MODULATION OF PHOSPHOLIPID AND CHOLESTERYL ESTER TRANSFER ACTIVITIES BY ALPHA-TOCOPHEROL ADMINISTRATION IN THE HYPERLIPIDEMIC HAMSTER

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Phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP) are key enzymes in the lipid metabolism and play critical roles in cardiovascular diseases. Our goal was to evaluate the activities of PLTP and CETP in the serum of hyperlipidemic (HL) hamsters and the effect of alpha-tocopherol administration in this animal model. Male Golden Syrian hamsters received standard chow or chow supplemented with 20% butter and either 0.1%, or 0.5% cholesterol. Alpha-tocopherol was administrated by gavage for 4 weeks to HL and control hamsters. Sera from animals were analyzed for changes in: lipid parameters, apoE concentration, PLTP and CETP activities. Hamsters fed a HL diet for 8 weeks developed significantly increased cholesterol, triglycerides and LDL-C levels, while the ratio of HDL/total cholesterol was significantly decreased. PLTP and CETP activities were significantly increased in all HL hamsters, but PLTP activity was less modulated by the high serum cholesterol levels. ApoE levels were also increased in the sera from HL hamsters, but were not correlated with the increased PLTP activity. Alpha-tocopherol administration had no significant effect on PLTP activity or apoE concentration, but significantly inhibited CETP activity, probably by reducing serum cholesterol.

Key words: Phospholipid transfer protein; Cholesteryl ester transfer protein; High fat diet; Hyperlipidemia; Alpha-tocopherol.

INTRODUCTION

The transfer of lipids between lipoproteins, and between lipoproteins and the cell membranes is mainly regulated in human serum by the activity of phospholipid transfer protein (PLTP) and cholesteryl ester transfer protein (CETP). PLTP and CETP are products of the lipid transfer/LBP gene family, that also includes the lipopolysaccharide binding protein (LBP) and bactericidal/permeability increasing protein (BPI).

Increased PLTP and CETP activities are risk factors for patients with cardiovascular diseases[1,2]. Both lipid transfer proteins play an important role in the remodeling of HDL particles and in HDL metabolism; they also exert important effects on the metabolism of apolipoprotein B 100- containing lipoproteins[3]. There are numerous data concerning the *in vitro* transfer of lipids exerted by these two proteins, but their overall impact on the lipoprotein metabolism *in vivo* and on the atherosclerotic process are complex and remain poorly understood.

An important property of PLTP is to catalyze exchange/transfer processes of alpha-tocopherol (vitamin E) between lipoproteins and cells membranes[4]; this process affects the oxidative status of the atherogenic lipoproteins[5] and of various tissues[6-9].

PLTP activity was identified in all the species studied so far, while CETP is absent in some of the species[10,11], for example it is not naturally occurring in the mouse, the most frequently used animal model for atherosclerosis studies[12]. Unlike mice, and much like humans, hamsters possess both transfer activities in plasma and develop atherosclerosis by administration of hyperlipidemic (HL) diets[13]. These characteristics make this animal a suitable model to study the modulation of lipid transfer activities *in vivo* during hyperlipidemia.

Our goal was to evaluate the modulation of the PLTP and CETP activity in the sera of hamsters fed high fat diets, and the effect of alpha-tocopherol administration on their activity.

METHODS AND MATERIALS

Animals

Male Golden Syrian hamsters (*Mesocricetus auratus*), (n=16, 100120 g body weight) received standard chow (control, n=8) or chow supplemented with 20% butter and 0.1% cholesterol for 8 weeks, followed by an additional 12 weeks feeding with 20% butter and 0.5% cholesterol (diet, n=8). Alpha-tocopherol (500 mg/kg b.w.) was administrated daily by gavage for 4 weeks, starting after 16 weeks of diet. For the gavage experiments, hamsters were randomly distributed in 4 groups, each consisting of 4 animals. Blood samples were collected from the retro-orbital sinus under isoflurane anesthesia, after 12 hours of fasting. All procedures were develope performed in accordance with institutional ethical guidelines regarding animal care.

Serum parameters assays

Serum was isolated by keeping the blood samples to clot at 37^{o}C, followed by centrifugation at 2000g at 4°C for 5 minutes. The following serum parameters were assayed: cholesterol, triglycerides, LDL-C, HDL-C, using commercially available kits.

Total peroxyl radical-trapping potential (TRAP)

TRAP was determined as described by Niculescu *et al.*[14]. Briefly, serum was incubated with DCFH-DA (2, 7-dichlorofluorescein-diacetate), on ice, and the reaction was started by adding AAPH [2,2'-diazobis (2-amidinopropane) dihydrochloride]. The reaction between the free radicals hydrophilic source (AAPH) and DCFH leads to oxidation products spectrophotometrically detectable (at 504 nm). The TRAP values were calculated as:

$$TRAP = (T_{Serum}/T_{Trolox}) \times serum\ dilution\ factor \times 2 \times [Trolox];$$

where, T_{Serum} is the length of the first lag phase, due to the serum, T_{Trolox} is the length of the second lag phase, due to Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxilic acid) and 2 is the number of the peroxyl radicals molecules trapped by each Trolox molecule. The resulting TRAP values were expressed as free radicals, in μmoles, trapped by 1 liter of serum.

Transfer protein activities

PLTP and CETP activities were assessed in serum using commercially available kits from BioVision (California, USA). Activity Assay Kits use a donor molecule containing a fluorescent self-quenched phospholipid or a neutral lipid that is transferred to an acceptor molecule in the presence of PLTP or CETP, respectively. PLTP or CETP-mediated transfer of the fluorescent molecule to the acceptor molecule results in an increase in fluorescence (Excitation: 465 nm; Emission: 535 nm).

Western blot analyses of serum apolipoprotein E

Hamster serum (0.5 μl) was mixed with Laemmli buffer and subjected to 10% SDS-PAGE, transferred onto nitrocellulose and the protein was detected using specific rabbit anti-hamster apoE IgG from Abcam (Cambridge, UK), followed by goat anti rabbit IgG-HRP to visualize the apoE protein. The bands were quantified by densitometry using the software TL100 from Nonlinear Dynamics (Newcastle, UK).

Statistical Analysis

Results are expressed as mean ± SD. The statistical analyses were performed using **SPSS 10**. For p values <0.05, the population means are statistically different; * p<0.05, ** p<0.01, *** p<0.001.

RESULTS

Hamsters fed a chow diet supplemented with 0.1% cholesterol and 20% butter develope mild hyperlipidemia as reflected in serum lipid parameters: higher cholesterol (chow diet 116 ± 6 mg/dl, high fat diet 211 ± 41 mg/dl; p<0.001) and triglycerides (chow diet 127 ± 40 mg/dl, high fat diet 219 ± 65 mg/dl; p<0.05), and lower HDL-cholesterol per total cholesterol ratio (chow diet 0.67 ± 0.09; high fat diet 0.53 ± 0.08; p<0.05). Hamsters fed the chow diet supplemented with 0.5% cholesterol and 20% butter had a more advanced stage of hyperlipidemia. Some of the serum lipid values were highly increased compared with the corresponding values in the group fed 0.1% cholesterol and 20% butter: cholesterol from 211 ± 41 mg/dl (0.1% CHOL) to 646 ± 123 mg/dl (0.5% CHOL), p<0.001, triglycerides from 219 ± 65 mg/dl (0.1% CHOL) to 743 ± 286 mg/dl (0.5%CHOL), p=0.001; while a decrease was observed for: HDL-cholesterol per total cholesterol ratio from 0.53 ± 0.08 (0.1% CHOL) to 0.21 ± 0.08, p<0.001; and TRAP values from 614 ± 90 μM (0.1% CHOL) to 401 ± 55 μM (0.5% CHOL), p=0.001 (Table 1).

Table 1

Serum parameters of hamsters fed for 8 weeks with standard chow diet and chow diet supplemented with 0.1% or 0.5% cholesterol and 20% butter[1, 2]

Serum parameters	Chow (n=6)	0.1% CHOL (n=6)	0.5% CHOL (n=6)	p-value*	p-value**
Cholesterol (mmg/dl)	116 ± 6	211 ± 41	646 ± 123	<0.001	<0.001
Triglycerides (mg/dl)	127 ± 40	219 ± 65	743 ± 286	<0.05	0.001
LDL-Chol (mg/dl)	69 ± 11	74 ± 26	96 ± 61	NS	NS
HDL-Chol (mg/dl)	78 ± 9	112 ± 26	134 ± 48	<0.05	NS
LDL-Chol/Chol	0.60 ± 0.11	0.35 ± 0.11	0.17 ± 0.12	<0.01	<0.05
HDL-Chol/Chol	0.67 ± 0.09	0.53 ± 0.08	0.21 ± 0.08	<0.05	<0.001
TRAP (μM)	694 ± 45	614 ± 90	401 ± 55	NS	0.001

[1] Values are means ± SD. Asterisks indicate differences among groups: * hamsters fed chow diet and hamsters fed chow diet supplemented with 0.1% cholesterol and 20% butter; ** hamsters fed chow diet supplemented with 0.1% cholesterol and 20% butter and hamsters fed chow diet supplemented with 0.5% cholesterol and 20% butter.
[2] To convert values for total cholesterol, HDL cholesterol and LDL cholesterol from mg/dL to mmol/L, divide by 38.65. To convert triglycerides to mmol/L, divide by 88.54.

PLTP and CETP activities were assayed in serum from hamsters fed standard chow supplemented with 0.1% cholesterol and 20% butter for 8 weeks and with 0.5% cholesterol and 20% butter up to 20 weeks. Both lipid transfer activities were increased as a function of time, but after 8 weeks of diet PLTP activity reached a plateau that was maintained even after 12 weeks of increased cholesterol concentration in the diet (0.5%), (Fig. 1A). In contrast, CETP activity had a constant increase, which continued even after the cholesterol concentration in the diet was increased to 0.5% (Fig. 1B).

In hamsters fed 0.1% cholesterol and 20% butter PLTP and CETP activities are highly correlated (r = 0.813), (Fig. 2A).

Both lipid transfer activities were also positively correlated with serum cholesterol levels: r = 0.929 for PLTP activity and r = 0.811 for CETP activity (Fig. 2, B and C).

Apolipoprotein E (apoE) levels were assayed by semiquantitative Western blot analysis in serum from hamsters fed chow diet or high fat diets. As illustrated in Figure 3A, the diet containing 0.1% cholesterol and 20% butter did not induce an increase in serum apoE compared with the control group. A 3.5 fold increase in apoE levels was observed after 8 weeks of chow diet supplemented with 0.5% cholesterol and 20% butter, compared with hamsters fed chow diet (Fig. 3B).

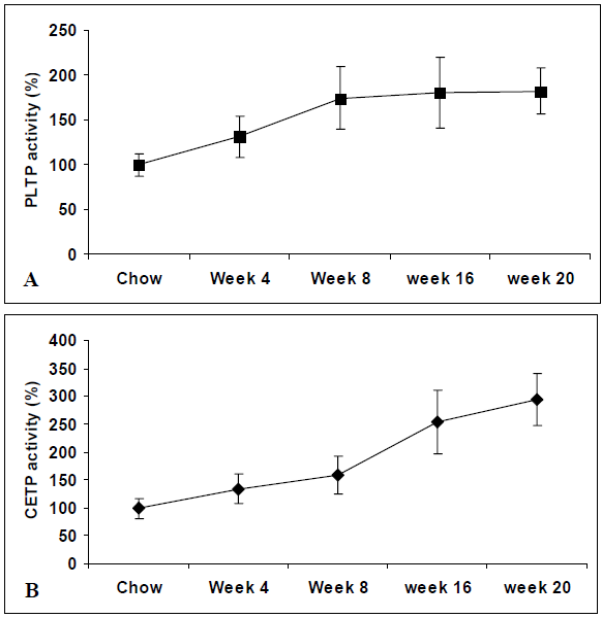


Fig. 1. Variation of PLTP and CETP activities in the serum of hyperlipidemic hamsters along the weeks of diets: (A) PLTP activity; (B) CETP activity; hamsters were fed standard chow supplemented with 0.1% cholesterol and 20% butter for 8 weeks and with 0.5% cholesterol and 20% butter up to 20 weeks. Values are expressed as percentage (%) of the control group activities at the same time of diet. Bars represent means ± SD of 6 animals.

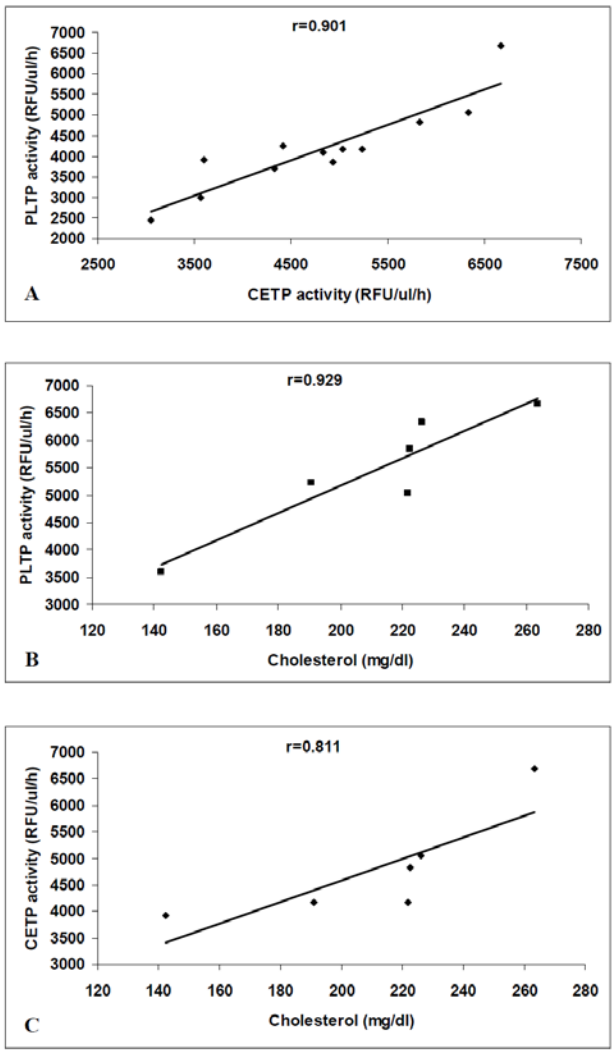
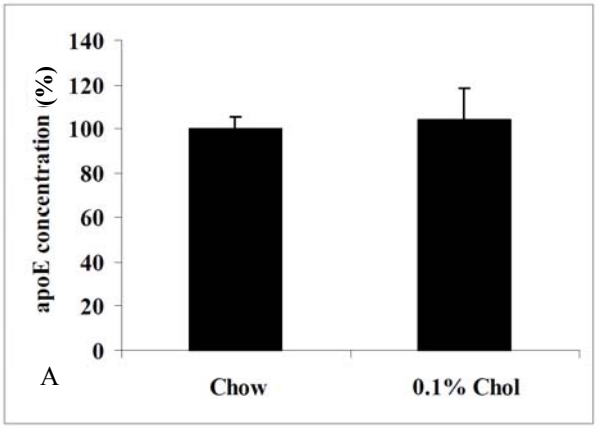


Fig. 2. Correlation between PLTP and CETP activities and cholesterol concentration in serum from hamsters fed 0.1% cholesterol and 20% butter diet: (A) correlation between PLTP and CETP activities week 4 and week 8, r = 0.813; (B) correlation between PLTP activity and cholesterol concentration week 8, r = 0.929; (C) correlation between CETP activity and cholesterol concentration week 8, r = 0.811.



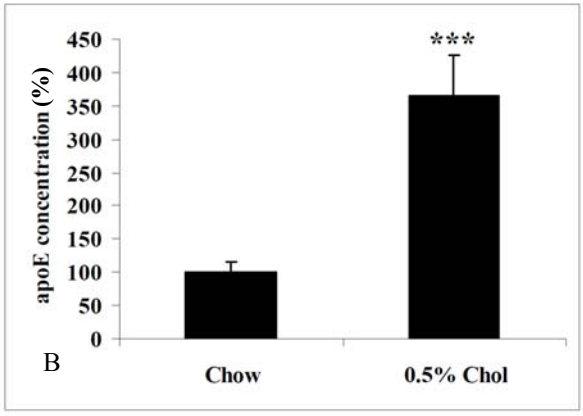


Fig. 3. Western blot analysis of ApoE in the sera from control and hyperlipidemic hamsters; effect of cholesterol concentration in the high fat diet: (A) ApoE protein expression in sera from hamsters fed chow diet supplemented with 0.1% cholesterol and 20% butter; (B) ApoE protein expression in sera from hamsters after 8 weeks of chow diet supplemented with 0.5% cholesterol and 20% butter. Values represent percent of the apoE band quantification using TL-100 software. Bars represent means ± SD of 3 to 5 animals.

Alpha-tocopherol was administrated to hamsters fed chow diet and chow diet supplemented with 0.5% cholesterol and 20% butter. As illustrated in Figure 4, after 4 weeks of administration alpha-tocopherol induced a statistically significant increase of the antioxidant potential of the serum, as shown by the increased TRAP values: from 531 ± 18 μM in the control group to 655 ± 15 in the treated group, $p<0.001$ (chow diet) and from 357 ± 31 μM in the control group to 438 ± 31 μM in the treated group, $p<0.05$ (high fat diet). In hamsters fed high fat diet alpha-tocopherol administration for 4 weeks induced a significant decrease ($p<0.05$) in serum total cholesterol levels, from 568 ± 72 mg/dl in the control group to 379 ± 106 mg/dl in the treated one (Fig. 5).

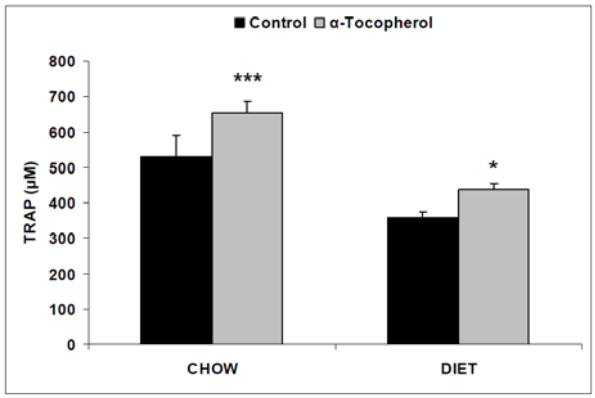


Fig. 4. TRAP values in the sera from control and HL hamsters receiving alpha-tocopherol for 4 weeks. Bars represent means ± SD of 4 animals.

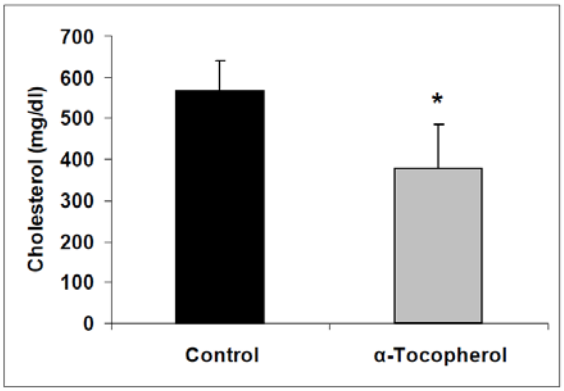


Fig. 5. Effects of alpha-tocopherol administration on serum cholesterol concentration in HL hamsters. Serum cholesterol concentration was determined in hamsters fed fat diet receiving alpha-tocopherol by oral gavage for 4 weeks. Bars represent means ± SD of 4 animals.

As depicted in Figure 6A, alpha-tocopherol treatment for 4 weeks had no significant effect on serum PLTP activity, neither in hamsters fed chow diet, nor in hamsters fed the high fat diet. In hamsters fed chow diet, alpha-tocopherol also failed to induce a modification in CETP activity, but a decrease of 40% in CETP activity ($p<0.01$) was observed in the treated HL hamsters, as compared with the non-treated HL group (Fig. 6B).

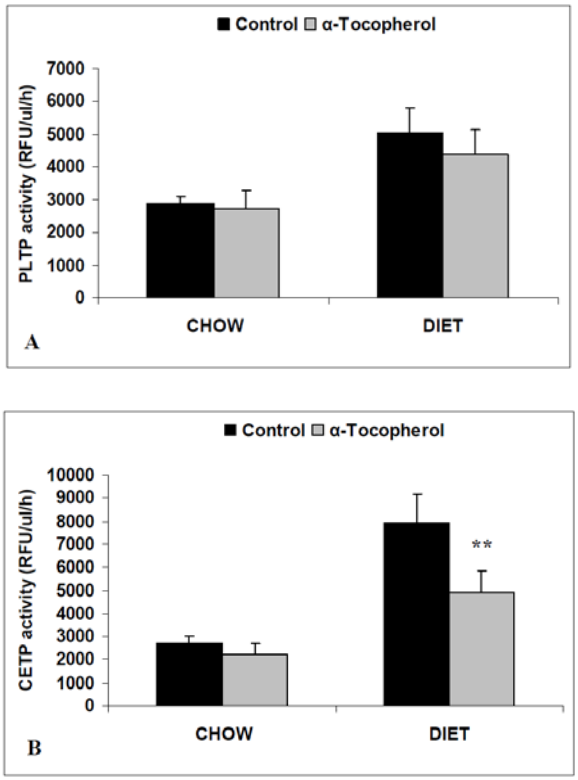


Fig. 6. Effect of alpha-tocopherol administration on PLTP and CETP activities from the sera of hyperlipidemic hamsters. Alpha-tocopherol was administered to hamsters fed chow or fat diets for 4 weeks (A) PLTP activity; (B) CETP activity; Bars represent means ± SD of 4 animals.

DISCUSSION

The present study aimed to investigate the *in vivo* modulation of PLTP and CETP activities. We used the hyperlipidemic Golden Syrian hamster, an animal model that develops atherosclerotic lesions similar to humans after receiving high fat diets[13]. It has been previously shown by Dorfman *et al.* that both PLTP and CETP activities are increased in Golden Syrian hamsters fed chow diet supplemented with 0.1% cholesterol and 10% butter for 6 weeks[15]. Our results show also an increase in PLTP and CETP activities. Moreover, we observed a positive correlation between the two lipid transfer activities at 4 and 8 week of chow diet supplemented with 0.1% cholesterol and 20% butter. From this point on, the correlation is lost as the PLTP activity reached a plateau, and the activity remained constant even after 12 weeks of 0.5% cholesterol in the diet.

In contrast, CETP activity continuously increased until the end of our experiments. *In vivo*, PLTP activity seems to be regulated in a more complex manner compared to that of CETP, which make them respond differently to variations in serum cholesterol levels. In human plasma, PLTP exists in two forms: one with high activity and the other with low activity[16]. These two forms are associated with macromolecular complexes of different apparent sizes: 512kDa for the low activity form and 160kDa for the high activity form, respectively[17]. Mechanisms for the co-existence of the two forms of PLTP are currently unknown.

Several groups have reported a correlation between serum PLTP activity and serum apoE concentration[18, 19]. In our experiment, we observed that apoE increases in the sera of hamsters fed chow diet supplemented with 0.5% cholesterol and 20% butter, but not in those fed with chow diet supplemented with lower (0.1%) cholesterol and 20% butter. The increase in serum apoE seems to coincide with the plateau level of PLTP activity. Further experiments are necessary to evaluate the relation between apoE and PLTP.

The observed responses of PLTP and CETP activities to increased serum cholesterol levels can be explained by differences in the regulation of their gene expression or by certain serum factors that can modulate their activities. However, detailed mechanisms remain to be established. The correlation between the transfer activities and the cholesterol concentrations are in accordance with the fact that the synthesis of PLTP and CETP are

under the control of Liver X receptors[20, 21]. It is well known that Liver X receptors are highly activated in hypercholesterolemia by the formation of oxysterols[22], as is the case with HL hamsters.

In vitro and *in vivo* experiments support the role of PLTP in alpha-tocopherol metabolism. It was shown that PLTP regulates the bioavailability of alpha-tocopherol in the atherogenic lipoproteins[5]. In addition, PLTP deficiency decreases liver alpha-tocopherol content, increases the hepatic oxidant status, and substantially enhances the reactive oxygen species (ROS)-dependent degradation of newly synthesized apoB-100, via a post-endoplasmic reticulum process[9]. Numerous studies have shown that alpha-tocopherol can modulate signal transduction and gene expression[23], affecting in this way the function of different tissues. Recently, it was shown that incubation of cultured hepatocytes with alpha-tocopherol alters the expression of the genes that encode enzymes which catalyze key steps in the de novo biosynthesis of cholesterol, and that these effects are not due to alpha-tocopherol's antioxidant effect[24]. In regard to these data, we also investigated whether alpha-tocopherol can modulate the serum phospholipid transfer activity, in both normal and hyperlipidemic hamsters. After 4 weeks of alpha-tocopherol administration, the serum antioxidant potential was increased in both normal and hyperlipidemic hamsters, demonstrating that alpha-tocopherol was effectively assimilated and active *in vivo*. In parallel, we observed an inhibition of CETP activity, this result being in agreement with a previous report by Shen *et al.*[25]. Our hypothesis is that the lowering of CETP activity is probably due to the decrease of serum cholesterol levels induced by alpha-tocopherol in the treated group. Supporting data obtained in treated hamsters fed chow diet show no significant reduction neither in CETP ($p=0.158$) activity, nor in serum cholesterol level ($p=0.215$), but an increase of the antioxidant potential ($p < 0.001$). This observation supports the hyperlipidemic status as a need for the efficient response to alpha-tocopherol treatment, its hypocholesterolemic effect being a pre-requisite. PLTP activity was not significantly decreased by alpha-tocopherol administration neither in hamsters fed chow diet, nor in hamsters fed the high fat diet. As our data demonstrate, increase of serum cholesterol concentration over 210 ± 40 mg/dl does not significantly affect PLTP activity. After 4 weeks of alpha-tocopherol supplementation, cholesterol values in HL hamsters decreased from 568 ± 72 mg/dl to 379 ± 106 mg/dl, which most probably is insufficient to induce a decrease in PLTP activity.

CONCLUSIONS

PLTP and CETP activities are increased in HL hamsters, but the two transfer activities appear to be modulated differently by the serum cholesterol levels. PLTP activity is less responsive to variations of serum cholesterol concentrations, in contrast with CETP activity. Alpha-tocopherol administration to HL hamsters has a positive effect, by the reduction serum CETP activity, probably due to the decrease of the serum cholesterol concentration.

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