. The α-T exchange/transfer (α-TET) activity was purified by multiple-step column chromatography. It gave a single band in PAGE with an apparent molecular mass of 75 kDa, and was found to be identical with the phospholipid transfer protein (PLTP). PLTP catalysed α-T exchange between different lipoprotein classes, as well as the transfer of α-T from artificial liposomes to high-density lipoproteins. The α-TET activity measured with a newly developed assay in ten healthy people was 2.45 ± 0.88 nmol·ml^{-1}·h^{-1}. α-TET activity was negatively correlated with plasma low-density lipoprotein-cholesterol ($r = -0.75$; $P < 0.01$). It is concluded that human PLTP catalyses exchange/transfer processes of α-T between lipid compartments. This factor may be of relevance in atherogenesis and tumour initiation and growth.

INTRODUCTION

Vitamin E (tocopherol) is an important anti-oxidant in biological systems [1]. It not only inhibits peroxidation of membrane lipids but also plays an important role in protecting plasma lipoproteins against oxidative modification [2–6]. α-Tocopherol (α-T) very efficiently scavenges lipidperoxyl radicals and thereby prevents the lipid peroxidation process in an uninhibited chain reaction. As a consequence of scavenging peroxyl radicals, α-T is converted into a tocopheroxyl radical. The efficiency of α-T as a lipophilic anti-oxidant *in vivo* seems to be due to the fact that it can be readily recycled within the membrane by interaction of the tocopheroxyl radical with anti-oxidants present in the aqueous phase, such as ascorbic acid, thioctic acid and other thio-compounds [4,7].

Among other factors, free radicals, lipid peroxidation and imbalances between the anti-oxidants and pro-oxidants have been suggested to be key mediators of the pathophysiological processes occurring during atherogenesis and carcinogenesis. In the last decade evidence has accumulated that a crucial and causative role in the pathogenesis of atherosclerosis is played by the oxidative modification of low-density lipoproteins (LDL) [reviewed in 8]. Since LDL oxidation is basically a lipid peroxidation process it follows that its anti-oxidant content should have a major impact on its resistance to oxidation. Quantitatively, the major anti-oxidant of LDL is α-T, with approximately 11 nmol/mg of protein. Other potential anti-oxidants (e.g. carotenoids, ubiquinol-10) are present in amounts 10- to 300-fold lower than α-T [3]. Investigations from subjects not supplemented with vitamin E revealed a very strong intersubject variation in the protection of LDL from oxidation [3,9,10]. This variation is caused partly by variations in the α-T content of LDL, but is also dependent on other not yet fully identified variables: the fatty acid composition [11] and the ubiquinol-10 content [12] are likely to be of importance. On the other hand, supplementation studies in animals and humans have clearly shown that oxidation resistance of LDL can be increased by increasing its α-T content [8,9,13,14]. Epidemiological studies suggest that a high dietary intake of vitamin E reduces the risk of coronary heart disease [15]. Such a protective effect however is not necessarily mediated by the anti-oxidant action. For example, α-T inhibits smooth-muscle cell proliferation probably by modulating protein kinase C activity [16]. It should however also be mentioned here that under certain circumstances vitamin E may even act as a pro-oxidant for LDL and contribute to the propagation of lipid peroxidation if no aqueous anti-oxidants are present [17]. Such a situation, however, has not been demonstrated so far to occur *in vivo*.

Lipid peroxidation also seems to be involved in the initiation and promotion of carcinogenesis [18,19], a process which may be prevented by anti-oxidants. In fact there is evidence that vitamin E may influence the development of several forms of cancer. It can inhibit the proliferation of several malignant cell lines including human osteosarcoma and neurosarcoma cells, also here by interfering with the action of protein kinase C [20–22].

Vitamin E homoeostasis in living cells and the mechanism by which it incorporates into cell membranes or enters the cells is poorly investigated. It is readily absorbed from the intestine and transported to the liver by chylomicrons [23]. Liver possesses an α-T binding protein that is specific for (2*R*,4′*R*,8′*R*)-α-tocopherol (from here on named α-T) [24]. This protein facilitates the incorporation of α-T into nascent very-low-density lipoproteins (VLDL). Because of the specificity of the binding protein, α-T is the major form of vitamin E found in human plasma. In the

Abbreviations used: α-T, (2*R*,4′*R*,8′*R*)-α-tocopherol; α-TET, α-tocopherol exchange/transfer; BHT, butylated hydroxytoluene; CETP, cholesterol ester transfer protein; HDL, high-density lipoproteins; HSA, human serum albumin; PCCAT, phosphatidylcholine:cholesterol acyl transferase; LDL, low-density lipoproteins; PLTP, phospholipid transfer protein; VLDL, very low density lipoproteins; VDL*, LDL*, HDL*, lipoprotein fractions radiolabelled with [^{14}C]α-T; RBC, red blood cells; PC, phosphatidylcholine.

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circulation, VLDL are partially converted into smaller particles with a relatively short half-life of some hours, or into LDL with a half-life of 3–5 days. During these metabolic processes an active exchange and transfer of core lipids and of surface components between high-density lipoproteins (HDL) and lipoproteins of lower density take place [24–27]. Also lipid-soluble vitamins including α-T have been shown to move actively between lipoproteins of different density classes [28]. α-T, in addition, readily exchanges between lipoproteins and erythrocytes [29] as well as between many other cells. The most efficient donor of α-T to cells seems to be HDL [29], and thus the speed by which HDL gains anti-oxidant from VLDL or LDL is of great importance for an effective supply of α-T to different organs.

The current opinion is that α-T transfer from VLDL to other lipoproteins or to cells is independent of specific mediators as it equilibrates between lipoproteins relatively fast [28,30,31]. A factor which would promote α-T exchange/transfer (α-TET) processes in plasma has not been demonstrated so far [32]. It has been shown that the cholesterol ester transfer protein (CETP), which facilitates the exchange and transfer of neutral lipids as well as of phospholipids between different lipoprotein classes, plays no role in exchange/transfer-processes of α-T between plasma lipoproteins [29].

Human plasma lipid transfer protein (PLTP) has been purified to homogeneity and cloned [33,34]. It consists of a plasma protein with an estimated molecular mass of 75–78 kDa, which is distinct from CETP, and facilitates the transfer of surface lipids, such as phospholipids, but not of core lipids among plasma lipoproteins [35]. Recently, it has been shown that PLTP has the ability to convert HDL_3 into larger and smaller particles and thus it is also considered as the putative conversion factor of human plasma [33].

We present data which indicate that human PLTP [31] facilitates the exchange of α-T between lipoproteins and between lipoproteins and cell membranes, as well as the transfer of α-T from liposomes to HDL.

EXPERIMENTAL

Materials and methods

Isolation of lipoproteins and labelling with [^{14}C]α-T

Blood was drawn from fasting normolipemic volunteers, allowed to clot at room temperature for 30 min and centrifuged at 3000 *g* for 10 min. Na_2EDTA (0.5 mg/ml), and *p*-chloromercuribenzene sulphonic acid (PCMBS) (2 mmol/l) were immediately added to serum in order to block the activity of phosphatidylcholine: cholesterol acyl transferase (PCCAT). At these concentrations the reagents had no influence on the α-TET activity as tested in preliminary experiments. Serum lipoproteins were prepared by equilibrium density-gradient ultracentrifugation in a Beckmann SW-41 swinging bucket rotor as described earlier [36]. In most of the experiments, serum was heat-inactivated by incubation for 1 h at 55 °C under nitrogen. To avoid oxidation of lipoproteins during the preparation, all salt solutions and buffers were flushed with nitrogen. VLDL, LDL and HDL_3 fractions were isolated in the density ranges < 1.006, 1.025–1.055 and 1.125–1.21 g/ml, respectively. In control experiments the separation of lipoproteins was performed in the presence of 1 mM butylated hydroxytoluene (BHT) and 50 mM ascorbic acid. The fractions were > 98% pure as monitored by agarose gel electrophoresis and immunochemical methods [37].

In order to label lipoproteins with [^{14}C]α-T, 20 ml of fresh human serum was mixed with 4.2 μCi of α-T in 170 μl of ethanol (uniformly ^{14}C-labelled α-T, specific activity 12.4 Ci/mol, Amersham Laboratories/GB). The mixture was incubated under nitrogen for 3 h at 37 °C followed by lipoprotein separation as described above. Most of the radioactivity (80%) was recovered in the lipoprotein fractions whereas the remaining 20% was distributed in the intermediate fractions between the main lipoprotein bands and the $d > 1.22$ g/ml bottom fraction. The lipoproteins were concentrated to approx. 10 mg/ml by dialysis against a 50% (w/w) polyethylene glycol solution (20 kDa, Fluka/Buchs) in buffer A (0.15 M NaCl/0.05 M $NaHCO_3$/1 mg/ml Na_2EDTA, pH 7.4) and stored at 4 °C under nitrogen in the dark. This yielded lipoproteins with a specific activity of 25000–50000 c.p.m./mg protein. The labelled lipoproteins were used within 7 days.

The $d > 1.22$ (1.23) g/ml fractions were isolated from human or pig sera. Human serum was adjusted to $d = 1.22$ g/ml and pig serum to $d = 1.23$ g/ml by the addition of solid NaBr and centrifuged at 15 °C for 30 h at 160000 *g* in a Beckmann Ti-70 fixed-angle rotor. The tubes were sliced just below the HDL_3 layer, the lipoprotein-free fraction was aspirated by a syringe and dialysed exhaustively against buffer A.

α-Tocopherol exchange assay

Radiolabelled VLDL, LDL or HDL with [^{14}C]α-T (here designated as VLDL*, LDL*, HDL*) in buffer A were mixed with the unlabelled acceptor lipoprotein together with different potential sources of α-TET-activity. Buffer A, or buffer A containing 40 mg/ml of human serum albumin (fatty acid-poor HSA from Sigma), served as a blank. To block the PCCAT activity, PCMBS (2 mmol/l) was added and the mixture was incubated for up to 24 h at 37 °C. A representative assay mixture was composed of 600 μl of α-TET activity, 1.2 mg of [^{14}C]α-T-labelled lipoprotein and 1.2 mg of acceptor lipoprotein, made up to 1.2 ml with buffer A. Aliquots of the incubation mixture (100 μl) were taken out at given time intervals and mixed with 200 μl of a 9.5% polyethylene glycol solution (Quantolip A for HDL-cholesterol (HDL-C) determination from Immuno Corp., Vienna) [38]. The precipitated lipoproteins consisting of VLDL or LDL were removed by centrifugation in an Eppendorf centrifuge (10 min, 8000 *g*) and washed once with buffer A. The radioactivity in the precipitate as well as in the supernatant (containing all HDL) was counted. In all experiments zero-time exchange rates were recorded and subtracted as a blank; they amounted to < 5% of the maximally observed α-T exchange.

For the estimation of α-TET activity in serum we developed a quick and practical assay: VLDL and LDL of human sera were precipitated with Na-phosphotungstate, centrifuged and an equal mass of [^{14}C]α-T-labelled-LDL (specific activity: 26–31 c.p.m./μg LDL) was added to the supernatant which contained all the endogenous HDL. Briefly, 1 ml of fresh human serum was mixed with 2 μmol/l of PCMBS, 25 μl of 2 M $MgCl_2$ and 100 μl of 4% Na-phosphotungstate [38], incubated for 10 min at room temperature and centrifuged at 5000 *g* for 5 min. The supernatant containing HDL_{2+3} as well as practically all the α-TET-activity was dialysed against buffer A for 3 h at room temperature, and then mixed with [^{14}C]α-T-labelled LDL. The amount of added LDL was chosen to give an approximate HDL:LDL mass ratio of 1. The lipoprotein mass of LDL and HDL was calculated from LDL-C and HDL-C by multiplying by a factor of 3.2 or 5, respectively. The mixture was incubated for 24 h at 37 °C, 100 μl aliquots were withdrawn at time intervals of 0, 0.5, 1, 2, 4, 12 and 24 h and precipitated with 9.5% polyethylene glycol. The distribution of radioactivity between the precipitated LDL and the HDL in the supernatant was determined by liquid scintillation counting.

In some experiments we also measured the exchange rate of α-T between lipoproteins and red blood cells (RBCs). For this assay, RBCs were purified from fresh EDTA blood by washing six times with Tris-buffered saline (pH 7.4). A 400 μl aliquot of sedimented RBCs was mixed with the indicated amount of [^{14}C]α-T-labelled lipoproteins and incubated for up to 24 h at 37 °C in sealed tubes under nitrogen with mild shaking. Thereafter, RBCs were separated from lipoproteins by centrifugation, washed twice and the distribution of radioactivity between the cells and the supernatant was determined.

Calculation of α-TET activities and of τ/2

The α-TET activity in different sera was calculated according to Barter and Jones [39] as described in detail by Groener et al. [40] from the equations:

$$\ln A = -FMt;$$

$$A = \frac{S_{D,t} - S_{D,eq.}}{S_{D,0} - S_{D,eq.}}$$

$$M = \frac{M_D + M_A}{M_D \times M_A},$$

where S_D = specific activity of α-T in the donor lipoprotein; t indicates the time-point at which the activities were measured; eq. = time at equilibrium (in most cases 24 h); 0 = zero time; F = α-TET activity in nmol·ml^{-1}·h^{-1}, i.e. the rate of exchange of α-T between donor and acceptor lipoprotein; M = pool size of α-T in donor (D) and acceptor (A) lipoprotein in the incubation mixture in nmol.

F was evaluated graphically by plotting ln A against time. From the slope (k) of this plot, F was calculated according to: $k = -FM$: $F = -k/M$. Since F calculated in this way, according to definition, is dependent on the α-T pool in the incubation mixture, the values were normalized to 10 nmol of α-T per ml of serum, yielding the term F_n. F_s is used to denote the spontaneous exchange in the absence of α-TET activity.

The half-time for reaching equilibration of α-T between donor and acceptor lipoproteins ($\tau/2$) was calculated from the same plot (ln A versus time) according to: $\tau/2 = -\ln 2/k$.

Purification of the PLTP

PLTP was isolated from the lipoprotein-depleted $d > 1.21$ g/ml fraction using the following chromatographies: Butyl-toyopearl 650 (CM), Heparin–sepharose, MonoS 5/5 cation exchange and MonoQ 5/5 anion exchange. Chromatographic conditions were essentially the same as recently described [33], but the CM-Toyopearl 650 was replaced by MonoS and the Superose 6 HR gel-filtration step was omitted. The purified protein at a final concentration of 0.1–0.2 mg/ml was kept at 4 °C in 25 mM Tris/HCl buffer, pH 7.4, containing 1 mM EDTA. The activity of PLTP was monitored during the purification by measuring [^{14}C]phosphatidylcholine ([^{14}C]PC) transfer from phospholipid vesicles to HDL_3 [33].

PLTP activity was measured as described previously [33]. In short, 10 mmol of egg PC, 1 mCi [^{14}C]dipalmitoyl PC and 20 nmol BHT were dried under nitrogen and emulsified thereafter by ultrasonication in 1 ml of 10 mM Tris/HCl buffer, pH 7.4, containing 150 mM NaCl and 1 mM EDTA (referred to as Tris buffer hereafter). After centrifugation for 10 min at 15000 rev. min^{-1} (19000 g), the optically clear supernatant was used for PLTP assay. The assay contained 250 mg of HDL-protein, 150 nmol of labelled liposomes, 4–25 μl of sample and Tris buffer to a final volume of 400 μl. The mixture was incubated at 37 °C for up to 120 min and the reaction was stopped by adding 0.3 ml of a heparin/$MnCl_2$/NaCl solution, followed by centrifugation for 10 min at 15000 rev. min^{-1} (19000 g). A 0.5 ml volume of the supernatant containing HDL but no liposomes was used for radioactivity determination. The results are expressed as nmol of PC transferred from PC liposomes to HDL per ml of substrate per hour and are corrected for spontaneous transfer, i.e. transfer in the absence of PLTP activity.

In some experiments, α-T transfer from liposomes to HDL was also measured. This assay was carried out practically in an identical way as the PLTP assay except that 1 μCi of [^{14}C]-αT plus 10 μmol of unlabelled α-T were used for preparing the liposomes. All other steps in α-T transfer measurements were identical with those described for the phospholipid transfer.

The CETP activity in different sera was measured according to Groener et al. [40] by monitoring the transfer of [^{14}C]cholesteryl ester from HDL to VLDL/LDL following 3 h incubation at 37 °C.

Quantification of α-T

α-T was measured by h.p.l.c. as described [3]. Briefly, 400 μl of the sample was mixed with 400 μl of methanol and extracted with 800 μl of hexane. The extract was dried under N_2 and the residue was redissolved in 100 μl of ethanol. A volume of 20 μl was injected into the h.p.l.c. system and separated on an ODS-2 column at a flow rate of 1 ml/min and fluorometric detection at 335 nm with excitation at 292 nm.

Other analytical methods

The ^{14}C radioactivity was measured in an LKB-Wallac β-counter using Ready Save (Beckmann) as a scintillator. All counts were corrected for background. In order to avoid bioluminescence, samples were allowed to stand in the counter for at least 2 h before counting.

Immunodiffusion, immunoelectrophoresis and PAGE were performed as described [33,36–38]. Phospholipids, triglycerides and cholesterol were quantified enzymically using commercial kits from Boehringer Mannheim. Protein was measured according to Lowry et al. [41] using HSA as standard. All chemicals were p.a. reagents from Merck (Darmstadt, Germany) if not stated otherwise.

Statistical analysis

Univariate analyses included unpaired Student's t-test and Pearson's correlation coefficients using SYSTAT, Inc. (Evanston, IL, U.S.A.) statistical package for personal computers.

RESULTS

α-T concentration in the sera of a normolipidemic study group ($n = 10$) varied between 24.0 and 47.7 μmol/l (mean ± S.D.: 31.2 ± 7.2 μmol/l) and correlated positively with total cholesterol ($r = 0.84$, $P = 0.002$) and triglycerides ($r = 0.85$; $P = 0.001$). The distribution of α-T between VLDL/LDL/HDL in 14 h fasting sera was 1:1.9:1.4. This distribution was strongly dependent upon the relative lipoprotein content of these sera.

First the kinetics of α-T exchange between LDL and HDL was studied. For this purpose, LDL and HDL were purified by ultracentrifugation and radiolabelled with [^{14}C]α-T. We assumed that the radioactive label formed a homogeneous fraction in the lipoproteins with the endogenous α-T pool. In order to test this,

Table 1 Exchange of α-T between LDL and HDL in the presence of human or pig lipoprotein-free sera

LDL and HDL were isolated from individual sera and radiolabelled with [^{14}C]α-T. LDL* was mixed with HDL and vice versa in the original ratio, and adjusted to the original concentration by adding $d > 1.22$ g/ml bottom fraction from human serum (H-Bottom) or $d > 1.23$ g/ml bottom fraction from pig serum (P-Bottom) or buffer A or 4% HSA respectively. The mixture was incubated for 24 h and $\tau/2$ was determined as described in the Materials and methods section. The results are means (±S.D.) from five experiments performed with five different lipoprotein- and bottom-fractions. *Significantly different from corresponding incubations with buffer A or 4%-HSA ($P < 0.001$).

	$\tau/2$ (min)			
Incubation	Buffer A (ref.)	4% HSA	H-Bottom	P-Bottom
LDL* → HDL	154 (5)	151 (6)	95 (4)*	84 (3)*
HDL* → LDL	141 (4)	138 (6)	82 (3)*	71 (2)*

two experiments were performed. (1) Radiolabelled LDL and HDL were dialysed for 24 h against buffer A and the specific radioactivity was measured at time 0 and after 24 h. We found no change of the specific activity of α-T within the experimental error (±5%); less than 2% of radioactivity was lost during dialysis. (2) LDL from pooled human serum was spiked with cold α-T and the lag-time during Cu^{2+}-mediated oxidation using 5 mM $CuCl_2$ was measured at 234 nm [42]. Native LDL with an α-T content of 9.5 nM α-T per mg of LDL-protein exhibited a lag-time of 65 min; the spiked LDL with 13.9 nM α-T/mg had a lag-time of 81 min. From these two experiments we concluded that the [^{14}C]α-T incorporated into LDL by our method behaves in a comparable manner to the endogenous α-T pool.

The isolated lipoproteins were reconstituted to original serum concentrations: approx. 1.6 mg of LDL-C plus 0.51 mg of HDL-C per ml of the $d > 1.22$ g/ml bottom fraction of the parent serum (native or heat inactivated) or of pig serum were mixed and incubated for up to 24 h. HSA (4%) or buffer A were used as controls. These studies were performed in parallel with either [^{14}C]α-T-labelled LDL (LDL*) plus cold HDL or vice versa. The results of these experiments performed with five individual sera are summarized in Table 1. The exchange of [^{14}C]α-T radioactivity between HDL and LDL versus time is shown in a typical experiment (Figure 1). From these data it is apparent that the addition of $d > 1.22$ bottom fraction to the lipoprotein incubation accelerates the [^{14}C]α-T equilibrium by a factor of 1.7–2 as compared with spontaneous α-T equilibrium. The final distribution of α-T between HDL and LDL, however, was the same whether or not $d > 1.22$ g/ml bottom fraction was present.

It is essential for the validity of our assay that no oxidation of α-T takes place during the experiment, otherwise we might measure the exchange of any oxidation product of α-T. In order to test whether this was the case, the amount of α-T present in the whole assay mixture before and after 24 h incubation was measured by h.p.l.c. There was no change within the experimental error of ±5%. In addition, $\tau/2$ measurements, similar to those shown in Table 1 were performed with lipoproteins isolated in the presence of 1 mM BHT and 50 μM ascorbic acid. These two anti-oxidants were also added at the same concentration to the assay mixture. In an experiment using $d > 1.22$ g/ml bottom fraction from human serum as the source of exchange activity and LDL* → HDL as donor and acceptor lipoproteins respectively, without anti-oxidants, $\tau/2$ values of 90 and 92 min were found. The same assay in the presence of BHT and ascorbic acid yielded $\tau/2$ values of 91 and 93 min.

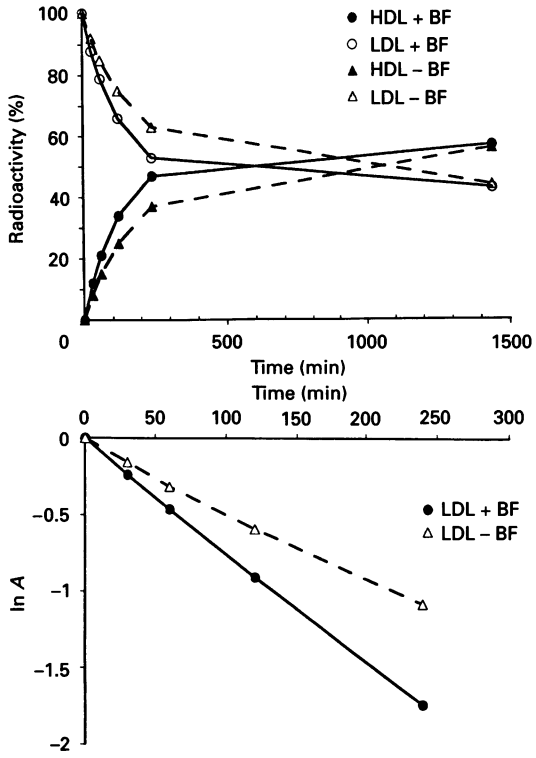

Figure 1 Typical experiment of α-T exchange between HDL and LDL in the presence (+) or absence (−) of $d > 1.22$ bottom fraction (BF) of human serum as a source of α-TET activity

LDL-C (1.6 mg) and 0.51 mg of [^{14}C]α-T-labelled HDL-C (31 c.p.m./μg) were incubated for 24 h at 37 °C in the presence of buffer A or of 600 μl of LPDS from human serum. The decay of radioactivity in HDL and the increase of radioactivity in LDL was monitored as described in the Materials and methods section. (**a**) Changes in radioactivity in relation to incubation time; (**b**) plot of ln *A* versus time; for details of the calculation of the α-TET activities see Materials and methods section.

Next, the effect of various enzyme inhibitions on the α-TET activity was studied: (a) heating the serum for 1 h at 55 °C before isolating the $d > 1.22$ ($d > 1.23$) g/ml lipoprotein free bottom fraction; (*b*) addition of 2 mmol/l of phenylmethanesulphonyl fluoride (PMSF), or (*c*) addition of 2 mmol/l of PCMBS to the bottom fractions. α-TET activity was almost completely abolished by heating the serum, whereas 2 mmol/l of PMSF or PCMBS had no effect (Table 2).

When HDL acted as the donor lipoprotein, the equilibration of the labelled α-T was faster than when LDL was the donor. Human and pig sera exhibited comparable α-TET activities. Heat inactivation of the serum for 1 h at 55 °C abolished the α-TET activity in the $d > 1.22$ ($d > 1.23$) g/ml bottom fraction. When fresh serum instead of heat-inactivated serum was used as a source of LDL* and HDL* for incubation with buffer A, an appreciable amount of α-TET activity bound to the lipoprotein fractions, notably to HDL (experiments not shown). In subsequent experiments, therefore, only heat-inactivated serum was used for the preparation of donor and acceptor lipoproteins.

From this part of the study we concluded that there is also some spontaneous exchange of α-T between LDL and HDL in the absence of serum factors. If, however, human or pig lipoprotein-free bottom fraction is added to the incubation

Table 2 Influence of heat and of hydroxyl- and thiol-group blocking agents on the α-TET activity

Human serum or pig serum was incubated for 1 h at 55 °C followed by the isolation of the $d > 1.22$ g/ml ($d > 1.23$ g/ml) bottom fraction (BF). Alternatively, the human or pig bottom fractions were isolated from fresh serum and 2 mmol/l or PMSF or PCMBS were added before assaying the α-TET activity. The experiments were performed as described in Table 1. The results are means (±S.D.) of two experiments carried out in duplicate. References are given in parentheses. *Significantly different from corresponding experiment with buffer A ($P < 0.01$).

Source of α-TET activity	τ/2 (min)	
	LDL* → HDL	HDL* → LDL
Human serum		
Buffer A	166 (6)	136 (5)
$d > 1.22$ BF: fresh serum	98 (3)*	78 (2)*
$d > 1.22$ BF: heat inactivated	152 (5)	125 (3)
$d > 1.22$ BF + 2 mmol/l PMSF	101 (3)*	80 (2)*
$d > 1.22$ BF + 2 mmol/l PCMBS	96 (3)*	76 (3)*
Pig serum		
Buffer A	166 (5)	136 (4)
$d > 1.23$ BF: fresh serum	77 (3)*	67 (2)*
$d > 1.23$ BF: heat inactivated	160 (6)	128 (4)
$d > 1.23$ BF + 2 mmol/l PMSF	75 (2)*	64 (2)*
$d > 1.23$ BF + 2 mmol/l PCMBS	79 (2)*	69 (1)*

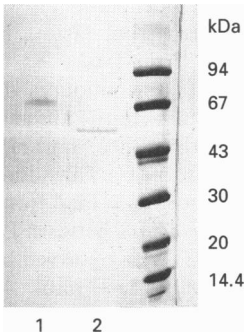

Figure 2 Electrophoresis of purified PLTP

Purified PLTP in the presence (lane 1) and absence (lane 2) of 1% mercaptoethanol was electrophoresed in a 5–25% polyacrylamide gradient gel containing 0.1% SDS. The molecular mass of the standards in kDa is shown.

mixture, the equilibration time of α-T is reduced by up to 50%. From these results we postulated the existence of a serum factor, which can catalyse the exchange of α-T between the two lipoprotein classes. This factor is heat-labile but resistant to Ser hydroxyl-group and thiol-group blocking agents.

To test if this protein fraction is the recently characterized PLTP we replaced the $d > 1.22$ bottom fraction by PLTP. PLTP was purified from human serum as described in the Materials and methods section. An SDS/PAGE pattern of the preparation used is shown in Figure 2. It displayed one band with a molecular mass of approximately 75000 Da in the presence of mercaptoethanol; without mercaptoethanol the protein migrated faster than albumin.

In order to ascertain that PLTP and α-TET activities are mediated by the same protein, the following experiments were carried out. PLTP was purified from $d > 1.21$ g/ml bottom fraction as described [33] and its specific activity with respect to

Table 3 Inhibition of PC and α-T transfer rates by heat and different substances

The $d > 1.22$ g/ml bottom fraction (BF) of a human serum pool was compared for PC transfer and for α-T transfer activity. The assay is described in the Materials and methods section. The results are expressed as a percentage of the activity observed in fresh bottom fraction and are means of triplicate analyses.

Sample	PC transfer activity	α-T transfer activity
BF	100	100
BF heated for 1 h at 45 °C	66	64
BF heated for 1 h at 50 °C	41	43
BF heated for 1 h at 56 °C	3	0
BF + 2 mmol/l PMSF	102	99
BF + 2 mmol/l PCMBS	98	97

Table 4 Exchange of α-T between VLDL, LDL and HDL in the presence and absence of PLTP

[^{14}C]α-T-labelled lipoproteins (VLDL*, LDL*, HDL*) were incubated with cold lipoproteins in buffer A or in buffer A containing 600 units of PLTP, and the half-equilibration time (τ/2) of α-T was determined as described in the Materials and methods section. The results are mean values of three independent incubations (±S.D.).

Incubation	τ/2 (min)	
	Buffer A	600 units of PLTP
VLDL* → HDL	233.4 (11.3)	111.1 (4.3)
HDL* → VLDL	151.6 (8.4)	58.1 (1.6)
LDL* → HDL	120.2 (93.0)	39.5 (1.8)
HDL* → LDL	87.4 (4.2)	21.6 (1.0)

PC transfer or α-T transfer from liposomes to HDL was followed. Both specific activities increased in parallel with the degree of purification (data not shown). In addition, $d > 1.22$ g/ml bottom fraction of human serum was inactivated by heat, 2 mmol/l of PMSF or 2 mmol/l of PCMBS, and the influence on α-T transfer activity and PLTP activities were measured in parallel (Table 3). In these experiments there was very good correlation of the inactivation of PC transfer and α-T transfer activity which led us to assume that PLTP and α-TET are identical.

The specific PLTP-activities of the preparations used in subsequent experiments ranged from 1500–5500 units/ml (1 unit catalyses the transfer of 1 nmol of PC from PC-vesicles to HDL_3 per hour). Equal masses of donor and acceptor lipoproteins were incubated with PLTP. Incubations with 4% HSA or with buffer A served as controls. The experiments were carried out as follows: either [^{14}C]α-T-labelled lipoproteins (VLDL* or LDL*) were incubated with equal amounts of cold HDL, or [^{14}C]α-T-labelled HDL* was incubated with VLDL or LDL. The incubation mixture consisted of 1 mg of [^{14}C]α-T-labelled donor lipoprotein, 1 mg of acceptor lipoprotein, 50–300 μl of PLTP solution (PLTP activity 600 units) and made-up to 1 ml with buffer A. In most cases, 2 ml of these mixtures were prepared, incubated at 37 °C and at the indicated time intervals 100 μl aliquots were withdrawn and the distribution of ^{14}C radioactivity was determined. The results of these experiments are listed in Table 4.

α-T equilibration was significantly faster in the presence of PLTP as compared with the control incubations. The τ/2

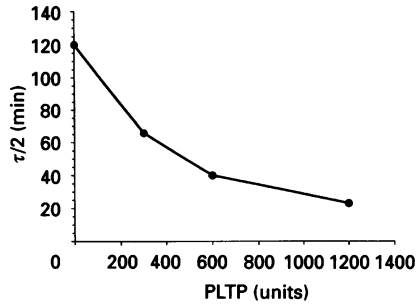

Figure 3 Half-equilibration time ($\tau/2$) as a function of the PLTP concentration

[^{14}C]α-T-labelled LDL was mixed with HDL and incubated with increasing amounts of purified PLTP, and $\tau/2$ was measured as described in the Materials and methods section. The points are means of one duplicate analysis.

Table 5 Phospholipid and α-T transfer from liposomes to HDL

A 5 ml volume of sample was mixed with PC or with α-T liposomes and with HDL, incubated for 45 min at 37 °C and the transfer rate was determined as described in the Materials and methods section. Samples consisted of freshly prepared $d > 1.22$ g/ml bottom fractions from five male normolipemic donors (20–23 years) and of two batches of purified PLTP. The values are means of triplicate determinations (±S.D.).

Sample	PC-transfer nmol·ml^{-1}·h^{-1}	α-T transfer	Ratio
$d > 1.22$ bottom			
1	5420 (260)	1129 (51)	4.80
2	4837 (145)	1037 (62)	4.66
3	6667 (266)	1371 (49)	4.86
4	5783 (321)	1212 (84)	4.77
5	5983 (251)	1270 (45)	4.71
PLTP			
1	7582 (197)	1644 (95)	4.61
2	9655 (391)	2015 (51)	4.79

observed with VLDL* + HDL in our system was 233 min, and the addition of 600 units of PLTP reduced this time to 111 min. If, on the other hand, α-T equilibration was studied in HDL* + VLDL, $\tau/2$ values were 151 and 58 min respectively. If LDL* was incubated with HDL, the values for $\tau/2$ in the absence and presence of 600 units of PLTP were 120 and 39 min respectively. The shortest $\tau/2$ was observed if HDL* was incubated with LDL plus 600 units of PLTP (21.6 min). From these experiments we concluded that α-T is most efficiently exchanged with other lipoproteins if present in HDL, irrespective of whether PLTP is present. In the presence of 600 units of PLTP, however, the speed of the exchange is increased by a factor of 3–4.

The specific activity of [^{14}C]α-T at equilibrium (after 24 h incubation) in the donor/acceptor lipoproteins was identical in all cases, whether the incubation was performed with or without added PLTP; only the equilibration time decreased significantly in the presence of PLTP (results not shown).

In order to study the concentration-dependence of α-TET activity, increasing amounts of PLTP were added to a mixture of LDL* and HDL, and half-equilibration times were measured (Figure 3). In the absence of added PLTP, $\tau/2$ was 121.1±4.3 min. Increasing amounts of PLTP from 300 to 1200 units reduced $\tau/2$ in a dose-dependent manner to 23.4±1.2 min. In a similar way we tested the PLTP concentration-dependence of $\tau/2$ in the incubation of HDL* with LDL and obtained comparable results (not shown).

Transfer of α-T from liposomes to HDL

We were also interested to see whether PLTP catalyses the transfer of α-T from artificial liposomes to HDL in a similar manner to PC. For this part of our study, two separate liposome substrates were prepared, one with PC and the other with α-T, as described in the Materials and methods section. With these substrates the transfer rates of PC and of α-T were measured using the $d > 1.22$ bottom fraction of five fresh human sera samples, as well as of two different PLTP preparations as a source of activity. The results are displayed in Table 5. With PC liposomes, the $d > 1.22$ bottom fractions exhibited a transfer rate ranging between 4837 and 6667 nmol·ml^{-1}·h^{-1}. The corresponding α-T transfer rates were 1037–1371 nmol·ml^{-1}·h^{-1} and the mean ratio of PLTP:α-T activity was 4.76. With purified PLTP PC:α-T transfer ratios of 4.61–4.79 were obtained. We take this as an additional indication of the identity of α-TET and PLTP activities in human serum.

Experiments with RBC

In order to test whether PLTP facilitates α-T exchange also between lipoproteins and cells, experiments were carried out with RBC. Freshly isolated RBC from fasting human blood were incubated for 24 h with [^{14}C]α-T-labelled lipoproteins in the presence or absence of different amounts of PLTP or human $d > 1.22$ g/ml bottom fraction. In the absence of PLTP, the $\tau/2$ values obtained with VLDL*, LDL* and HDL* were 457, 309 and 221 min respectively (Table 6). The addition of 290 units of PLTP/ml had little influence on α-TET from VLDL* to RBC, but stimulated significantly α-TET between LDL* and HDL* to RBC. A very efficient α-TET was observed between HDL* and RBC at 580 units PLTP/ml ($\tau/2$, 97 min).

α-TET activity in ten normolipemic volunteers

To obtain a rough estimate of the variations of α-T exchange activities, fasting sera of five male and five female healthy volunteers were tested and F_n values (α-TET activity normalized

Table 6 Influence of PLTP on the exchange of α-T between lipoproteins and RBC

Washed, sedimented RBC (400 μl) were mixed with 1 mg of VLDL*, LDL* or HDL* containing 4.7–5.2 nmol of α-T, various amounts of purified PLTP, or alternatively $d > 1.22$ g/ml bottom fraction from human serum, and made-up to 1 ml with buffer A. In control experiments PLTP was omitted. Values of $\tau/2$ were calculated as described in the Materials and methods section. The results are means of three different experiments (±S.D.).

Amount of PLTP added	$\tau/2$ (min) RBC + VLDL*	RBC + LDL*	RBC + HDL*
0 (Buffer A)	457 (12.2)	309 (10.1)	221 (7.0)
290 units	450 (15.4)*	248 (7.2)†	151 (6.7)†
580 units	401 (9.8)†	215 (6.6)†	97 (4.1)†

* Not significantly different from control experiment (buffer A).
† Significantly different from control experiment (buffer A); $P < 0.002$.

Table 7 α-TET activities of ten healthy volunteers as compared with PLTP and CETP activities

The serum of five male (m) and five female (f) healthy persons was collected and immediately assayed for α-TET, PLTP and CETP activities as described in the Materials and methods section. TC, total cholesterol; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; TG, triglycerides; F_n, α-TET activity normalized to 10 nmol of α-T per ml of serum; F_s, spontaneous α-TET activity.

	TC (mg/l)	LDL-C (mg/l)	HDL-C (mg/l)	TG (mg/l)	α-T (μmol/l)	[^{14}C]α-T in LDL at equil. (%)	PLTP (μmol · ml^{-1} · h^{-1})	CETP activity (nmol · ml^{-1} · h^{-1})	α-TET activity, F_n (nmol · ml^{-1} · h^{-1})	$F_n - F_s$ (nmol · ml^{-1} · h^{-1})
R.M. (f)	2280	980	340	4810	47.7	53	5.79	23.6	3.72	2.82
S.I. (f)	2200	1130	450	3090	35.8	56	6.26	20.9	3.43	2.53
G.G. (f)	1910	1160	570	920	23.6	57	5.56	24.3	2.63	1.73
S.M. (f)	1910	1000	800	620	24.1	59	7.38	19.7	3.20	2.30
W.I. (f)	1900	900	870	630	30.6	59	6.27	23.0	4.54	3.64
K.G. (m)	1980	1370	470	720	32.4	59	4.61	22.5	3.27	2.37
I.A. (m)	2050	1400	530	510	30.7	59	5.64	19.9	3.39	2.49
M.E. (m)	2040	1310	400	1640	33.3	57	6.54	15.0	2.97	2.07
L.G. (m)	1760	1040	490	1140	24.1	57	6.44	14.6	4.71	3.81
G.H. (m)	2130	1560	320	1270	29.8	49	7.53	24.7	1.67	0.77
Mean	2016.00	1185.00	524.00	1535.00	31.20	56.50	6.20	20.82	3.35	2.45
S.D.	156.70	215.70	182.10	1379.60	7.16	3.24	0.87	3.61	0.88	0.88

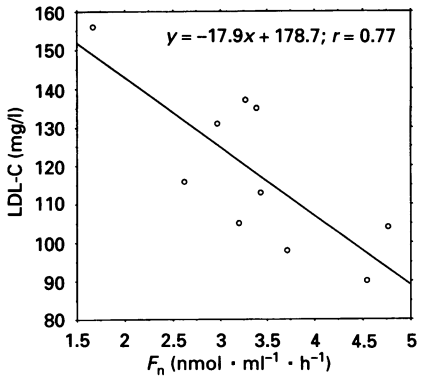

Figure 4 Correlation of the α-TET activity with LDL-C

α-TET activity, F_n from Table 7 was correlated with LDL-C. The correlation was statistically significant ($P < 0.01$).

to 10 nmol of α-T per ml of serum) were calculated as outlined in the Materials and methods section (Table 7). Two of the subjects studied were hypertriglyceridemic, and two others had HDL-C values above the 90th percentile of the local population. The mean F_n values of these subjects were 3.35 ± 0.88 nmol · ml^{-1} · h^{-1} (min. 1.67; max. 4.71), indicating large interindividual variation. The corresponding $F_n - F_s$ (α-TET activity corrected for spontaneous exchange) were 2.45 ± 0.88 nmol · ml^{-1} · h^{-1}. PLTP and CETP activities of the ten serum samples were also determined. A statistical evaluation of the results yielded no correlation of α-TET activity either with PLTP or with CETP. A significant negative correlation was seen between α-TET activity and LDL-C ($r = 0.77$; $P < 0.01$) (Figure 4).

DISCUSSION

It has been reported previously that in normal fasting sera, α-T distributes between VLDL, LDL and HDL in a ratio of 1:1.7:1 [43,44]. In our ten volunteers the distribution was 1.0:1.9:1.4. These ratios are different from those published by Behrens et al. [45] who found a ratio of 1:9.4:8.4. It should be borne in mind that α-T distribution among lipoprotein classes in a given individual varies according to the lipoprotein mass contained in different density classes, the metabolic state, the α-T uptake in the diet and other factors.

The principal aim of the present study was: (1) to assess whether there is any serum factor which might facilitate exchange/transfer processes of α-T between lipoproteins; (2) if this factor exists, is it identical with a known serum lipid exchange/transfer protein; (3) does this factor catalyse also net transfer in an artificial system; and (4) are there interindividual differences in α-TET activities.

First, however, control experiments were carried out in order to ascertain, that (a) the added [^{14}C]-α-T distributed homogeneously within the lipoproteins and (b) that α-T is not oxidized during the 24 h incubation period. Since dialysis of labelled lipoproteins did not alter the specific radioactivity of α-T, the amount of α-T present in the assay mixture did not change during incubation, and since the addition of anti-oxidants had no influence on the observed $\tau/2$, we concluded that (a) the radiolabel distributes homogeneously in the lipoproteins and (b) no oxidation of α-T occurs during the assay.

Experiments to address aim (a) were performed with LDL and HDL_3 since these lipoproteins are readily accessible in sufficient amounts with adequate purity and homogeneity. We observed that radiolabelled α-T readily equilibrated between both fractions in the absence of serum, irrespective whether the label was in LDL or HDL, with an equilibration $\tau/2$ of roughly 140–150 min. Under the conditions tested, $\tau/2$ was approx. 10% shorter if the label resided in HDL instead of LDL. The addition of serum albumin to the LDL–HDL mixture had no effect. However, the $d > 1.22$ g/ml bottom fraction reduced $\tau/2$ in a concentration-dependent manner down to 15 min. To exclude that the increase of the exchange rate is caused by CETP, the $d > 1.23$ g/ml bottom fraction of pig sera, which is known to have very little if any CETP activity, was also used as a source of α-TET activity. Pig bottom fraction was found to contain α-TET activity in amounts comparable with human bottom fraction. From this we concluded that CETP is not responsible for the acceleration in α-T exchange. This is in agreement with findings of Granot et al. [29] who reported on a CETP-independent exchange/transfer of α-T between lipoproteins.

To characterize the factor responsible for the increased α-TET activity, human lipoprotein-free bottom fraction was fractionated by a combination of hydrophobic interaction, heparin–sepharose, cation-exchange and anion-exchange chromatographies. The protein obtained, with an apparent molecular mass of about 75 kDa, was recently characterized as PLTP [33]. When PLTP was added to our assay system in amounts up to 1200 units/ml, $\tau/2$ of α-T was reduced to 23 min without reaching a plateau (Figure 3). The effect of higher PLTP concentrations could not be investigated in our particular test system. There are three reasons why we believe that α-TET activity is mediated by PLTP. (1) Isolated PLTP which was virtually 100% pure exhibited the highest specific α-TET activity. (2) During purification the specific PLTP and the α-T transfer activities correlated very well. (3) The ratios of α-T transfer:PC transfer in $d > 1.22$ g/ml bottom fractions of five normolipemic sera ranged between 4.71 and 4.86 and were comparable to the ratios observed in purified PLTP (Table 4). The observation that this ratio was not 1 may have several reasons. One is certainly related to the differences in the structure and size between PC- and α-T-liposomes; since α-T is reported to be more hydrophobic than PC [32], it is likely that the size and the overall shape of the two liposomes is different, in fact we found in some preliminary experiments that PC liposomes of different size reacted markedly differently with PLTP (M. Jauhiainen, unpublished work). Also, the transport through the aqueous phase, provided this is the mechanism by which PLTP acts, should be faster with PC than with α-T. These assumptions certainly need to be verified by further experiments.

In the present work we mainly measured α-T exchange using naturally occurring lipoproteins which can be assumed to be already in equilibrium with respect to their α-T content. We have also shown that PLTP promotes α-T net transfer from artificial liposomes to HDL in a similar manner to transfer of PC. In order to be of physiological relevance, it would be necessary for such a net transfer to take place *in vivo* as well. Although our experiments were not designed to address this question, we do assume a physiological importance of PLTP for α-T transfer. As mentioned above, vitamin E enters the blood stream via chylomicrons and VLDL, lipoproteins which are partly taken up rapidly by the liver after hydrolysis, and partly enter the LDL pool. In our test system, the spontaneous exchange of α-T between VLDL or LDL and HDL was comparatively slow in the absence of PLTP (Table 3). The same was true for the spontaneous exchange between VLDL and RBC. Spontaneous and especially PLTP-mediated α-T exchange was considerably faster between HDL and RBC. Thus in an open system where all lipids and lipoproteins are metabolized at different rates, a faster equilibration of α-T between lipoproteins and between lipoproteins and cells, mediated by PLTP, should yield an actual net transfer if the donor lipoprotein is catabolized faster than the acceptor.

It should also be remembered that free-radical formation and lipid peroxidation is a fast and continuing process in living organisms, occurring not only in plasma but also in cell membranes and intracellularly. We rationalize that the action of PLTP is relevant *in vivo*, as some lipoprotein fractions, in particular HDL, enter the circulation most probably devoid of α-T [46]. Yet HDL has been demonstrated to serve as a protective factor in many pathological processes [47], in particular in preventing the oxidation of LDL. In a recent report, Bowry et al. [48] demonstrated that HDL carries the highest amount of hydroperoxides of all lipoproteins in human plasma, suggesting that *in vivo* HDL lipids may be more rapidly oxidized than those in LDL or alternatively that HDL may serve as a sink for oxidized lipids. As mentioned before, oxidized α-T is rapidly regenerated by ascorbic acid and other substances [4,7], suggesting that the circulating tocopherol pool may turn over only slowly. In fact it has been shown recently that α-T is eliminated with a half-life of 80.6 ± 19.2 h [31]. This in fact is not the case with γ-tocopherol, which after intravenous infusion is almost completely eliminated within 24 h [44,49]. Thus any mechanism which speeds up the supply of HDL with dietary vitamin E may prevent the initial steps in the lipid auto-oxidation cascade and protect from atherogenesis and probably other diseases. We have shown here that PLTP may act as such a catalyst in α-T equilibration.

It has been reported previously and confirmed also in this study that α-T exchange between LDL and RBC membranes (in the absence of serum factors) is relatively slow, whereas HDL are more effective α-T donors [29]. In our assay the $\tau/2$ of α-T exchange between VLDL and RBC in the absence of activators was 7.5 h and could be reduced to 6.7 h in the presence of 580 units of PLTP. The $\tau/2$ of α-T exchange between HDL and RBC on the other hand was 3.6 h in the absence of PLTP and was reduced to 1.6 h by PLTP. In an on-going study we have found that PLTP also facilitates α-T delivery to cultured human skin fibroblasts (G. M. Kostner, K. Oettl, M. Jauhiainen, C. Ehnholm, H. Esterbauer and M. Dieplinger, unpublished work). These findings underline the potential physiological significance of PLTP and the important role of HDL for supplying cells with α-T. In that respect it is noteworthy that patients suffering from type-I hyperlipoproteinaemia who are known to have very low HDL concentrations exhibit reduced cellular contents of α-T despite the fact that serum α-T levels are increased [50].

We also made preliminary measurements of the α-TET activities in ten volunteers. This was performed with an assay which allows in the shortest possible time the separation of VLDL and LDL by phosphotungstate-precipitation followed by incubation of the supernatant with [^{14}C]α-T-labelled LDL. Ultracentrifugation could not be used for this first step since appreciable amounts of PLTP are present in the HDL_3 as well as in the intermediate fraction between HDL_3 and $d > 1.22$ g/ml fraction [33] and, in addition, since ultracentrifugation caused a variable loss of α-TET activities of up to 50%. As the HDL content of the phosphotungstate supernatant is subject to interindividual fluctuation, variable amounts of labelled LDL were added to keep the LDL:HDL ratio at unity. We are aware that the LDL:HDL mass ratio of 1 does not reflect the situation *in vivo* but for practical reasons this ratio was chosen; in previous experiments we ascertained that the measured α-TET activities expressed in F_n were not dependent on the actual HDL concentration in the different sera but only on the LDL:HDL ratio.

Using this assay, α-TET activities in ten healthy individuals varied in the range 1.67–4.71 nmol·ml^{-1}·h^{-1}, demonstrating interindividual variation by a factor of almost 5. Against our expectation α-TET activities were not significantly correlated with PLTP activities. The lack of correlation between α-TET and PLTP activities might be explained by the gross differences of the two assay procedures. In the PLTP assay, originally described by Jauhiainen et al. [33] and also adopted here, the $d > 1.21$ g/ml lipoprotein-free serum is incubated with artificial liposomes as donor and with pooled HDL as acceptor in a mass ratio of approx. 1:16. In the α-TET assay, endogenous HDL is incubated with radiolabelled LDL at a ratio of 1:1. Therefore, in the latter, possible interindividual differences in the structure and composition of HDL should have an influence on the observed values. If, on the other hand $d > 1.22$ g/ml bottom fractions of five human sera were assayed in comparable systems, PC transfer and α-T-transfer correlated very well (Table 4).

Among the other parameters listed in Table 7, α-TET activity

was correlated only to plasma LDL-C ($r = -0.75$, $P < 0.01$). The physiological significance of this correlation needs to be confirmed in further work.

In summary, we have shown that α-T equilibrates spontaneously between different lipoprotein fractions and between lipoproteins and cells and that HDL plays a major role in the latter process. PLTP, recently shown to cause HDL conversion [33], was found to accelerate the exchange reactions between lipoprotein classes and in particularly between HDL and cell membranes. A role of this protein in atherosclerosis, tumour initiation and promotion and other diseases is proposed.

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