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Plasma phospholipid transfer protein prevents vascular endothelium dysfunction by delivering α -tocopherol to endothelial cells

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ABSTRACT α -tocopherol, the most potent antioxidant form of vitamin E, is mainly bound to lipoproteins in plasma and its incorporation into the vascular wall can prevent the endothelium dysfunction at an early stage of atherogenesis. In the present study, the plasma phospholipid transfer protein (PLTP) was shown to promote the net mass transfer of α -tocopherol from high density lipoproteins (HDL) and α -tocopherol-albumin complexes toward α -tocopherol-depleted, oxidized low density lipoproteins (LDL). The facilitated transfer reaction of α -tocopherol could be blocked by specific anti-PLTP antibodies. These observations indicate that PLTP may restore the antioxidant potential of plasma LDL at an early stage of the oxidation cascade that subsequently leads to cellular damages. In addition, the present study demonstrated that the PLTP-mediated net mass transfer of α -tocopherol can constitute a new mechanism for the incorporation of α -tocopherol into the vascular wall in addition to the previously recognized LDL receptor and lipoprotein lipase pathways. In *ex vivo* studies on rabbit aortic segments, the impairment of the endothelium-dependent arterial relaxation induced by oxidized LDL was found to be counteracted by a pretreatment with purified PLTP and α -tocopherol-albumin complexes, and both the maximal response and the sensitivity to acetylcholine were significantly improved. We conclude that PLTP, by supplying oxidized LDL and endothelial cells with α -tocopherol through a net mass transfer reaction may play at least two distinct beneficial roles in preventing endothelium damage, i.e., the antioxidant protection of LDL and the preservation of a normal relaxing function of vascular endothelial cells.—Desrumaux, C., Deckert, V., Athias, A., Masson, D., Lizard, G., Palleau, V., Gambert, P., Lagrost, L. Plasma phospholipid transfer protein prevents vascular endothelium dysfunction by delivering α -tocopherol to endothelial cells. *FASEB J.* 13, 883–892 (1999)

Key Words: acetylcholine · aorta · lipid transfer protein · oxidized LDL · vitamin E

IN THE EARLY steps of atherosclerosis, oxidized low density lipoproteins (LDL)² can impair the endothelium-dependent arterial relaxation (1–3). Recent investigations reported that lysophosphatidylcholine (4, 5) and cholesterol oxides (6) can mimic the inhibitory effect of oxidized LDL, and these compounds were shown to significantly impair the release of nitric oxide (i.e., the main endothelium-derived relaxing factor (7, 8)) by human vascular endothelial cells (9). Interestingly, the endothelial dysfunction mediated by oxidized LDL in cholesterol-fed animals can be counteracted by the dietary intake of lipophilic antioxidants (10, 11) that leads to the rise in the antioxidant status of the vascular tissue (12–14). Keaney and co-workers (12) proposed that both β -carotene and α -tocopherol in the vascular wall might preserve the endothelium-dependent arterial relaxation by protecting nitric oxide (NO) from inactivation by superoxide anion rather than by reducing the susceptibility of LDL to undergo oxidative transformations. Since the beneficial effect of α -tocopherol was shown to be strictly dependent on its tissue incorporation (14), the transfer of dietary α -tocopherol from the plasma compartment to endothelial cells appears to constitute a major determinant of its ability to prevent endothelium dysfunction. Because of its hydrophobicity, α -tocopherol cannot be solubilized in aqueous fluids and it is mainly transported in association with lipoproteins in the plasma compartment (15–18). All plasma lipoproteins can constitute α -tocopherol vehicles, and the contribution of distinct plasma lipoprotein

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² Abbreviations: ACh, acetylcholine; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; EC₅₀, concentration required to produce a half-maximal relaxing effect; Emax, maximal relaxation; EDTA, ethylenediaminetetracetic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase; NE, norepinephrine; PLTP, phospholipid transfer protein; TBS, Tris-buffered saline.

fractions to α -tocopherol transport actually depends on their relative proportions in one given plasma sample (15, 19).

In contrast to the intracellular transport of α -tocopherol that involves a unique and specific factor (i.e., the α -tocopherol transfer protein) (20), the incorporation of extracellular α -tocopherol into the tissues would relate to the combination of several processes rather than to one single, specialized pathway. In earlier studies, both the LDL receptor (21) and the lipoprotein lipase (LPL) (22) were shown to facilitate the tissue incorporation of plasma α -tocopherol as part of LDL and triglyceride-rich lipoproteins, respectively. More recently, Kostner and co-workers demonstrated that the plasma phospholipid transfer protein (PLTP), which is known to catalyze the exchange of phospholipids and other amphipathic compounds (23–26), can also mediate the rapid exchange of radiolabeled α -tocopherol between lipid structures in the intravascular compartment (27). Although these observations suggest a new molecular mechanism for α -tocopherol transfer, their relevance to the delivery of α -tocopherol to endothelial cells and to the prevention of vascular endothelium dysfunction is unknown.

In a first part of the present study, we searched for the ability of PLTP to promote the net mass transfer of α -tocopherol between lipoproteins and cultured endothelial cells. To this end, alterations in lipoprotein and cellular α -tocopherol contents were assessed through the direct quantitation of α -tocopherol mass. In a second part of the study, the impact of PLTP-mediated α -tocopherol fluxes on the impairment of the endothelium-dependent arterial relaxation by oxidized LDL was addressed.

MATERIALS AND METHODS

Chemical compounds

Acetylcholine (ACh), butylated hydroxytoluene (BHT), ethylenediaminetetraacetic acid (EDTA), (\pm)-arterenol hydrochloride (NE), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Anti-PLTP polyclonal antibodies

Specific anti-PLTP antiserum was obtained by immunization of a New Zealand White rabbit with purified human PLTP, and the serum immunoglobulin G (IgG) fraction was prepared by using a protein A column (protein A Sepharose 4 Fast Flow, Pharmacia, Uppsala, Sweden).

Isolation of LDL and HDL particles

Fresh citrated plasma from normolipidemic subjects was provided by the Centre de Transfusion Sanguine (Hôpital du Bocage, Dijon, France). LDL were isolated as the $1.019 < d < 1.063$ g/ml plasma fraction by sequential ultracentrifugation

at 45,000 rpm (149,000 *g*) in a 70-Ti rotor in an L7 ultracentrifuge (Beckman, Palo Alto, Calif.), with one 24 h spin at the lowest density and one 22 h spin at the highest one. The LDL fraction was then washed with one 6-h, 90,000-rpm (561,000 *g*) spin at the density of 1.063 g/ml in an NVT-90 rotor in an XL-90 ultracentrifuge (Beckman). High density lipoproteins (HDL) were isolated as the $1.063 < d < 1.210$ g/ml plasma fraction at a speed of 55,000 rpm (223,000 *g*) in a 70-Ti rotor in an L7 ultracentrifuge; two 20-h spins were conducted at the lowest density and one 30-h spin was performed at the highest density. The HDL fraction was finally washed with one 8-h spin at the density of 1.21 g/ml, at a speed of 90,000 rpm (561,000 *g*) in an NVT-90 rotor in an XL-90 ultracentrifuge. Densities were adjusted by the addition of solid potassium bromide (KBr). The isolated lipoproteins were dialyzed overnight against a Tris-buffered saline solution (TBS) (10 mmol/l Tris, 150 mmol/l NaCl, pH 7.4).

Oxidation of lipoproteins

The oxidation of LDL and HDL was performed by incubating freshly prepared lipoproteins adjusted to 2 g protein/l in TBS with a copper sulfate solution (final concentration, 5 μ mol/l) for a few minutes up to 24 h at 37°C. Oxidation was stopped by the addition of EDTA (final concentration, 200 μ mol/l) and BHT (final concentration, 20 μ mol/l). '90 min-oxidized' LDL or HDL were dialyzed against TBS buffer prior to be used for α -tocopherol transfer experiments. For vascular reactivity experiments, '24 h-oxidized' LDL preparations were dialyzed overnight against Krebs' buffer with the following composition (in mmol/l): NaCl 119, KCl 4.7, KH_2PO_4 1.18, MgSO_4 1.17, CaCl_2 2.5, EDTA 0.027, glucose 11, and NaHCO_3 25. Lipoprotein fractions were used within 2 days after their preparation.

Preparation of α -tocopherol-BSA complexes

α -Tocopherol-BSA complexes were prepared by dissolving 100 mg of α -tocopherol in 5 ml ethanol. 200 μ l of α -tocopherol solution was gradually added in drops to 1.8 ml of a continuously stirred solution of human serum albumin (30 mg/ml) in TBS buffer, pH 7.4. The resulting mixture was sonicated for 1 min in an ice bath with a probe-type sonifier and incubated for 1 h at 37°C. At the end of the incubation, the mixture was sonicated 3×1 min in ice, centrifuged for 2 min at 3000 *g*, and filtered through a 22- μ m filter (Millipore). An homogeneous preparation of α -tocopherol-BSA complexes was finally obtained by gel filtration on a Superose 6 HR column on a fast protein liquid chromatography (FPLC) system (Pharmacia). α -tocopherol-BSA complexes were kept at 4°C and used within 24 h after their preparation.

Endothelial cell culture

The human umbilical vein endothelial cell line ECV-304 was obtained from the American Type Culture Collection (ATCC, Rockville, Md.) (28). Endothelial cells were seeded at 10^4 cells/cm² in T75 flasks (Falcon) containing 15 ml of culture medium constituted by Medium 199 with Glutamax (Life Technologies, Inc., Eragny, France) supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim, Meylan, France) and with antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin; Life Technologies, Inc.). Cells were incubated in a humidified atmosphere of air/CO₂ (95:5 v/v) at 37°C. Confluent monolayers of cells taken at 7 days of culture were used in subsequent experiments.

Preparation of PLTP active fraction

PLTP was purified from fresh citrated human plasma. All purification steps were performed on a FPLC system (Pharmacia), according to the sequential procedure previously described (29). Briefly, the $d > 1.21$ g/ml plasma proteins were isolated by a 24-h, 55,000 rpm ultracentrifugation step performed in a 70-Ti rotor in an L7 ultracentrifuge. The resulting infranant was fractionated successively by hydrophobic interaction chromatography on a phenyl-Sepharose CL-4B column (Pharmacia), by affinity chromatography on a heparin-Ultrogel A4R column (Sepracor, Villeneuve-la Garenne, France), and by anion exchange chromatography on a MonoQ HR 5/5 column (Pharmacia). This procedure allowed to obtain a PLTP preparation with a specific phospholipid transfer activity corresponding to an ~ 1000 -fold increase as compared with total plasma. The PLTP preparation was dialyzed against TBS or Krebs' buffer in experiments involving cultured endothelial cells and arterial segments, respectively.

α -Tocopherol net mass transfer experiments

Lipoprotein incubation protocol

Freshly isolated native or mildly, 90 min-oxidized LDL (1 g/l of LDL protein) were incubated for 1 h at 37°C with either plasma HDL (0.5 g/l of HDL protein; 10 μ mol/l of α -tocopherol) or α -tocopherol-BSA complexes (30 μ mol/l of α -tocopherol) in the absence or in the presence of purified PLTP (10 μ g/ml). Alternatively, 90 min-oxidized HDL (0.5 g/l of HDL protein) were incubated for 1 h at 37°C with native plasma LDL (1 g/l of LDL protein) in the absence or in the presence of purified PLTP. Control mixtures were maintained at 4°C. At the end of the incubation, the density was adjusted to 1.068 g/ml and mixtures were ultracentrifuged for 3 h at a speed of 100,000 rpm (350,000 *g*) in a TLA-100.2 rotor in a Beckman TL-100 ultracentrifuge. The $d < 1.068$ g/ml fraction containing LDL and the $d > 1.068$ g/ml fraction containing HDL were recovered in 200- μ l and 500- μ l volumes, respectively.

Endothelial cell incubation protocol

The complete culture medium was replaced by serum-free medium just before the experiments. α -tocopherol-BSA complexes (10 μ mol/l of α -tocopherol) or native plasma HDL (0.5 g/l of cholesterol) were added to the culture medium. PLTP was added in various amounts to the medium and endothelial cells were incubated at 37°C for up to 2 h. At the end of the incubation, the medium was poured off and the cells were scraped from the flasks and washed thoroughly with TBS buffer.

α -tocopherol assay

Lipids were extracted in darkness with an ethanol-hexane solution (1:3, v/v). The hexane fraction was evaporated under nitrogen and recovered in a mixture of methanol-acetonitrile-chloroform (25:60:15, v/v/v). Endothelial cells were sonicated for 10 min in a cold water bath prior to the lipid extraction. Finally, α -tocopherol was assayed by high performance liquid chromatography (HPLC) according to the method described by Miller and Yang (30). The chromatographic analysis was performed by using a Gold HPLC System (Beckman), on a 220 \times 4.6 mm Spheri-5 RP 18 column (Brownlee) that was connected to a diode array

detector (model 168; Beckman). α -tocopherol-acetate was added to each sample as an internal standard before the extraction, and the α -tocopherol contents were determined from the ratio of the peak area of the sample to that of the internal standard.

Determination of vascular reactivity

Preparation of blood vessels

New Zealand White rabbits of either sex, weighing ~ 3 kg were killed by an overdose of pentobarbital sodium via the marginal ear vein. The descending aorta was rapidly removed and transferred into a Krebs' solution bubbled with 95% O₂ plus 5% CO₂. The aortas were cut into 3 mm rings and suspended horizontally between two wire hooks in 20-ml jacketed organ baths containing oxygenated Krebs' solution that was maintained at 37°C. Arterial rings were then preincubated for 1 h with either no addition, α -tocopherol-BSA complexes (10 μ mol/l of α -tocopherol), or α -tocopherol-BSA complexes plus PLTP (10 μ g/ml). At the end of the preincubation period, the organ baths were rapidly washed with Krebs' buffer, and the aortic segments were then incubated for 2 h with oxidized LDL or Krebs' buffer, as indicated. After washout, aortic segments were precontracted with norepinephrine (NE) (0.3 μ mol/l) and progressively relaxed with cumulative doses of acetylcholine (ACh). Alterations of the isometric tension in response to these vasoactive compounds were monitored according to the general procedure previously described (6).

Data analysis

The maximal relaxation (E_{max}) induced by ACh and expressed as a percentage of the contraction to NE (0.3 μ mol/l) was determined from experimental data. EC₅₀ values, corresponding to the concentration required to produce a half-maximal relaxing effect, were calculated after fitting each curve according to a sigmoidal equation of the form

$$Y = P1 + P2 / (1 + e^{P3(\log X - P4)})$$

in which Y is the relaxing effect, expressed as a percentage of the contraction to NE (0.3 μ mol/l); X is the corresponding agonist concentration; P1, the lower plateau response; P2, the range between the lower and the maximal plateaus of the concentration-effect curve; P3, a negative curvature index indicating the slope independently of the range; and P4, log EC₅₀ (31).

Other analyses

Protein concentrations were measured with the bicinchoninic reagent (Pierce) according to Smith et al. (32). The assay was performed on a COBAS-FARA centrifugal analyzer (Roche).

Statistical analysis

Data are expressed as mean \pm SE. The statistical significance of differences between data means was determined by use of analysis of variance with a factorial or a repeated measures design, as appropriate.

RESULTS

Net mass transfer of α -tocopherol between lipoproteins

In a first attempt to evaluate the potential role of PLTP in mediating the net mass transfer of α -tocopherol to

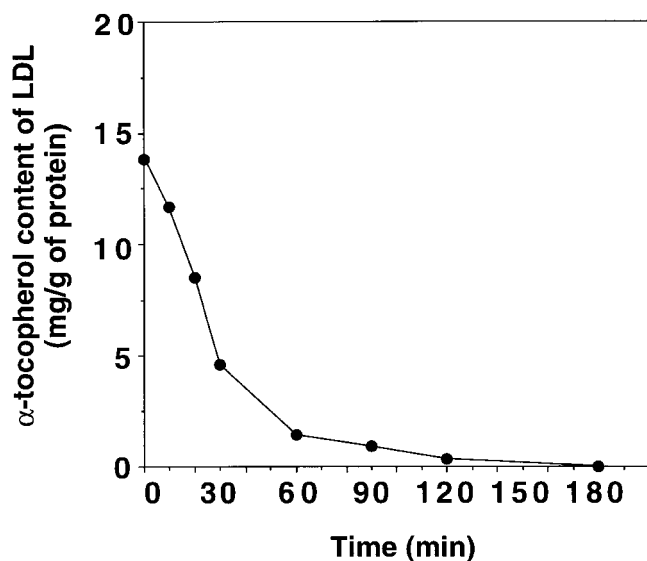


Figure 1. Time-dependent decrease in α -tocopherol content of LDL during oxidation in the presence of copper sulfate. Freshly isolated, native LDL (2 g/l of LDL protein) were incubated for up to 180 min at 37°C in the presence of copper sulfate (5 μ mol/l). At the end of the incubation period, oxidation was stopped by adding BHT (final concentration, 20 μ mol/l) and EDTA (final concentration, 200 μ mol/l). The α -tocopherol content of LDL was determined after lipid extraction, as described under Materials and Methods.

lipoproteins, freshly isolated native plasma LDL and HDL were incubated in the presence or in the absence of purified human PLTP (see Materials and Methods). A 1-h incubation at 37°C did not produce net movements of α -tocopherol between HDL and native LDL whether PLTP was added or not, and the α -tocopherol to protein ratio in LDL remained constant under the various experimental conditions studied (control LDL, 17.5 ± 0.3 mg/g; LDL incubated at 37°C with HDL, 18.1 ± 0.7 mg/g; LDL incubated at 37°C with HDL and purified PLTP, 17.7 ± 1.1 mg/g; $n=3$, *n.s.*).

In subsequent experiments, lipoproteins were progressively depleted in α -tocopherol through an oxidation step in the presence of copper sulfate. As shown in **Fig. 1**, a time-dependent reduction in the α -tocopherol content of LDL was observed, with an $\sim 95\%$ decrease in LDL α -tocopherol content after 90 min of oxidation. In contrast to observations made with native LDL, a net flux of α -tocopherol from native HDL toward α -tocopherol-depleted LDL occurred during a 1-h incubation in the presence of purified PLTP. Indeed, whereas the incubation with HDL alone did not significantly modify the α -tocopherol content of oxidized LDL, the α -tocopherol to protein ratio significantly rose from 0.8 ± 0.1 mg/g in oxidized LDL up to 2.2 ± 0.1 mg/g when oxidized LDL were incubated for 1 h at 37°C with plasma HDL and purified PLTP ($P < 0.0001$) (**Fig. 2**). An even greater PLTP-mediated enrichment of oxidized LDL could be obtained when α -tocopherol-BSA complexes were used as an α -tocopherol source. Under

these experimental conditions, the net mass transfer of α -tocopherol to oxidized LDL was markedly facilitated during a 1-h incubation at 37°C in the presence of PLTP, with an ~ 3 -fold increase in the α -tocopherol to protein ratio in oxidized LDL incubated with both α -tocopherol and PLTP as compared with oxidized LDL incubated with α -tocopherol alone (**Fig. 2**). Although native LDL constituted less efficient α -tocopherol donors as compared with native HDL, a PLTP-mediated α -tocopherol net mass transfer was also shown to occur from native plasma LDL toward α -tocopherol-depleted HDL in which a 70% drop in the α -tocopherol content was induced by oxidation in the presence of copper sulfate. In the incubated native LDL/oxidized HDL mixtures, the α -tocopherol to cholesterol ratio of oxidized HDL significantly rose from 11.4 ± 0.3 mg/g in the absence of purified PLTP to 13.7 ± 0.4 mg/g in the presence of purified PLTP (10 μ g/ml) ($P < 0.05$; $n=3$).

Effect of anti-PLTP antibodies on the net mass transfer of α -tocopherol between lipoproteins

Figure 3 shows the effect of anti-PLTP immunoglobulins on the PLTP-facilitated transfer of α -

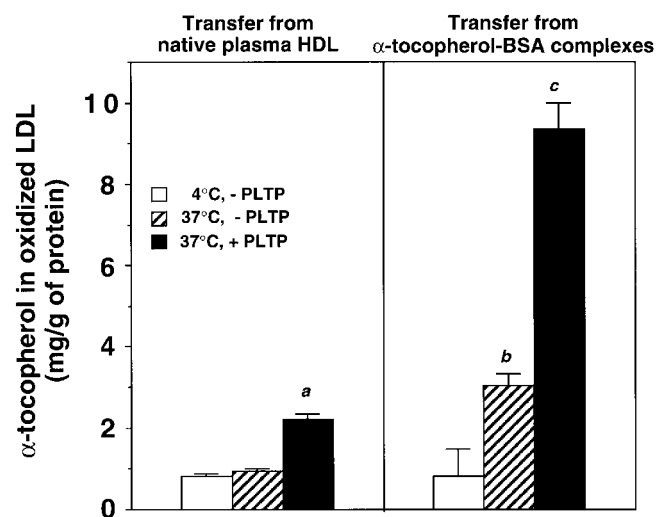


Figure 2. PLTP-mediated net mass transfer of α -tocopherol to oxidized LDL. α -Tocopherol-depleted LDL (final concentration, 1 g/l of LDL protein) that were oxidized for 90 min in the presence of copper sulfate (see Materials and Methods) were mixed with either native plasma HDL (final concentration, 0.5 g/l of HDL protein, 10 μ mol/l of α -tocopherol) or α -tocopherol-BSA complexes (30 μ mol/l of α -tocopherol), and mixtures were supplemented (closed bars) or not (opened and hatched bars) with purified PLTP (10 μ g/ml). Samples were either maintained at 4°C (opened bars) or incubated at 37°C for 1 h (hatched and closed bars), and the α -tocopherol content of LDL was subsequently determined as described under Materials and Methods. Vertical bars represent means \pm SE of triplicate determinations and are representative of three distinct experiments. *a* $p < 0.0001$ vs. 4°C and 37°C mixtures with no purified PLTP added; *b* $p < 0.05$ vs. 4°C mixture; *c* $p < 0.001$ vs. 4°C and 37°C mixtures with no purified PLTP added.

copherol from native HDL donors toward oxidized LDL acceptors. The 51% and 77% increases in the α -tocopherol content of LDL observed in the presence of 10 and 15 $\mu\text{g}/\text{ml}$ of purified PLTP, respectively, were completely abolished when PLTP activity was blocked by anti-PLTP antibodies.

Net mass transfer of α -tocopherol to cultured vascular endothelial cells

The role of PLTP in facilitating the incorporation of α -tocopherol to vascular endothelium was investigated in a second part of the study. Since HDL are susceptible to influence the metabolism of endothelial cells not only through their α -tocopherol content, but also through their lipid content, apolipoprotein composition or paraoxonase activity (33–37), subsequent studies with cultured cells or arterial segments were conducted by using α -tocopherol-BSA complexes as α -tocopherol donors. In preliminary experiments, the endothelium incorporation of α -tocopherol was evaluated with cultured ECV-304 human vascular endothelial cells that were incubated with α -tocopherol-BSA complexes in the absence or in the presence of purified PLTP (see Materials and Methods). Whereas the α -tocopherol content of

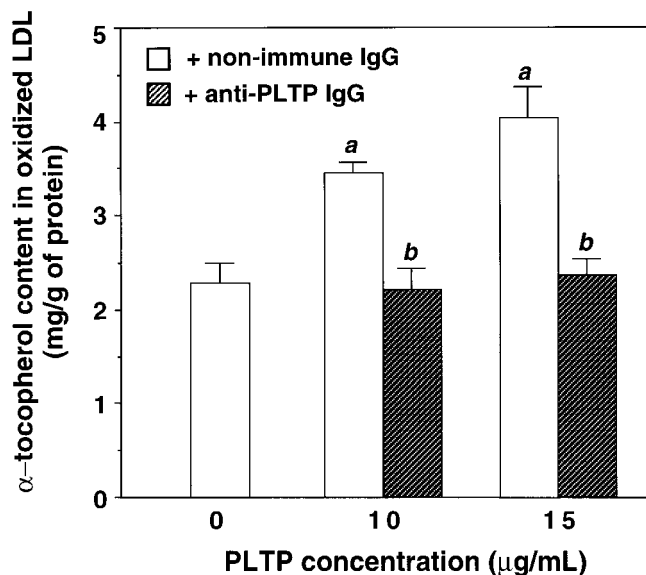


Figure 3. Effect of anti-PLTP polyclonal immunoglobulins on the net mass transfer of α -tocopherol from HDL to oxidized LDL. Mildly oxidized LDL (1 g/l of LDL protein) and native plasma HDL (0.5 g/l of HDL protein) were incubated for 1 h at 37°C with a purified PLTP fraction (final concentration, 0, 10 or 15 $\mu\text{g}/\text{ml}$) that was pretreated with either nonimmune (opened bars) or anti-PLTP (hatched bars) IgG. At the end of the incubation period, the α -tocopherol content of LDL was determined as described under Materials and Methods. Vertical bars represent means \pm SE of triplicate determinations from one experiment. *a* $p < 0.01$ vs. mixtures with no purified PLTP added; *b* $p < 0.01$ vs. counterparts treated with nonimmune IgG.

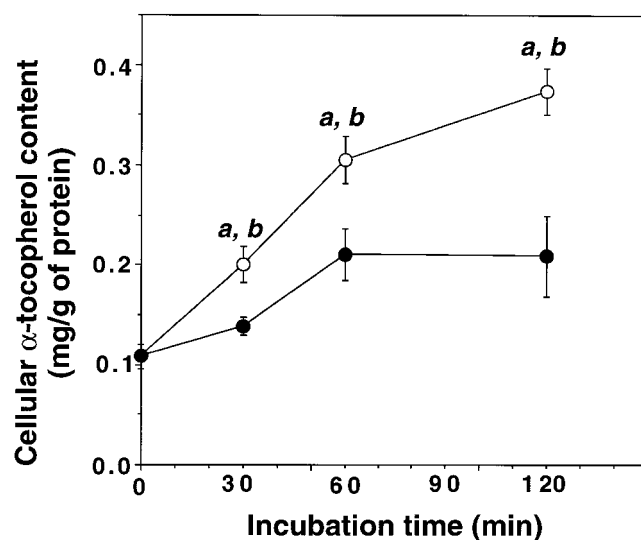


Figure 4. Time course of the PLTP-mediated net mass transfer of α -tocopherol from α -tocopherol-BSA complexes to vascular endothelial cells. Confluent monolayers of ECV-304 endothelial cells were incubated at 37°C in 5 ml of serum-free medium containing α -tocopherol-BSA complexes (α -tocopherol concentration, 10 $\mu\text{mol}/\text{l}$) and in the absence (closed circles) or in the presence (opened circles) of purified PLTP (10 $\mu\text{g}/\text{ml}$). At the end of the incubation, the culture medium was removed and cells were scraped for α -tocopherol assay (see Materials and Methods). Points are means \pm SE of five determinations and are representative of two distinct experiments. *a* $p < 0.05$ vs. homologous mixtures with no purified PLTP added; *b* $p < 0.01$ vs. nonincubated control.

endothelial cells was not modified over a 2-h incubation in the sole presence of α -tocopherol, the addition of purified PLTP induced a time-dependent net transfer of α -tocopherol toward cultured cells (Fig. 4). In another set of experiments, purified PLTP was shown to increase the α -tocopherol content of cultured cells in a concentration-dependent manner, reaching the statistical significance with the 7.5 and 12.5 $\mu\text{g}/\text{ml}$ PLTP doses (Fig. 5). It is noteworthy that consistent observations were made when HDL, instead of α -tocopherol-BSA complexes were used as α -tocopherol donors, and Fig. 6 shows a concentration-dependent effect of purified PLTP in promoting the cellular incorporation of α -tocopherol.

Effect of α -tocopherol and PLTP on vascular reactivity

Rabbit aortic rings pretreated with various concentrations of oxidized LDL were precontracted with NE and they were progressively relaxed with cumulative additions of acetylcholine as described under Materials and Methods. Whereas the contractile response of arterial segments to NE did not differ significantly after the various preincubation protocols (results not shown), significant alterations in the relaxing response to acetylcholine were noted. In agreement with previous studies (14, 33), oxidized LDL were

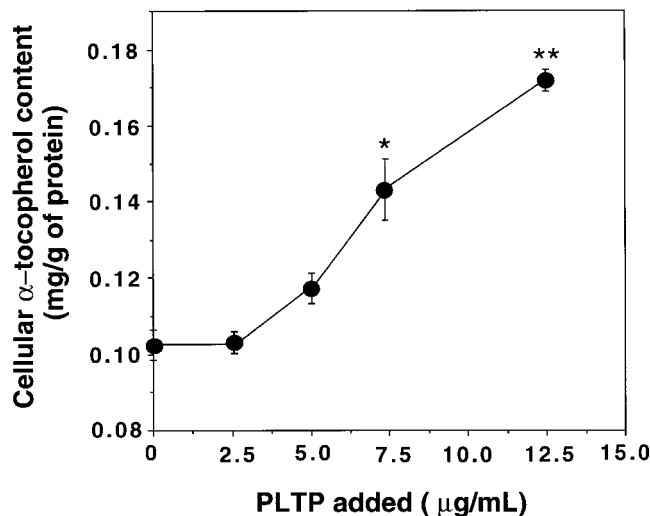


Figure 5. Concentration-dependent effect of purified PLTP on the net mass transfer of α -tocopherol from α -tocopherol-BSA complexes to vascular endothelial cells. Confluent monolayers of ECV-304 endothelial cells were incubated for 1 h at 37°C in 5 ml of serum-free medium containing α -tocopherol-BSA complexes (α -tocopherol concentration, 10 μ mol/l) and purified PLTP (concentration range, 0–12.5 μ g/ml). At the end of the incubation, the culture medium was removed and cells were scraped for α -tocopherol assay (see Materials and Methods). Points are means \pm SE of triplicate determinations and are representative of three distinct experiments. * P <0.01 and ** P <0.001 vs. mixtures with no PLTP added.

shown to inhibit the acetylcholine-mediated, endothelium-dependent arterial relaxation in a concentration-dependent manner (Fig. 7). This was accompanied by a progressive decrease in the maximal arterial relaxation (E_{max}) as well as by a progressive increase in the ACh dose required to obtain a half-maximal relaxing effect (EC_{50}) (Fig. 7, insert). In subsequent experiments, arterial rings were pretreated with α -tocopherol in the presence or in the absence of purified PLTP prior to be incubated with highly oxidized LDL (LDL protein, 0.5 mg/ml). Whereas the deleterious effect of oxidized LDL on the relaxing response to acetylcholine was not modified by α -tocopherol alone, it was markedly counteracted by a combination of α -tocopherol and PLTP pretreatments (Fig. 8). As shown in Fig. 9, significantly higher maximal relaxation and significantly lower EC_{50} values were measured with oxidized LDL-treated arteries that were pretreated with both α -tocopherol and PLTP as compared with oxidized LDL-treated arteries that were pretreated with α -tocopherol alone. With the combination of α -tocopherol and PLTP, the relaxation curve of oxidized LDL-treated rings was shifted toward the control curve that was obtained with untreated arterial segments (Fig. 8), and a significant restoration of the sensitivity to ACh was observed (Fig. 9). In the absence of α -tocopherol-BSA complexes, PLTP alone had no effect on the oxidized LDL-mediated

impairment of the endothelium-dependent arterial relaxation (results not shown). The combination of α -tocopherol and PLTP appeared to exert its beneficial effect only on oxidized LDL-treated arteries since pretreatments of control aortic rings with α -tocopherol and PLTP did not significantly alter the subsequent normal relaxing response to ACh (results not shown).

DISCUSSION

The present study describes the role of PLTP in supplying oxidized lipoproteins and endothelial cells with α -tocopherol through a net mass transfer reaction that may help to prevent disorders that are known to constitute key steps of atherogenesis, i.e., LDL oxidation and vascular endothelium dysfunction.

α -tocopherol, the most potent antioxidant form of vitamin E is a lipophilic compound that is mainly bound to lipoprotein particles in human plasma (15–18). Although on a theoretical basis the hydrophobic α -tocopherol molecules are unlikely to skip spontaneously from one lipid structure to another through an aqueous phase, α -tocopherol is known to exchange rapidly between lipoprotein particles in the plasma compartment (19), indicating that a specific mechanism of redistribution of α -tocopherol between plasma lipoproteins must exist. Whereas earlier studies dem-

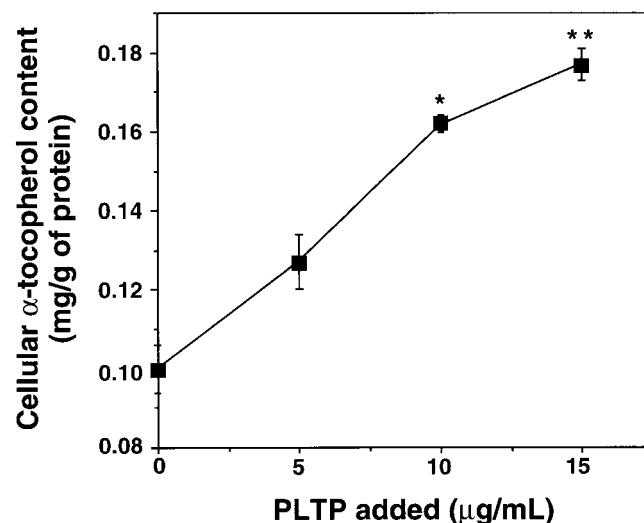


Figure 6. Concentration-dependent effect of purified PLTP on the net mass transfer of α -tocopherol from HDL to vascular endothelial cells. Confluent monolayers of ECV-304 endothelial cells were incubated for 1 h at 37°C in 5 ml of serum-free medium containing freshly isolated native plasma HDL (cholesterol concentration, 0.5 g/l) and purified PLTP (concentration range, 0–15 μ g/ml). At the end of the incubation, the culture medium was removed and cells were scraped for α -tocopherol assay (see Materials and Methods). Points are means \pm SE of triplicate determinations and are representative of two distinct experiments. * P <0.05 and ** P <0.01 vs. mixtures with no PLTP added.

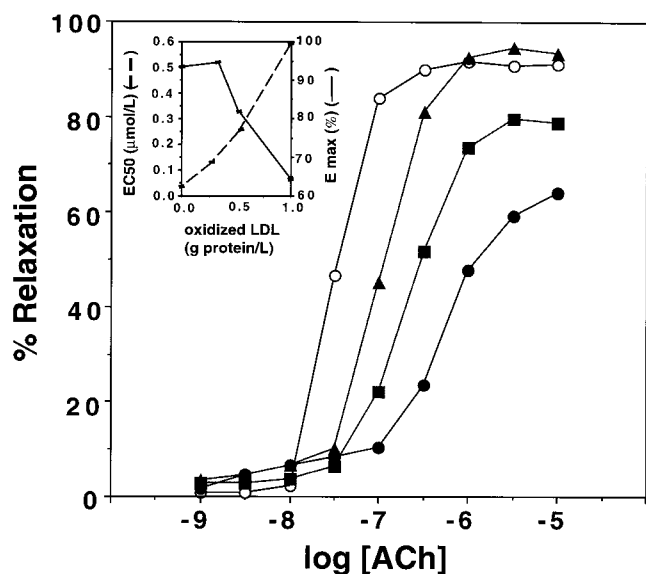


Figure 7. Dose-dependent effect of oxidized LDL on the acetylcholine-induced endothelium-dependent relaxation of rabbit aorta. Graph shows cumulative concentration-response curves to ACh of aortic rings that were incubated for 2 h in the absence (opened circles) or in the presence of oxidized LDL (final concentration, 0.25 mg/ml (closed triangles), 0.5 mg/ml (closed squares), or 1 mg/ml (closed circles) of LDL protein) and precontracted with 0.3 $\mu\text{mol/l}$ NE. Insert shows the dose-dependent effect of oxidized LDL on the maximal arterial relaxation to ACh (E_{max}) and on the ACh concentration required to obtain a half-maximal relaxing effect (EC_{50}). E_{max} and EC_{50} values were calculated as described under Materials and Methods. Each point represents the mean of two determinations.

onstrated that the plasma cholesteryl ester transfer protein (CETP) is not involved in the exchange of α -tocopherol between plasma lipoproteins (38), the related PLTP was recently described as an α -tocopherol exchange factor (27). In typical experiments involving isolated HDL and LDL, PLTP was shown to accelerate the exchange of radiolabeled α -tocopherol, reaching the equilibrium state after a few hours of incubation (27). In fact, the final distribution of the radiolabeled α -tocopherol tracer between HDL and LDL after long incubation periods was shown to be virtually the same whether a PLTP source was added or not (27), suggesting that plasma PLTP may only accelerate the equilibration of initial pools through bidirectional transfer reactions without forcing net flux toward one given lipoprotein class. Since Romanchik and co-workers (39) did not observe net mass transfers of α -tocopherol among plasma lipoproteins when human plasma was incubated *in vitro*, the α -tocopherol contents of plasma lipoproteins may be mostly at equilibrium *in vivo* in the fasting state. Consistently, the present study reported no PLTP-mediated alterations in lipoprotein α -tocopherol contents in incubations containing native LDL and HDL fractions. However, with regard to nonequibrated α -tocopherol pools, the present report provides direct evidence for the ability of PLTP to promote net

mass transfers of α -tocopherol between lipid structures. In fact, three complementary observations could be made under appropriate experimental conditions: *i*) the redistribution of α -tocopherol from α -tocopherol-rich HDL toward mildly oxidized, α -tocopherol-depleted LDL, *ii*) the redistribution of α -tocopherol from LDL toward mildly oxidized, α -tocopherol-depleted HDL and *iii*) the net mass transfer of α -tocopherol from α -tocopherol-BSA complexes to vascular endothelial cells. Given the mean PLTP concentration in human plasma ($\sim 4 \mu\text{g/ml}$) (39a) and the degree of purity of the PLTP preparation (corresponding to ~ 1000 -fold purification), the amounts of PLTP used in these studies were within, or even below the range of physiological concentrations. Overall, the present study describes a new mechanism for the tissue incorporation of α -tocopherol, in addition to the previously recognized pathways involving the LDL receptor (21) or the lipoprotein lipase (22). In contrast to the LDL receptor and LPL pathways that only deal with LDL and triglyceride-rich lipoproteins, respectively, PLTP

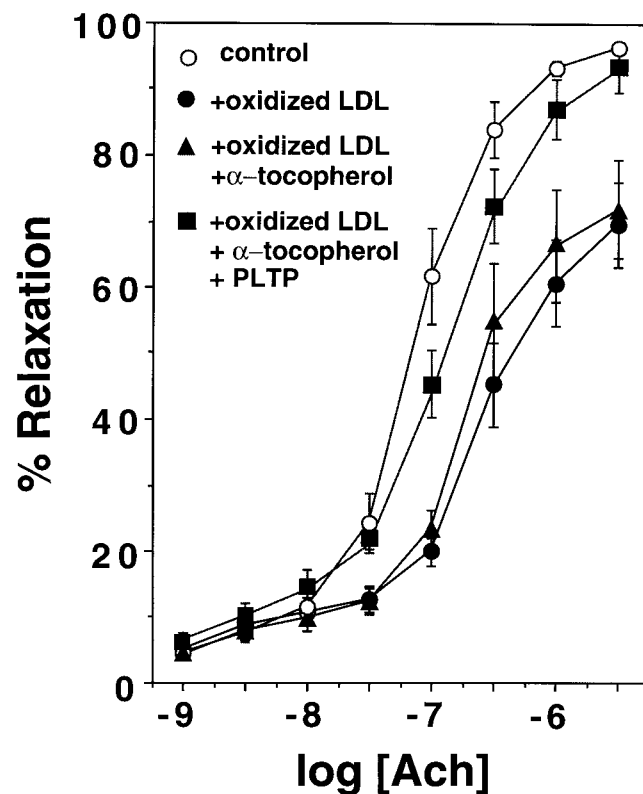


Figure 8. Effect of α -tocopherol and PLTP on the concentration-response curve of rabbit aorta to acetylcholine. Rabbit aortic rings were preincubated for one hour with either Krebs' buffer (closed and opened circles), α -tocopherol-BSA complexes (α -tocopherol, 10 $\mu\text{mol/l}$; closed triangles), or α -tocopherol-BSA complexes plus PLTP (α -tocopherol, 10 $\mu\text{mol/l}$; PLTP, 10 $\mu\text{g/ml}$; closed squares). Aortic rings were subsequently washed and they were treated (closed symbols) or not (opened symbols) with oxidized LDL (LDL protein, 0.5 mg/ml). Graph shows cumulative concentration-response curves to ACh after a precontraction with 0.3 $\mu\text{mol/l}$ NE. Each point represents the mean \pm SE of 12 arterial segments.

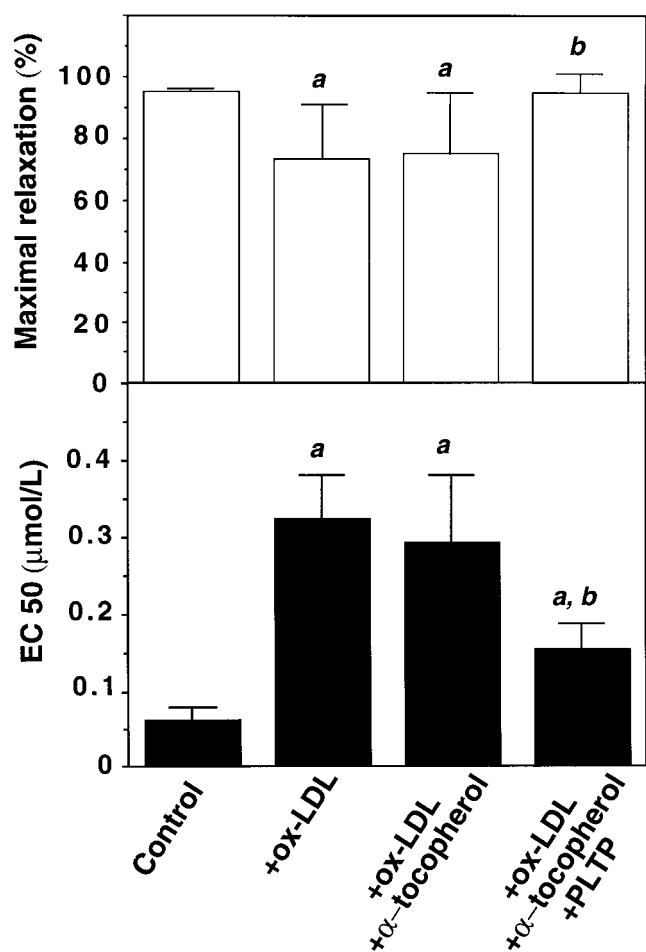


Figure 9. Effect of α -tocopherol and PLTP on maximal relaxation and sensitivity of rabbit aortic rings to acetylcholine. Experimental conditions are as described in the legend to Fig. 6. The maximal relaxation to ACh (E_{max}) and the concentration required to produce half-maximal relaxing effect of ACh (EC_{50}) were determined as described under Materials and Methods. Vertical bars are means \pm SE of data obtained with 12 arterial segments. *a* $p < 0.05$ vs. control; *b* $p < 0.05$ vs. oxLDL and oxLDL + α -tocopherol values.

can interact with HDL, and it is actually mainly bound to this lipoprotein fraction in plasma (40). Since HDL constitute one of the main reservoirs of vitamin E in the plasma compartment (41), the PLTP-mediated α -tocopherol transfer may well represent a quantitatively important pathway for the α -tocopherol tissue incorporation. Interestingly, an highly efficient, selective uptake of α -tocopherol by cultured HepG2 cells was recently reported, and it was shown to be independent on holoparticle internalization (42). The role of PLTP in the latter process deserves further investigation.

It must be noted that only net movements, and not equimolecular exchanges of α -tocopherol between lipid structures are susceptible to alter secondarily their resistance against oxidative injury. The present study brought arguments in favor of the pathophysiological relevance of the net mass transfers of α -tocopherol mediated by PLTP. First, our *in vitro* observations with oxidized LDL suggest that plasma PLTP could restore

the antioxidant potential of circulating LDL at an early stage of the oxidation cascade that subsequently leads to deleterious cellular damages. Indeed, it is now well established that oxidized LDL, unlike native LDL, critically contribute to atherosclerosis by means of numerous deleterious effects, including cellular cholesterol accumulation, foam cell formation (43), vascular coagulant activities (44), endothelial cell pinocytosis (45) and endothelium dysfunction (1–3). Biochemical studies evidenced that lipid peroxidation in LDL is observed only after the antioxidant defenses, including α -tocopherol, have been lost (46–48). It results that the preservation of the α -tocopherol content of LDL particle through the PLTP-mediated transfer reaction may constitute an efficient mean to break the chain reaction of lipid peroxidation. Interestingly, the protective effect of the PLTP-mediated α -tocopherol transfer reaction against the occurrence of vascular events that accompany atherogenesis may not be limited to the sole protection of circulating LDL against an oxidative stress. Indeed, the present study demonstrated that the α -tocopherol transfer activity of PLTP can also protect the vascular endothelial cells against the deleterious effect of oxidized LDL that leads to the impairment of the endothelium-dependent arterial relaxation.

In previous studies, Kearney et al. (12, 14) reported that the diet-induced increase in the α -tocopherol content of the vascular tissue is associated with a resistance to the endothelium dysfunction induced by oxidized LDL. In addition, the abnormalities in endothelium-dependent control of vascular tone (that are known to develop early in the course of atherogenesis (49) as well as after myocardial ischemia (50)) have been shown to disappear in hypercholesterolemic rabbits when fed a β -carotene-supplemented diet (i.e., another lipophilic dietary antioxidant), despite a persistent weaker resistance of circulating LDL to *ex vivo* oxidation (12). However, given the multiplicity of the biological effects of α -tocopherol (i.e., inhibitions of free radical tissue damage (51–53), leukocyte adhesion to endothelial cells (54, 55), monocyte transmigration (56), and smooth muscle cell proliferation (57–59)), definitive conclusions concerning the ability of lipophilic antioxidants to reverse endothelium dysfunction cannot be easily drawn from dietary experiments. Nevertheless, it remains that this effect is strictly dependent on antioxidant tissue incorporation, and in accordance with Kearney et al. (14), we did not observe alterations in vascular reactivity when α -tocopherol was added alone into the organ bath medium. In fact, the present *ex vivo* study extends previous observations made after dietary interventions (10–14), and it brings direct evidence for the beneficial effect of α -tocopherol incorporation on arterial relaxation. More precisely, the maximal response and

the sensitivity to acetylcholine tended to be normalized in the present study when arterial segments were pretreated with both α -tocopherol and PLTP, indicating that the PLTP-mediated α -tocopherol incorporation has the potential to counteract the deleterious effect of oxidized LDL in *ex vivo* experiments. It is noteworthy that α -tocopherol and PLTP were washed prior to incubation of aortic rings with oxidized LDL, indicating that the α -tocopherol/PLTP combination exerts its beneficial effect directly on the vessel rather than indirectly through the antioxidant protection of exogenous LDL. The molecular mechanism of the protective effect of the PLTP-mediated α -tocopherol incorporation might relate in some way to its ability to inhibit the oxidized LDL-induced stimulation of protein kinase C as found in hypercholesterolemic rabbits fed an α -tocopherol-supplemented diet (14). The relevance of the PLTP-mediated incorporation of α -tocopherol to the cholinergic receptor-mediated signal transduction and protein kinase C activity deserves further attention.

In conclusion, the PLTP-mediated net mass transfer of α -tocopherol provides a new pathway by which α -tocopherol can integrate the vascular wall, in addition to the previously described LDL receptor and LPL pathways. The present study indicates that PLTP may play at least two distinct beneficial roles in preventing endothelium damage at an early stage of atherogenesis, i.e., the antioxidant protection of LDL and the preservation of a normal relaxing function of vascular endothelial cells. The discovery of the ability of purified human PLTP to supply the vascular endothelium with α -tocopherol *ex vivo* provides a new and convenient tool for future investigation of the molecular mechanisms of the effect of α -tocopherol on the endothelial function. **FJ**

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