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Effect of supplementation of smoking men with plain or slow release ascorbic acid on lipoprotein oxidation

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Objective: To study the effects of two month ascorbic acid supplementation on *in vitro* lipoprotein oxidation resistance and on *in vivo* lipid peroxidation, and to compare the absorption of two ascorbic acid preparations.
Design: Randomized, single blinded and placebo-controlled clinical trial.
Setting: Men, aged 36–65 y, smoking 11–40 cigarettes daily.
Subjects: Sixty-two subjects were recruited by newspaper advertisements and randomized. Fifty-nine subjects completed the study.
Intervention: Subjects were randomized into three groups to receive 250 mg BID of plain or slow release ascorbic acid tablets or placebo daily for two months. In the pharmacokinetic part of the study, the absorption of the ascorbic acid preparations was followed for 12 h.
Main outcome measures: Plasma malondialdehyde (MDA) concentration and the oxidation resistance of VLDL + LDL. For the pharmacokinetic study, the area under the plasma concentration curve (AUC) of ascorbic acid.
Results: Plasma reduced ascorbic acid increased by 32% in the plain ascorbate group and by 54% in the slow release group during a two month supplementation. Plasma MDA increased in the plain ascorbic acid group compared with placebo group ($P < 0.05$), but there were no significant differences in the changes in lipoprotein oxidation reactions induced by copper or by hemin and H_2O_2. Plasma reduced and total ascorbic acid AUC values were significantly higher in both plain and slow release ascorbate groups compared with placebo group.
Conclusions: Oral supplementation of 500 mg of ascorbic acid daily for two months alone without any other antioxidant does not appear to have protective effect on either *in vitro* lipoprotein oxidation resistance or *in vivo* lipid peroxidation in smoking men, but might even promote the formation of MDA.
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Descriptors: ascorbic acid; lipid hydroperoxides; lipoprotein oxidation; pharmacokinetics; vitamin C

Introduction

Ascorbic acid (vitamin C) is a water-soluble, chain-breaking antioxidant in plasma. It reacts directly with superoxide, hydroxyl radicals and singlet oxygen. Ascorbic acid added to whole plasma *in vitro* effectively protects against peroxyl radical production induced by water-soluble azo-initiators (Frei *et al*, 1989). Also *in vitro*, ascorbic acid has been shown to inhibit low-density lipoprotein (LDL) oxidation by an azo-initiator, activated neutrophils or cigarette smoke (Friel, 1991), and by copper or human macrophages (Jialal and Grundy, 1991). There is some epidemiologic evidence suggesting that low vitamin C plasma levels are associated with increased risk of coronary heart disease (Salonen *et al*, in press). Because oxidative modification of LDL has been suggested an important step in the atherosclerotic process (Witztum, 1994; Salonen *et al*, 1992; Salonen, 1995), the antioxidative properties of ascorbic acid make it a potential candidate agent in the prevention of atherosclerosis progression.

One *in vivo* supplementation study with a small number of subjects suggested that ascorbic acid may protect against LDL oxidation induced by smoking, as measured by content of thiobarbituric acid reactive substances in LDL (LDL TBARS) (Harats *et al*, 1990). Recently, Fuller *et al* (1996) reported that LDL oxidation susceptibility, as measured by thiobarbituric acid reactive substances (TBARS) after copper induction was reduced during a four week oral supplementation with a high dose of ascorbic acid (1000 mg/d). An uncontrolled study demonstrated that supplementation with ascorbic acid increased the resistance of the combined fraction of very-low density lipoprotein (VLDL) and LDL to oxidation induced by copper (Rifici *et al*, 1993). The present study was undertaken to compare the 12 h pharmacokinetics of two ascorbic acid preparations and to investigate the possible sparing effect of ascorbic acid on plasma α-tocopherol level. We also studied the effects of a two month ascorbic acid supplementation on lipoprotein oxidation resistance and on lipid peroxidation.

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Subjects and methods

Subjects

Sixty-two men, smoking regularly, were recruited by newspaper advertisements. Exclusion criteria included regular intake of any drug with antioxidative properties or acetylsalicylic acid, intake of other investigational products within the last three months, severe obesity (body mass index > 31 kg/m^2), insulin dependent (type 1) diabetes, cancer, severe cardiovascular disease or other severe diseases. Subjects with concomitant illness such as fever, vomiting or diarrhea for more than 72 h during the last week before the trial were also excluded. One subject did not come to the baseline visit and two subjects missed the two month visit. The remaining 59 subjects were 36–65 y old and smoked 11–40 cigarettes daily. All subjects gave a written informed consent. The study protocol was approved by the Research Ethics Committee of the University of Kuopio. The study was conducted according to the Good Clinical Practice procedures and Declaration of Helsinki (World Medical Assembly, 1989).

Study design

The study was one part of our block randomized, single blinded and placebo controlled MASI study (Multiple Antioxidant Supplementation Intervention study), in which 142 healthy, smoking men from Eastern Finland were randomized into seven groups to receive different antioxidant supplements and placebo. The supplementation in vitamin C block comprised two tablets of 250 mg of plain ascorbic acid, 250 mg of slow release ascorbic acid with a matrix of hydroxypropyl methyl cellulose, or placebo daily (one with the morning and one with the evening meal). The subjects did not use any other antioxidant supplements and they were advised to keep their health habits unchanged during the two month study period. Plasma or blood antioxidant levels, lipoprotein oxidation resistance and plasma content of malondialdehyde (MDA) and electronegatively charged LDL (LDL^-) were measured at the beginning and at the end of the two month follow-up period. Before the first dose, venous blood sample was drawn after a fast of 10 h. The two month follow-up sample was drawn approximately 10–12 h after the last supplement.

Pharmacokinetic trial

Five subjects from each group (plain ascorbic acid, slow release ascorbic acid and placebo) were invited to stay for the 12 h pharmacokinetic study to test the absorption of two ascorbic acid preparations and the direct effect of plain or slow release ascorbic dose on plasma α-tocopherol levels and on lipoprotein oxidation resistance. The first blood sample was drawn before breakfast with a dose of 250 mg of ascorbic acid or placebo. Breakfast consisted of bread, margarine, salami, cheese, milk and coffee or tea. After the drug dose, blood samples for ascorbic acid and α-tocopherol determinations were taken every two hours. Samples for lipoprotein oxidation resistance and antioxidant capacity measurements were drawn in six hours from the start. Lunch, containing the same meal as breakfast plus white rice and meat stew, was served after the second sample, and dinner (equal to lunch) after the fifth sample. The last blood sample was drawn after 12 h from the ascorbic acid dose. Coffee was available and smoking was allowed during the pharmacokinetic study. A sweet cookie was served after the fourth sample. All meals were free of ascorbic acid. The pharmacokinetic trial was repeated after two month supplementation period with 250 mg BID ascorbic acid or placebo daily.

Measurement of plasma or blood antioxidants

Reduced ascorbic acid was stabilized from heparin plasma with metaphosphoric acid immediately after plasma separation. Plasma for total ascorbic acid determination was treated with homocysteine before stabilization with metaphosphoric acid. Ascorbic acid was determined with a high-performance liquid chromatographic (HPLC) method (Nyyssönen *et al*, 1988). Dehydroascorbic acid (DHA) concentration was calculated by subtracting the reduced ascorbic acid from the total ascorbic acid. Heparin plasma for α-tocopherol, β-carotene and lycopene determination was extracted with ethanol and hexane and measured by a reversed phase HPLC method by using α-tocopherol acetate as an internal standard (Porkkala-Sarataho *et al*, 1996). Plasma total Q10 (coenzyme Q10) was determined by using an HPLC method after reduction of plasma oxidized ubiquinone to ubiquinol by sodium dithionite (Lang *et al*, 1986).

Total (GSH) and oxidized (GSSG) glutathione of whole blood was determined by an enzymatic recycling reaction (Baker *et al*, 1990). Sulfosalicylic acid was used to precipitate proteins and to stabilize fresh EDTA blood samples. Production of 5-thio-2-nitrobenzoic acid was monitored photometrically at 414 nm in a microtiter plate reader (iEMS, Labsystems, Helsinki, Finland). GSSG content of samples was measured after trapping reduced glutathione with 2-vinylpyridine.

Measurement of lipids, ferritin, lipid oxidation resistance and antioxidative capacity

Serum cholesterol and triglycerides were determined with enzymatic colorimetric tests (Boehringer Mannheim, Mannheim, Germany). Serum LDL cholesterol was assessed after LDL precipitation with polyvinylsulfate (Boehringer Mannheim). Serum HDL cholesterol was measured from supernatant after magnesium chloride dextran sulphate precipitation (Finley *et al*, 1978). Serum ferritin was determined with an immunoradiometric assay (Bio-Rad, Hercules, CA).

Combined VLDL and LDL were isolated by ultracentrifugation of EDTA plasma, that had been frozen immediately after blood drawing and plasma separation and stored at -80°C for not more than a week. Copper- or hemin and hydrogen peroxide (H_2O_2)-mediated VLDL + LDL fraction oxidation method was previously described in detail (Nyyssönen *et al*, 1994). Briefly, for copper-induced VLDL + LDL oxidation, EDTA and other compounds with small molecular weight, including ascorbic acid, were removed chromatographically with small gel-filtration minicolumns. Copper chloride was added to start the formation of conjugated dienes, which was assessed spectrophotometrically at 234 nm. Maximal oxidation velocity and lag time were determined from the reaction kinetics.

Hemin + H_2O_2-induced VLDL + LDL oxidation was assayed photometrically at 405 nm in a microtiter plate reader. Lag time and maximal velocity of hemin degradation were determined (Nyyssönen *et al*, 1994). We did not use gel-filtration minicolumns for removing of EDTA from VLDL + LDL fraction after ultracentrifugation, because we found that only a minor part of ascorbic acid was present in VLDL + LDL fraction after gradient ultracentrifugation.

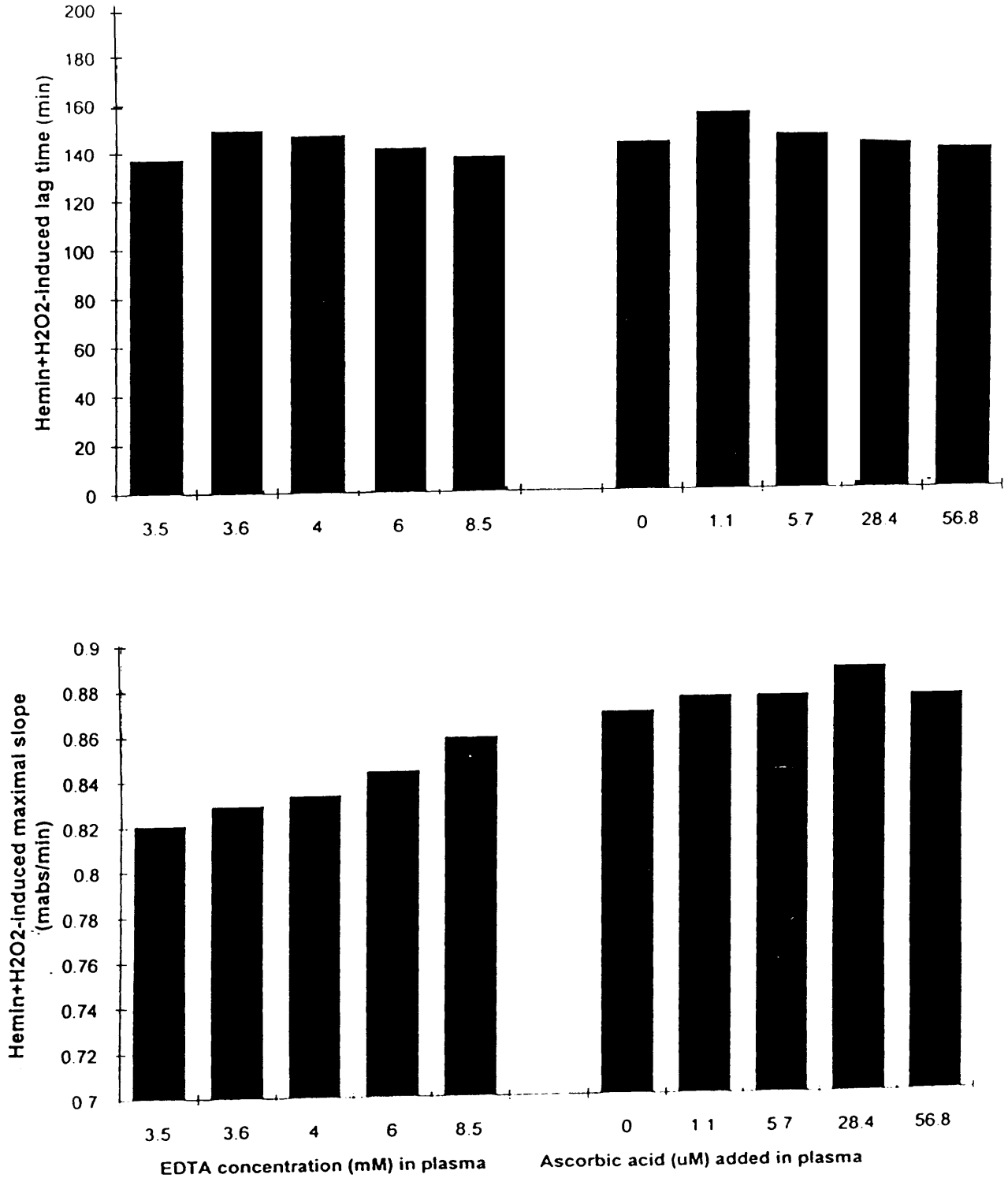

Figure 1 Effect of EDTA or ascorbic acid on hemin + H_2O_2-induced VLDL + LDL oxidation. Plasma contained approximately 3.5 mmol/l of 'endogenous' EDTA deriving from blood sampling tube (Venoject VT-053TKZ, Terumo Corp., Belgium). EDTA was added into aliquots of three plasma samples to final concentrations of 3.6, 4.0, 6.0 and 8.5 mmol/l. Ascorbic acid was added separately into three plasma samples containing 50–75 μmol/l of endogenous ascorbic acid. Added ascorbic acid concentrations were 1.1, 5.7, 28.4 and 56.8 μmol/l. The spiked plasma samples were ultracentrifuged for VLDL + LDL separation at 110 000 g in a Beckman XL-90 ultracentrifuge with Ti 50.4 rotor. The lag time and the maximal slope in hemin + H_2O_2-induced susceptibility to oxidation was then measured in separated VLDL + LDL fractions: VLDL + LDL as the concentration of 0.26 mmol/l of cholesterol was incubated in a microtiter plate well with 2.5 μmol/l of hemin and 50 μmol/l of H_2O_2. Reaction was followed by a microtiter plate reader (iEMS, Labsystems). Bars are means of three samples with EDTA or ascorbic acid additions.

The final ascorbic acid concentration in the hemin + H_2O_2-induced oxidation reaction was below 2 μmol/l as measured from five separated VLDL + LDL fractions (data not shown). The effect of varying concentrations of EDTA or ascorbic acid on lag time or maximal slope of hemin + H_2O_2-induced oxidation was tested (Figure 1). Known concentrations of EDTA or ascorbic acid were added in triplicate into plasma before ultracentrifugation, and the lag

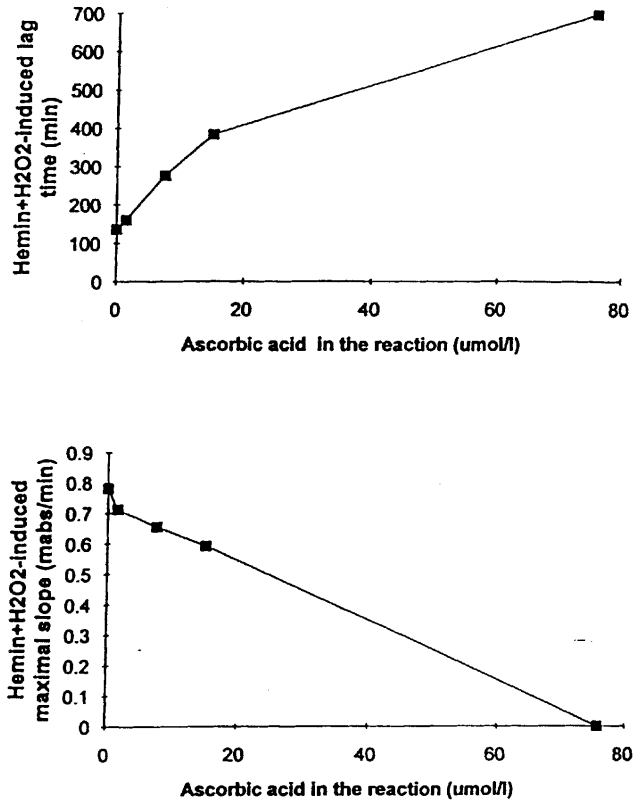

Figure 2 Effect of varying ascorbic acid concentrations in the reaction mixture of hemin + H_2O_2 induced VLDL + LDL oxidation. VLDL + LDL was first isolated from EDTA plasma by ultracentrifugation as described in the text of Figure 1. Ascorbic acid was added into the separated VLDL + LDL fraction to obtain the final concentrations of 0, 1.5, 7.6, 15, 76 and 380 μmol/l in the reaction mixture. Cholesterol, hemin and H_2O_2 concentrations in the reaction and the measurement equipment were as in Figure 1. With the ascorbic acid concentration of 380 μmol/l no reaction occurred during the available measuring time of 720 min.

time and the maximal slope of the hemin + H_2O_2-induced reaction were analyzed (Figure 1). Added ascorbic acid affected neither the lag time nor the maximal slope of the reaction. On the contrary, maximal slope increased with increasing EDTA concentrations in plasma. EDTA did not have any influence on hemin + H_2O_2-induced lag time (Figure 1). We also added ascorbic acid into the separated VLDL + LDL fraction and measured the lag time and maximal slope with known concentrations of ascorbic acid (Figure 2). Lag time was increased and maximal slope decreased by ascorbic acid in a concentration-dependent manner. Lag time increased nearly linearly when ascorbic acid concentration in the reaction mixture was increased from 0–76 μmol/l. We also tested ascorbic acid concentration of 380 μmol/l in the reaction mixture; that inhibited the oxidation reaction for over 720 min, which is the maximum measurement time with our absorbance reader. Maximal reaction velocity decreased with increasing ascorbic acid concentration. With the ascorbic acid concentration of 76 μmol/l, maximal slope was only weakly detectable (0.0001 mabs/min) (Figure 2).

Total antioxidative capacity of LDL (LDL TRAP) was measured after LDL precipitation from 1 ml of EDTA plasma with 7.5 ml of heparin, diluted to a concentration of 50 IU/ml in 64 mmol/l sodium citrate, pH 5.04. The precipitate was extracted with 2 ml of chloroform-methanol (1:1). One ml of the extract was evaporated and the remainder was dissolved with 500 μl of hexane-isopropanol (19:1). The hexane-isopropanol phase was divided for determinations of cholesterol and for chemiluminescent assay of chainbreaking antioxidants. Cholesterol was measured with a gas chromatography after adding 6-ketocholestanol as an internal standard and performing a mild saponification with potassium hydroxide. The other aliquot was exposed to peroxyl radicals produced by the thermal decomposition of 2,2-azobis(2,4-dimethylvaleronitrile) (AMVN). The reaction mixture contained 600 μl of chloroform-methanol (6:4), 50 μl of a mixture of 10 mmol/l luminol in borate buffer and methanol (1:1) and 50 μl of 25 mmol/l AMVN in benzene. The rate of peroxyl radical production in decomposition of AMVN was followed by the luminol enhanced chemiluminescence (Bio-Orbit 1251 Luminometer, Turku, Finland). The addition of 50 μl of sample in hexane-isopropanol to the reaction mixture extinguishes the chemiluminescence and its duration has linear correlation to the radical trapping capacity of the sample. The duration of the extinction was calibrated against known concentration of d-α-tocopherol. LDL TRAP results were expressed as pmol/nmol cholesterol in LDL without multiplying the results with the stoichiometric trapping factor (*n*) of α-tocopherol (Uotila *et al*, 1994).

Measurement of plasma MDA

MDA was measured from frozen EDTA plasma after thiobarbituric acid (TBA) reaction using a slight modification of the liquid chromatographic method described by Rabl *et al* (1992). The plasma samples were thawn out immediately before the assay and a volume of 100 μl was mixed with 100 μl water, 300 μl 0.15 mol/l phosphoric acid, 10 μl butylated hydroxytoluene (BHT; 0.2% methanolic solution) plus 100 μl 0.6% TBA and incubated at 95°C for 60 min. The chromagen was extracted with 1.25 ml of butanol-1 and analyzed by HPLC with fluorometric detection (excitation wavelength 525 nm, emission wavelength 550 nm). The MDA-TBA adduct was calibrated with tetramethoxypropane standard solutions, processed as the plasma samples.

Measurement of plasma LDL^-

For LDL^- determination, LDL was separated from frozen EDTA plasma by a short single-step ultracentrifugation. Plasma was adjusted to a density of 1.24 g/ml by potassium bromide and layered underneath a solution with density of 1.006 g/ml. The tube was centrifuged for 2.5 h at 417 000 g. The LDL fraction was removed with a needle and desalted by using gel permeation columns (HiTrap, Pharmacia, Uppsala, Sweden). The desalted LDL fraction was injected into an anion-exchange liquid chromatographic system where native and LDL^- were separated (Nyyssönen *et al*, in press). The chromatographic run was monitored at 280 nm and the area of LDL^- peak was expressed as percentage from total LDL.

Statistical methods

The area under the plasma concentration curve (AUC) from zero to the last observed point (12 h) was calculated by the trapezoidal rule (Gibaldi and Perrier, 1982) after the pretrial ascorbic acid values (baseline values before the first dose) were subtracted from the values obtained after supplementation. Non-parametrical Kruskal–Wallis one-way ANOVA test and Wilcoxon rank sum test were used to compare the heterogeneity of AUC and changes in lipoprotein resistance to oxidation between the groups in the pharmacokinetic study. Differences in changes during

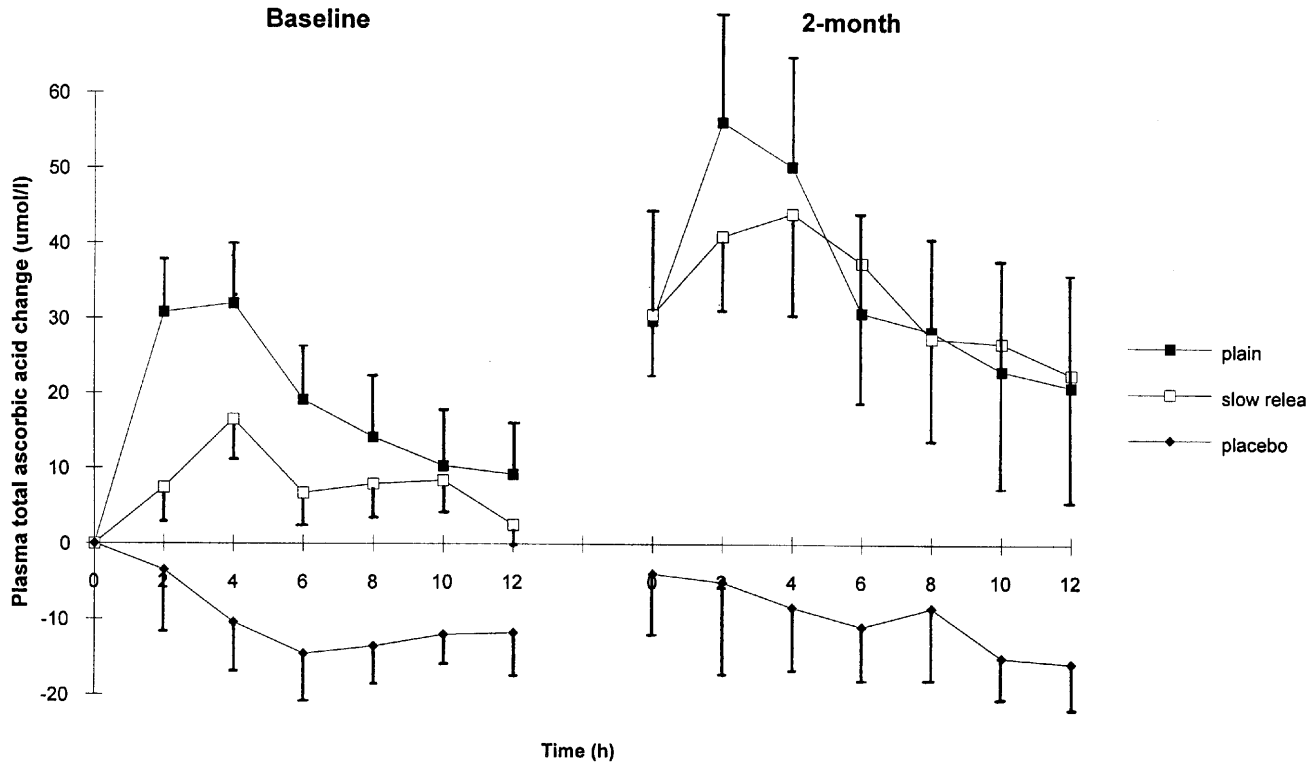

Figure 3 Changes in ascorbic acid concentrations in plasma (mean ± s.e.m., μmol/l) after the first 250 mg dose of plain or slow release ascorbic acid or placebo and on the steady state after two month supplementation. Baseline 0-point values are subtracted from other values obtained after the first dose.

Table 1 Twelve hour area under the plasma concentration curve (AUC) values and six hour-change in lipoprotein resistance to oxidation in pharmacokinetic groups. For AUC values, baseline value (the value before the first dose) was subtracted from each value of baseline and two month visits. Means of five subjects

	Plain	*Slow release*	*Placebo*	*P (Kruskal–Wallis) plain vs slow release vs placebo*	*P (Wilcoxon) plain vs slow release*
AUC (0–12 h) baseline (μmol × h/l)					
Plasma reduced ascorbic acid	183	96	−131	0.023	0.347
Plasma dehydroascorbic acid	40	1	11	0.651	0.754
Plasma total ascorbic acid	222	97	−120	0.006	0.175
Plasma α-tocopherol	−8	11	−21	0.145	0.117
AUC (0–12 h) two month (μmol × h/l)					
Plasma reduced ascorbic acid	360	381	−123	0.026	0.754
Plasma dehydroascorbic acid	39	−4	11	0.357	0.754
Plasma total ascorbic acid	400	377	−112	0.027	0.917
Plasma α-tocopherol	−34	−4	−24	0.878	0.602
Lag time change in hemin + H_2O_2-induced VLDL + LDL oxidation (0–6 h)					
Baseline (min)	39	4	28	0.177	0.076
Two month (min)	42	44	28	0.950	0.347
LDL TRAP change (0–6 h)					
Baseline (pmol/nmol cholesterol)	0.1	−1.6	0.6	0.557	0.347
Two month (pmol/nmol cholesterol)	0.6	2.4	−1.1	0.421	0.917

two-month supplementation between groups were tested with parametrical one-way ANOVA and Bonferroni post-hoc test with significance level 0.05 or analysis of covariance (MANOVA).

To separate the effect of α-tocopherol from that of serum lipids, lipid standardized α-tocopherol concentrations were used in the statistical analysis (Salonen *et al*, 1995).

Results

Pharmacokinetic trial

Plasma total ascorbic acid level before the first dose was 54.9 ± 26.6 μmol/l (mean ± s.d.) in the group supplemented with plain ascorbic acid, 54.2 ± 32.5 μmol/l in the group taking slow release tablets and 55.4 ± 24.0 μmol/l

in placebo group ($n = 5$ in each group). The individual baseline value was subtracted from the values determined after medication. After the first supplemented tablet, plasma total ascorbic acid concentration at 4 h elevated by 94% more in the plain ascorbic acid group than in the slow release ascorbic acid group (Figure 3). After the first dose, ascorbic acid plasma levels remained higher for 12 h in the plain group than in the slow release group. However, after two months of supplementation, the plasma levels (baseline 0-value subtracted) in these two groups were similar (Figure 3). Maximal ascorbic acid concentration during the two month visit was achieved in 2 h after the plain ascorbic acid and 4 h after the slow release ascorbic acid dose. There were no statistically significant differences in AUC values between the plain and slow release groups either at baseline or at two month visit. Plasma reduced and total ascorbic acid AUC values differed significantly between both ascorbic acid and placebo groups (Table 1). Plasma dehydroascorbic acid AUC values were higher in plain group than in slow release or placebo group. This difference, however, was not statistically significant.

There was a great interindividual variation in ascorbic acid AUC values. Plasma total ascorbic acid AUC at baseline varied in plain group from 54–390 μmol × h/l and in slow release group from 14–167 μmol × h/l. Plasma reduced ascorbic acid AUC values varied in plain group from −53–321 μmol × h/l and in slow release group from −14–268 μmol × h/l. Low or even negative reduced ascorbic acid AUC values were achieved in subjects with high increase in plasma DHA concentration together with or instead of increase in reduced ascorbic acid plasma concentration.

Plasma α-tocopherol level before the first dose of ascorbic acid was 28.0 ± 5.5 μmol/l (mean ± s.d.) in the group supplemented with plain ascorbic acid, 32.7 ± 13.3 μmol/l in the group of slow release ascorbic acid and 29.9 ± 5.7 μmol/l in placebo group. Ascorbic acid dose did not have any significant sparing effect on plasma α-tocopherol concentrations (Table 1).

Six hours after the ascorbic acid dose, blood sample was drawn for hemin + H_2O_2induced VLDL + LDL oxidation and for LDL TRAP determination. The 6 h change in lag time of hemin + H_2O_2-induced reaction were positive in ascorbic acid as well as in placebo groups indicating possible morning-afternoon variability in lipoprotein susceptibility to oxidation (Table 1). Similar increase was found at baseline and at two month visits. The changes did not differ significantly between the groups. There were no changes in LDL TRAP values between baseline and 6 h sample in any group.

Two-month supplementation

The subjects smoked 23 ± 6 (mean ± s.d.) cigarettes daily in plain, 19 ± 5 in slow release and 22 ± 9 in placebo group. The amount of their smoking did not change during the two month supplementation period.

The mean baseline concentrations of plasma ascorbic acid and dehydroascorbic acid were similar in plain, slow release and placebo groups (Table 2). During the two month supplementation period, the dose of 250 mg of ascorbic acid twice a day increased the plasma reduced ascorbic acid by 32% in the plain group and by 54% in the slow release group. In the slow release group, there was a subject with very low baseline reduced ascorbic acid value of 4.0 μmol/l. If this subject was excluded, the reduced ascorbic acid increase was 47% in the slow release group. Neither plasma dehydroascorbic acid concentrations nor other plasma antioxidants (α-tocopherol, β-carotene, lycopene and Q10) changed during the supplementation period. Blood GSSG/reduced GSH ratio increased slightly in both supplementation groups, but the increases were not statistically significant compared with the placebo group (Table 2).

Baseline serum LDL and HDL cholesterol and triglyceride concentrations were very similar in ascorbic acid and placebo groups. Serum LDL cholesterol and triglycerides decreased in slow release and placebo groups, but did not change in plain ascorbic acid group. This was probably due to a change towards lower fat in diet in slow release and placebo groups, even though the subjects were advised to keep their diet constant during the study. Serum ferritin decreased parallely in each group. This may be due to the loss of iron during blood drawing at baseline (75 ml for baseline tests and an additional 90 ml for pharmacokinetic tests, 5 men/group).

Of the lipid peroxidation measurements, the mean plasma MDA increased during the two month ascorbic acid supplementation. On the basis of parametric ANOVA, the change in plasma MDA concentration varied significantly between the three groups (Table 2). In the Bonferroni test, the MDA increase during supplementation was significantly greater in plain ascorbic acid group than in the placebo group ($P < 0.05$), but did not differ significantly between the slow release ascorbic acid and placebo groups. There was no significant difference in MDA change between the plain and slow release groups (Table 2). Change in neither the lag time nor the maximal slope of copper- or hemin + H_2O_2-induced oxidation of VLDL + LDL fraction differed significantly between placebo and supplementation groups. Copper-induced lag time decreased significantly in the slow release group compared with plain group, but this was due to the higher mean baseline value in this group than in other groups, and in covariance analysis with baseline lag time and plasma MDA as covariates the difference was not significant ($P = 0.105$). With the same covariates, the change in plasma MDA in plain ascorbic acid group remained significantly higher ($P = 0.013$) compared with the placebo group. LDL fraction TRAP values changed neither in the supplementated nor in placebo groups.

Measurement of LDL^- percentage is time-consuming. For this reason it was determined only in samples of those subjects who achieved the highest increases in plasma ascorbic acid concentration during two month supplementation. LDL^- percentage decreased in each group but there were no significant differences in the changes between the supplemented and placebo groups.

Discussion

Because of its hydrophilic character, ascorbic acid cannot directly act as an antioxidant for lipids. However, it has been shown to inhibit lipid peroxidation in isolated LDL *in vitro* in a number of studies (Frei, 1991: Jialal and Grundy, 1991; Esterbauer *et al*, 1992; Retsky *et al*, 1993). We also confirmed this finding for combined VLDL + LDL fraction. It has been suggested that the antioxidative effect of ascorbic acid is mediated in LDL via aqueous free radical scavenging and thus preventing oxidants from attacking and oxidizing LDL, or DHA or other its decomposition products may modify LDL, leading to decreased metal

Table 2 Baseline and two month values in ascorbic acid and placebo groups (mean ± s.d.). Statistical significances are for differences in changes between supplementation and placebo groups

Measurement	*Plain* ($n=19$)		*Slow release* ($n=20$)		*Placebo* ($n=20$)		*P for plain vs slow release vs placebo*	*P for plain vs slow release*
	baseline	post-treatment	baseline	post-treatment	baseline	post-treatment		
Plasma reduced ascorbic acid (μmol/l)	62.2 ± 21.2	82.5 ± 21.1	58.0 ± 27.2 ($n=18$)	89.1 ± 15.1 ($n=18$)	59.3 ± 18.4 ($n=18$)	54.8 ± 23.8 ($n=18$)	< 0.001	n.s.
Plasma dehydroascorbic acid (μmol/l)	1.9 ± 3.2 ($n=18$)	3.4 ± 5.7 ($n=18$)	2.1 ± 4.1 ($n=17$)	2.6 ± 2.4 ($n=17$)	2.0 ± 2.4 ($n=15$)	2.5 ± 3.4 ($n=15$)	n.s.	n.s.
Plasma total ascorbic acid (μmol/l)	62.5 ± 21.7 ($n=18$)	85.0 ± 19.2 ($n=18$)	60.0 ± 30.0 ($n=17$)	91.7 ± 15.3 ($n=17$)	60.9 ± 19.6 ($n=15$)	57.7 ± 25.7 ($n=15$)	< 0.001	n.s.
Plasma lipid standardized α-tocopherol	1.05 ± 0.13	0.87 ± 0.09	0.97 ± 0.20	0.84 ± 0.14	1.00 ± 0.13	0.86 ± 0.11	n.s.	n.s.
Plasma β-carotene (μmol/l)	0.27 ± 0.12	0.27 ± 0.11	0.31 ± 0.16	0.28 ± 0.12	0.27 ± 0.12	0.26 ± 0.14	n.s.	n.s.
Plasma lycopene (μmol/l)	0.23 ± 0.13	0.25 ± 0.16	0.30 ± 0.21	0.28 ± 0.20	0.21 ± 0.13	0.21 ± 0.12	n.s.	n.s.
Plasma coenzyme Q10 (μmol/l)	0.95 ± 0.43	1.06 ± 0.53	0.95 ± 0.42	0.90 ± 0.27	0.89 ± 0.33	0.88 ± 0.33	n.s.	n.s.
Blood GSSG/reduced GSH	0.047 ± 0.020	0.062 ± 0.021	0.045 ± 0.015	0.052 ± 0.019	0.049 ± 0.018	0.048 ± 0.013	n.s.	n.s.
Serum triglycerides (mmol/l)	1.57 ± 0.84	1.58 ± 0.77	1.87 ± 0.87	1.69 ± 0.94	1.66 ± 0.82	1.36 ± 0.76	n.s.	n.s.
Serum LDL cholesterol (mmol/l)	4.19 ± 0.81	4.20 ± 1.03	4.13 ± 0.81	3.75 ± 0.79	4.11 ± 0.94	3.85 ± 0.93	n.s.	< 0.05
Serum HDL cholesterol (mmol/l)	1.11 ± 0.24	1.18 ± 0.32	1.01 ± 0.20	1.03 ± 0.21	1.01 ± 0.16	1.10 ± 0.23	n.s.	n.s.
Serum ferritin (μg/l)	130 ± 107	122 ± 95	98 ± 84	91 ± 72	125 ± 102	115 ± 84	n.s.	n.s.
Plasma MDA (μmol/l)	0.39 ± 0.13	0.45 ± 0.15	0.39 ± 0.08	0.41 ± 0.10	0.40 ± 0.13	0.36 ± 0.12	< 0.05	n.s.
Copper induced VLDL + LDL oxidation								
Lag time (min)	76 ± 9	78 ± 5	81 ± 8	77 ± 11	74 ± 11 ($n=19$)	75 ± 8 ($n=19$)	n.s.	< 0.05
Maximal slope (mabs/min)	10.1 ± 0.8	10.4 ± 0.9	10.3 ± 1.3	10.7 ± 1.2	10.6 ± 2.4 ($n=19$)	10.6 ± 1.2 ($n=19$)	n.s.	n.s.
Hemin + H_2O_2 induced VLDL + LDL oxidation								
Lag time (min)	115 ± 40	117 ± 41	123 ± 65	125 ± 65	113 ± 40	113 ± 39	n.s.	n.s.
Maximal slope (mabs/min)	0.83 ± 0.19	0.82 ± 0.20	0.85 ± 0.22	0.86 ± 0.22	0.84 ± 0.22	0.85 ± 2.6	n.s.	n.s.
LDL TRAP (pmol/nmol cholesterol)	12.8 ± 2.6	13.0 ± 2.4	12.9 ± 3.8	13.5 ± 4.2	12.3 ± 3.4	12.1 ± 2.8	n.s.	n.s.
LDL^- (% of LDL)	16.4 ± 5.4 ($n=11$)	15.0 ± 5.5 ($n=11$)	21.3 ± 6.8 ($n=15$)	18.9 ± 4.5 ($n=15$)	18.9 ± 5.6 ($n=15$)	18.7 ± 3.8 ($n=15$)	n.s.	n.s.

n.s. = not significant.

binding to the LDL particle and increased resistance to oxidation (Retsky *et al*, 1993). Ames and coworkers (Frei *et al*, 1989, 1990) and Frei (1991) have reported that ascorbic acid is the first antioxidant consumed during lipid peroxidation in plasma and detectable lipid peroxidation starts only after all ascorbate has been consumed completely. They have even suggested that only ascorbate could prevent the initiation of lipid peroxidation.

In this present *in vivo* study, we supplemented 59 men for two months orally with either plain ascorbic acid, slow release ascorbic acid or placebo and did not find any antioxidative effect on *in vitro* lipid oxidation resistance or on total antioxidative capacity of LDL. In the contrary to our expectations, there was a significant increase in plasma lipid peroxidation products, determined by a chromatographic MDA-TBA measurement. The photometric or fluorometric measurement of MDA as TBARS have been criticized because of its unspecifity. TBA is known to react with numerous other substances to produce a pink or red colour that is measured in reaction mixture together with MDA (Knight *et al*, 1988). Some TBARS might be formed during the assay itself, because TBA reaction needs hot, even boiling, acidic environment, which may induce the formation of lipid hydroperoxides. A high plasma content of antioxidants, especially α-tocopherol, has been reported to decrease TBARS concentration either by preventing the lipid peroxidation *in vivo* or by extinguishing the TBA reaction (Sarkkinen *et al*, 1993; Meydani *et al*, 1991). In this study, we used BHT and EDTA to avoid artificial formation of lipid peroxides during the assay and chromatographic separation with fluorometric detection of MDA-TBA adduct to improve the measurement specificity. Most importantly, the baseline and post-treatment samples were analyzed in the same batch for all groups. It is possible that MDA concentration increases during freezing (Sarkkinen *et al*, 1993). This should have introduced a decrease of MDA in both placebo and the supplemented groups during two months. Thus the true increase MDA in the plain ascorbic acid group most likely was greater than the observed increase.

Total plasma MDA (or TBARS) has been determined in only few previous ascorbic acid supplementation studies. In a study by Harats and coworkers (1990), seven smokers were supplemented with 1000 mg of ascorbic acid daily. Plasma TBARS were measured at baseline before supplementation and after 40–48 h of total abstinence from smoking. The effect of acute smoking was examined at baseline by drawing a blood sample for TBARS measurement before and after smoking 5–7 cigarettes during 90 min. The trial of acute smoking was repeated after two week supplementation with ascorbic acid. The authors found a significant increase in plasma TBARS after 90 min smoking at baseline, but after two week ascorbic acid supplementation the acute increase of TBARS was insignificant. However, there was an increase of plasma TBARS before the rapid smoking test after two week supplementation with ascorbic acid. There was no control group in that study, and the statistical significance of the plasma TBARS increase in the ascorbic acid group was not reported. Their study suggests that ascorbic acid can prevent the acute effect of smoking on lipid peroxidation in plasma, but in accordance with our study, ascorbic acid supplementation for weeks or months might promote lipid peroxidation, as measured by TBARS.

Cigarette smoke is known to contain several reactive oxidants which induce the formation of lipid hydroperoxides (Frei, 1991). On the other hand, Chen and Loo (1995) found that the tar fraction of cigarette smoke may contain components with free radical scavenging potential. They studied the influence of cigarette smoke extract on LDL modification induced by either copper or a chemical azo-compound. Cigarette smoke extract inhibited the LDL modification even stronger than ascorbic acid, but caused structural modification of LDL at high concentrations. The effect of smoking on lipid peroxidation is complicated and may mostly be mediated by decreased water-soluble antioxidant levels (Frei, 1991).

Ascorbic acid is an important antioxidant, but paradoxically it may behave as prooxidant by promoting the reduction of ferric iron (Fe^{+3}) to ferrous iron (Fe^{+2}) (Stadtman, 1991; Salonen, 1993). Ferrous iron can then reduce H_2O_2 or O_2 to generate hydroxyl radical or superoxide anion that continues the radical chain reaction (Herbert *et al*, 1994). It has been suggested that supplements of vitamin C provide a constant supply of new reduced ascorbic acid, thus maintaining the cycle of ascorbate-driven repetitive free radical generation by iron (Herbert *et al*, 1994). In addition, ascorbate supplementation may enhance the absorption of nonheme iron in the diet (Sauberlich, 1994), thus increasing the circulating and body iron stores. In this study, however, serum ferritin concentrations did not increase during ascorbic acid supplementation.

Recently, Fuller *et al* (1996) investigated the effect of ascorbate supplementation on LDL copper-induced oxidation in smoking men or women. With a dose of 1000 mg/d for four weeks they reported significantly greater decrease in copper-induced TBARS than in a placebo group. The subjects followed a low ascorbate diet (≤ 30 mg/day) for two weeks before randomization to ascorbate or placebo groups. This resulted in low baseline plasma ascorbate levels (mean of 33 μmol/l in ascorbate and 41 μmol/l in placebo group), which probably enhanced absorption of ascorbate in intestine during the supplementation phase. However, at randomization, the mean of TBARS formation values was higher and conjugated dienes lag phase was shorter in the ascorbate group than in the placebo group. Consequently, the greater decrease of copper-induced oxidation in ascorbate group during the four week supplementation could have been in part due to regression towards the mean of these measurements.

In this present study, ascorbic acid supplementation for two months did not have any significant effect on the change in lipoprotein susceptibility to oxidation when differences in the baseline copper-induced lag time were controlled by covariance correction. However, it is also possible that the dose of 500 mg/d in our trial was not high enough to enhance the lipoprotein oxidation resistance. The mean plasma ascorbic acid increase of 32% in the plain group and 54% in the slow release group is much lower compared with the increase of 287% with 1000 mg/d in the study by Fuller and coworkers (1996), which was partly induced by low ascorbate diet before randomization.

We used frozen plasma samples for VLDL + LDL copper-induced oxidation measurements. We have preliminary data of 5 smoking men who were supplemented with 500 mg of ascorbic acid daily for 12 months and copper-induced VLDL + LDL oxidation was measured from fresh plasma. Lag time was 74 ± 6.5 min (mean ± s.d.) at baseline, and 73 ± 6.7 min after 12 month supplementation. Maximal slope was 11.2 ± 1.9 mabs/min at baseline and 11.0 ± 1.1 mabs/min in 12 months. This suggests, that freezing did not affect our

present finding concerning copper-induced lipoprotein oxidation susceptibility.

As observed in this study and by many authors (Nyyssönen *et al*, 1994; Fuller *et al*, 1996; Rifici *et al*, 1993), ascorbic acid is present only in minor concentration in the separated lipoprotein fraction and its antioxidative effect on *in vitro* oxidation tests is thought to be mediated through its protective action in plasma before lipoprotein separation. If ascorbic acid would be present in hemin + H_2O_2-induced lipoprotein oxidation method, it would extensively prolong the lag time and reduce the rate of the reaction as shown in Figure 2. Ascorbic acid and DHA have protected LDL against hemin + H_2O_2-mediated oxidation measured by decreased anodic electrophoretic mobility on agarose gel (Retsky and Frei, 1995). Also, the addition of ascorbate into the copper-induced LDL oxidation reaction has been previously shown to increase the lag time in a concentration-dependent manner (Esterbauer *et al*, 1989). However, Retsky and Frei (1995) have observed that added ascorbic acid may also behave as prooxidant, if transition metal-induced LDL oxidation is in advanced stage, where lipid peroxidation occurs primarily by a self-propagating mechanism, independent of initiating events.

Electronegatively charged LDL is a form of LDL that is supposed to be exposed to mild oxidation. It has been separated and chemically characterized in native human plasma (Cazzolato *et al*, 1991). Modified LDL is found to be more electronegatively charged than native LDL (Bittolo Bon *et al*, 1995) and thus it is possible to separate and measure by electrophoretic or chromatographic techniques. The LDL^- fractions separated with anion-exchange chromatography from human plasma have proved to have increased electrophoretic mobility compared with native LDL (Vedie *et al*, 1991). Previous studies have also shown increased electrophoretic mobility of human or rabbit atherosclerotic lesion LDL compared with normal intimal LDL or with plasma LDL (Ylä-Herttuala *et al*, 1989). Thus plasma LDL^- fraction may represent a mildly oxidatively modified LDL, the formation of which might be retarded by antioxidants. In this study, however, ascorbic acid supplementation did not have any effect on plasma LDL^- percentage of total LDL.

Previous reports suggest that ascorbic acid acts synergistically with α-tocopherol, which may partly be due to its sparing effect on α-tocopherol (Frei, 1991; Frei *et al*, 1989). Ascorbic acid has been shown by electron-spin-resonance spectroscopy to chemically reduce tocopheryl radical back to tocopherol (Niki, 1991; Sharma and Buettner, 1993). In LDL, ascorbic acid has been observed to reduce endogenous vitamin E radicals (α-tocopheryl and α-tocotrienoyl) after the formation of vitamin E chromanoxyl radicals was first induced by UV light irradiation (Kagan *et al*, 1992). Ascorbic acid supplementation of 1 g/d increased the levels of supplemented α-tocopherol and β-carotene in nonsmokers (Reaven *et al*, 1993). In this present study, smokers and ascorbic acid supplementation did not increase plasma α-tocopherol levels during either 12 h pharmacokinetic trial or two month supplementation. That is in agreement with all the previous results of supplementation studies in smokers (Fuller *et al*, 1996; Harats *et al*, 1990). Even though ascorbic acid is not able to increase the plasma α-tocopherol levels, it has been suggested that it can protect lipoproteins from oxidation by more complex mechanisms (Bowry *et al*, 1995). It is also suggested that prooxidant/antioxidant balance in arterial tissue is crucial in conditioning atherogenic processes in humans (Mezzetti *et al*, 1995). In a group of male patients undergoing coronary bypass surgery, the arterial tissue of smokers contained significantly less of vitamin E and C than that of non-smokers, and lipid peroxidation measured as fluorescent products of phospholipids was significantly higher in arterial tissue and plasma of smokers. There were also significant correlations between plasma and tissue vitamin E and C levels, which according to the authors indicates that arterial vitamin content is significantly influenced by plasma vitamin levels and intake with foods (Mezzetti *et al*, 1995).

We used daily doses of 500 mg, because in a recent study, the doses of 1 g or higher are not recommended (Levine *et al*, 1996). In the pharmacokinetic study, we used doses of 250 mg, because bioavailability was found to be incomplete for doses over 200 mg of vitamin C (Levine *et al*, 1996). The bioavailability of plain ascorbic acid and slow release ascorbic acid with hydroxyl propyl methyl cellulose appeared to be similar. The AUC values of ascorbic acid plasma concentration during the 12 h pharmacokinetic study did not differ significantly. The bioavailability of ascorbic acid preparations is likely due to the preparation itself or also intersubject variation in ascorbic acid absorption (Vidgren *et al*, 1992; Levine *et al*, 1996).

The results of this study indicate that oral supplementation of 500 mg of ascorbic acid daily for two months does not have protective effect on *in vitro* lipoprotein oxidation resistance or on *in vivo* lipid peroxidation in smoking men. On the basis of our findings, long-term ascorbic acid supplementation alone without any other antioxidant might even promote the formation of MDA. The reason for this is uncertain but may be mediated through ascorbic acid-metal ion catalyzed oxidation and generation of catalytic ferrous iron in the body.

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