Inhibition of rat lipoprotein lipid peroxidation by the oral administration of D003, a mixture of very long-chain saturated fatty acids

R. Menéndez, R. Más, A.M. Amor, N. Ledón, J. Pérez, R.M. González, I. Rodeiro, M. Zayas, and S. Jiménez

Abstract: Previous results have demonstrated that policosanol, a mixture of aliphatic primary alcohols isolated and purified from sugar cane wax, whose main component is octacosanol, inhibited lipid peroxidation in experimental models and human beings. D003 is a defined mixture of very long-chain saturated fatty acids, also isolated and purified from sugar cane wax, whose main component is octacosanoic acid followed by traicontanoic, dotriacontanoic, and tetracontanoic acids. Since very long-chain fatty acids are structurally related to their corresponding alcohols, we investigated the effect of oral treatment with D003 (0.5, 5, 50, and 100 mg/kg) over 4 weeks in reducing the susceptibility of rat lipoprotein to oxidative modification. The combined rat lipoprotein fraction VLDL + LDL was subjected to several oxidation systems, including those containing metal ions ($CuSO_4$), those having the capacity to generate free radicals 2,2-azobis-2-amidinopropane hydrochloride (AAPH), and a more physiological system (resident macrophages). D003 (5, 50, and 100 mg/kg) significantly inhibited copper-mediated conjugated-diene generation in a concentration-dependent manner. D003 increased lag phase by 53.1, 115.3, and 119.3%, respectively, and decreased the rate of conjugate-diene generation by 16.6, 21.5, and 19.6%, respectively. D003 also inhibited azo-compound initiated and macrophage-mediated lipid peroxidation as judged by the significant decrease in thiobarbituric acid reactive substance (TBARS) generation. In all the systems the maximum effect was attained at 50 mg/kg. There was also a parallel attenuation in the reduction of lysine amino groups and a significant reduction of carbonyl content after oxidation of lipoprotein samples. Taken together, the present results indicate that oral administration of D003 protects lipoprotein fractions against lipid peroxidation in the lipid as well in the protein moiety.

Key words: D003, very long-chain saturated fatty acids, lipoprotein lipid peroxidation.

Résumé : Les résultats antérieurs ont démontré que le policosanol, un mélange de alcools saturés qu'ont isolé et ont purifié de cire de la canne à sucre, dont le composant principal est l'octacosanol, que présent une activité inhibitrice sur le peroxydation des lipides dans les modèles expérimentaux et les êtres humains. D003, un mélange défini de acides gras saturés de très longue chaîne isolé et purifié de la cire de la canne à sucre aussi, dont le composant principal est l'acide octacosanoique suivie par le triacontanoique, le dotriacontanoique et l'acide tetraconanoioque. Comme les acides gras du très longue chaîne sont en rapport avec leurs alcools correspondants structurellement présentent une très grand similitude, nous enquêtons sur l'effet de traitement oral avec D003 (0,5, 5, 50 et 100 mg/kg) pendant 4 semaines dans réduire la susceptibilité des lipoprotéines du rat à l'oxydation. La fraction combinée des lipoprotéines VLDL + LDL a été soumis à plusieurs systèmes de l'oxydation qui incluent ceci que comprennent des ions du métal ($CuSO_4$), ceci qu'a la capacité de produit des radicaux libres (AAPH) et dans un système plus physiologique (macrophages résidant). D003 (0,5, 5, 50 et 100 mg/kg) inhibé, dans une manière concentration dépendante, la génération des dienes conjugués. Donc, D003 augmenté la phase latente par 53,1, 115,3 et 119,3 %, respectivement et provoque une diminution des taux de génération des dienes conjugués par 16,6, 21,5 et 19,6 %, respectivement. D003 aussi inhibé le peroxydation commence par azo-composé et par macrophages comme nous pouvons déterminer par la baisse significative dans génération du TBARS. Dans tous les systèmes, l'effet du maximum a été atteint à 50 mg/kg. il y avait aussi une atténuation parallèle dans la réduction des groupes du lysines des aminoacides et une réduction significative du contenu du groupes carbonyles après oxydation des échantillons des lipoprotéines. Pris ensemble, les résultats présents indiquent que l'administration orale de D003 protège des fractions de lipoprotéines contre le peroxydation lipidique dans les lipides comme dans les protéines.

Mots clés : D003, acides gras saturés de très longue chaîne, peroxydation lipidique des lipoprotéines.

Received 25 May 2001. Published on the NRC Research Press Web site at http://cjpp.nrc.ca on 28 January 2002.

R. Menéndez,[1] R. Más, A.M. Amor, N. Ledón, J. Pérez, R.M. González, I. Rodeiro, M. Zayas, and S. Jiménez. Laboratory of Biochemistry, Center of Natural Products, National Center for Scientific Research, P.O. Box 6412, Havana, Cuba.

[1]Corresponding author (e-mail: dalmer@ip.etecsa.cu).

 DOI: 10.1139/Y01-088

Introduction

Clinical trials have convincingly established that aggressive reduction of elevated cholesterol levels, particularly low-density lipoprotein cholesterol (LDL-C), reduces the progression of atherosclerosis and the incidence of coronary events (Lipid Research Clinical Program 1984; Frick et al. 1987; Scandinavian Simvastatin Survival Study Group 1994). However, other mechanisms beyond cholesterol lowering may contribute to the beneficial effect of lipid-lowering therapy.

Low-density lipoprotein (LDL) oxidation is considered to be a key process in early atherogenesis (Steinberg et al. 1989; Aviram 1995). LDL oxidation generates unsaturated aldehydes within the LDL particle, which are thought to bind to the amino acid side chain of apo B, leading to a increase of its affinity to the scavenger receptor (Steinbrecher 1987). Unlike the LDL receptor, the scavenger receptor is not regulated by intracellular cholesterol. Thus, oxidized LDL is avidly taken up by macrophages, leading to the formation of lipid-laden foam cells, the hallmark of early atherosclerotic lesions (Brown and Goldstein 1983). In addition to stimulating foam-cell generation, oxidized LDL (LDL_o) exerts other effects, which may contribute to the atherogenesis. Earlier steps of LDL oxidation render an LDL minimally oxidized (LDL_{mo}), which makes it unable to bind to the scavenger receptor of macrophages but able to induce serious damage to the endothelial cells, through the release of cytotoxic and proinflammatory products. In addition, LDL_o promotes the release of several growth factors that cause cellular migration and proliferation of smooth muscle cells (Rosenfeld et al. 1990) and impair endothelium-derived nitric-oxide production (Tanner et al. 1991; Cowan and Steffen 1995).

Policosanol is a mixture of aliphatic primary alcohols that are isolated and purified from sugar cane wax; its main component is octacosanol, followed by triacontanol and hexacosanol. Policosanol has proven to be an effective cholesterol-lowering agent in experimental models (Arruzazabala et al. 1994; Menéndez et al. 1997; Rodríguez-Echenique et al. 1994), healthy human volunteers (Hernández et al. 1993), and patients with type II hypercholesterolemia (Aneiros et al. 1995; Campilongo et al. 1997; Canetti et al. 1995; Castaño et al. 1998, 1999, 2000; Más et al. 1998, 1999).

In addition to its cholesterol-lowering effect, policosanol inhibits lipid peroxidation in experimental models (Fraga et al. 1997; Menéndez et al. 1999) and human beings (Fernández et al. 2001; Menéndez et al. 2000*a*, 2000*b*). Thus, oral administration of policosanol to rats over 4 weeks partially inhibited in vitro and in vivo liver microsomal lipid peroxidation (Fraga et al. 1997) and in vitro copper-induced lipoprotein peroxidation (Menéndez et al. 1999). Furthermore, inhibition of LDL oxidation was also observed after oral administration of policosanol within its therapeutic dosage to healthy human volunteers (Menéndez et al. 2000*a*), patients with type II hypercholesterolemia at high coronary risk (Menéndez et al. 2000*b*), and elderly hypercholesterolemic women (Fernández et al. 2001).

Nevertheless, policosanol fails to protect lipoprotein fractions against lipid peroxidation when directly added to the incubation mixture in a cell-free system. However, its antioxidant effects are observed after prolonged oral administration. It has been described that after oral administration of octacosanol, the main component of policosanol, it may be oxidized and degraded in vivo to fatty acids through β-oxidation and esterified with sterols and phospholipids (Kabir and Kimura 1993, 1995*a*, 1995*b*). In this regard, it has been shown that fatty-alcohol oxidation can be produced in cultured fibroblasts (Rizzo et al. 1988, 1990) and that β-oxidative chain shortened oxidation of very long-chain fatty alcohols can occur in vitro and in vivo (Singh and Poulos 1986; Singh et al. 1987; Wanders et al. 1987). Therefore, these facts are consistent with the hypothesis that the inhibition of lipid peroxidation exerted by policosanol could be caused not only by higher primary aliphatic alcohols but also by very long-chain fatty acids and chain-shortened secondary metabolites.

D003 is a defined mixture of very long-chain saturated fatty acids that have been isolated and purified from sugar cane wax (González et al. 1998). Octacosanoic acid is its main component ($C_{28}H_{56}O_2$, 40.0%), followed by triacontanoic ($C_{30}H_{60}O_2$, 21.7%), dotriacontanoic ($C_{32}H_{62}O_2$, 17.0%), and tetracontanoic ($C_{24}H_{68}O_2$, 10.2%) acids. Also, hexacosanoic ($C_{26}H_{52}O_2$, 1.0%), heptacosanoic ($C_{27}H_{54}O_2$, 0.7%), nonacosanoic ($C_{29}H_{58}O_2$, 2.1%), hentriacontanoic ($C_{31}H_{62}O_2$, 2.1%), tritriacontanoic ($C_{33}H_{66}O_2$, 2.0%), pentatriacontanoic ($C_{35}H_{70}O_2$, 1.0%), and hexacotriacontanoic ($C_{36}H_{72}O_2$, 1.2%) acids are present as minor components.

Since very long-chain fatty acids are structurally related to their corresponding alcohols, and different pharmacological effects of policosanol are not only exhibited by D003 but even greater effects have been reported (Mendoza et al. 2001; Molina et al. 2001), the aim of this work is to study the effect of D003 administered orally (0.5, 5, 50, and 100 mg/kg) to rats over 4 weeks on the susceptibility of rat lipoprotein lipid peroxidation. Because both LDL and very low density lipoprotein (VLDL) can be oxidized, and considering that rats have a small amount of LDL (Chapman 1980), we investigated the in vitro oxidation of lipoprotein fraction that contained both VLDL + LDL to copper-, azo compound-, and macrophage-mediated lipid peroxidation.

Materials and methods

Materials

Thiobarbituric acid (TBA), malondialdehyde bis (dimethyl acetate) (MDA), trinitrobenzelsulphonic acid (TNBS), and 2,4-dinitrophenzylhydrazine (DNPH) were obtained from Sigma (Sigma Chemical Co., St. Louis, Mo.). 2,2-Azobis-2-amidinopropane hydrochloride (AAPH) was purchased from Wako Chemical (Osaka, Japan). All other chemicals were of the best available quality. The ultracentrifuge was from Beckman (Beckman Instrument, Inc. Palo Alto, Calif.) and the Ultrospec-Plus spectrophotometer was from LKB (Pharmacia LKB Biotechnology, Uppsala, Sweden). Male Wistar rats (150–180 g) and male BalC mice (20–30 g) were purchased from the Centro Nacional para la Producción de Animales de Laboratorio (CENPALAB, La Habana, Cuba). D003 was generously supplied by the Analytical Chemistry Department of the Center of Natural Products after corroboration of its quality criteria specifications.

Animals treatment

Male Wistar rats were allowed free access to food and water. D003 was suspended in Acacia gum-water vehicle (10 mg/mL) and orally administered by gastric gavage (1 mL/kg). Five experimental groups were used: a control group receiving the vehicle and four treated groups receiving 0.5, 5, 50, and 100 mg of D003 per kilogram, respectively. All the experimental groups were treated for up to 4 weeks. The experiments were conducted in accordance with the ethical guidelines for investigations in laboratory animals and were approved by the Ethical Committee for Animal Experimentation of the National Center for Scientific Research (CNIC).

Lipoprotein oxidation

Lipoprotein from control and treated animals were prepared from fasted blood plasma by density gradient ultracentrifugation in a Beckman ultracentrifuge (Lasser et al. 1973). After centrifugation, VLDL + LDL fractions were removed, pooled, and dialyzed against a 200-fold volume of phosphate-buffered saline (PBS: 0.01 M phosphate, 0.15 M NaCl, pH 7.4) at 4°C in the dark for 24 h. The dialysis buffer was purged with nitrogen and changed twice during this period. To investigate the effects of the addition of D003 in vitro on copper- and AAPH-mediated lipid peroxidation, EDTA-free lipoprotein (100 μg) from nontreated rats was incubated in 1 mL quartz cells with 10 μM $CuSO_4$ and 5 mM AAPH. In controls, 10 μL of 0.4% Tween-20 was added, whereas treated groups received 10 μL D003 emulsions in 0.4% Tween-20 to ensure concentrations of 5, 50, and 100 μg/mL. It has been previously checked that 0.04% Tween-20 has no effect on lag time and propagation rate. To study the effect of oral treatment with D003 on rat lipoprotein lipid peroxidation, EDTA-free lipoprotein from control and treated rats was incubated with 10 μM $CuSO_4$ and 5mM AAPH. AAPH is a water-soluble azo compound that thermally decomposes and generates water soluble peroxyl radicals at a constant rate (Frei et al. 1988). The kinetics of copper-mediated oxidation were continuously monitored by measuring the formation of conjugated dienes at 234 nm, using an Ultrospec-plus spectrophotometer equipped with an automatic six-cell position changer. The absorption was measured at intervals of 10 min for a period of 5 h at 37°C. Results were expressed as lag phase, the rate of oxidation, and maximum conjugated-diene generation, according to Puhl et al. (1994).

The extent of AAPH-mediated oxidation was measured by the thiobarbituric acid reactive substances (TBARS) assay (Ohkawa et al. 1978). Oxidation was terminated by the addition of 10 μM butylated hydroxytoluene (BHT) and refrigeration at 4°C. Lipoprotein concentration was estimated by determining protein concentration with a modified Lowry procedure (Markwell et al. 1987).

Cell-mediated oxidation of the VLDL + LDL fraction was assessed as follows. After dialysis, EDTA-free VLDL + LDL samples were immediately subjected to a cell-mediated oxidation by resident macrophages that had been freshly isolated from male BalC mice according to de Whalley et al. (1990). Macrophages were isolated by peritoneal lavage of mice (20–30 g), using ice cold Dulbecco's PBS without Ca^{2+} and Mg^{2+}. The cells were plated in 12-well cluster plates at 1×10^6 cells/well and incubated at 37°C in CO_2, and the culture medium consisted of 9 vol. of Dulbecco's modified Eagle's medium (DMEM) and 1 vol. of fetal calf serum that contained 50 μg of gentamicin per millilitre. After 2 h, the macrophages were washed several times with DMEM and were immediately used for experiments. LDL + VLDL samples (100 μg/mL) were incubated up to 20 h with macrophages (macrophage-modified) or in cell-free wells (controls). The culture medium (0.5 mL/well) consisted of Ham's F-10 medium containing gentamicin (50 μg/mL). After incubation, EDTA and BHT were added (final concentrations of 20 μM and 2 mM, respectively) to avoid any further oxidation, and the medium was removed and centrifuged ($250 \times g$ for 10 min at 4°C) to remove any detached cells. LDL + VLDL in the medium were assayed for TBARS as described previously (Ohkawa et al. 1978). Calibration was done with MDA as a standard. TBARS were expressed as MDA equivalents (nmol MDA/mg cell protein).

Free amino groups were measured using TNBS (Steinbrecher 1987). Aliquots of lipoprotein fractions (60 μg) were mixed with 1 mL of 4% $NaHCO_3$ (pH 8.4) and 50 μL of 0.1% TNBS. Samples were incubated for 1 h at 37°C. Then, 100 μL of 10% sodium dodecyl sulphate (SDS) and 100 μL 1 N HCl were added and the absorbance at 340 nm was measured. The lysine reactivity was expressed as a percentage of the absorbance obtained from non-oxidized lipoproteins.

Carbonyl groups in oxidized VLDL + LDL fractions were determined according to Hazell et al. (1994). Lipoprotein fractions (final concentration 1.6 μM) were mixed with an equal volume of DNPH (1 mM in HCl). After incubation for 30 min at 50°C, 1.5 volume of 1 M NaOH was added and the absorbance at 430 nm was measured. An average molar absorption coefficient of 22 000 $M^{-1} \cdot cm^{-1}$ was used to estimate the carbonyl content.

Lipid profile of plasma lipoprotein

Cholesterol, phospholipid, and triglyceride content in the lipoprotein fraction of control and treated groups was determined using kits according to the manufacturer's instruction (Boehringer Mannheim) (cholesterol, No. 676535; tryglicerides, No. 238473; and phospholipids, No. 691844).

Statistical analysis

Data are presented as means ± SD. Statistical analysis was performed by two-tailed Mann Whitney *U* test. Differences with $p < 0.05$ were considered statistically significant.

Results

The effects of the addition of D003 in vitro on copper- and AAPH-mediated lipid peroxidation are shown in Table 1. When D003 was added at 0.5, 5, 50, and 100 μg/mL, no modification of lag time and propagation rate was observed. Also, D003 did not affect the accumulation of TBARS after AAPH-initiated lipid peroxidation.

The effect of in vivo administration of D003 on the subsequent susceptibility of rat lipoprotein was studied in several oxidation systems, including those containing metal ions ($CuSO_4$), those having the capacity to generate free radicals

Table 1. Effects of the addition of D003 in vitro on the susceptibility of VLDL + LDL lipoprotein fraction on copper- and AAPH-mediated lipid peroxidation.

	Copper mediated		
Treatment	Duration of lag time (min)	Propagation rate (Δ OD[a] $\times 10^{-3}$/min)	AAPH mediated (OD)
Control	98.81±11.00	17.46±1.40	0.72±0.10
D003 5 μg/mL	105.82±13.32	17.30±0.83	0.74±0.05
D003 50 μg/mL	103.82±10.77	17.10±0.98	0.68±0.13
D003 100 μg/mL	106.43±12.77	16.76±1.58	0.72±0.21

Note: EDTA-free lipoprotein (100 μg) isolated from nontreated animals were incubated with 10 μM $CuSO_4$ and 5 mM AAPH in the presence of vehicle (controls) and D003 (5, 50, 100 μg/mL). Results are means ± SD of three experiments.
[a]OD, optical density.

Table 2. Effect of pretreatment with D003 on the composition of VLDL + LDL lipoprotein fraction from rat plasma.

Treatment	Cholesterol (mg/mg protein)	Triglycerides (mg/mg protein)	Phospholipids (mg/mg protein)
Control	8.69±1.41	7.77±2.50	0.52±0.14
D003 0.5 mg/kg	8.28±1.27	6.81±1.54	0.43±0.22
D003 5 mg/kg	9.08±1.82	9.53±1.88	0.57±0.14
D003 50 mg/kg	9.60±1.69	8.32±1.13	0.48±0.14
D003 100 mg/kg	9.41±1.38	6.87±2.16	0.48±0.13

Note: Values are means ± SD, $n = 5$.

Table 3. Effect of pretreatment with D003 on the copper-mediated oxidation of VLDL + LDL as measured by the formation of conjugated dienes.

Treatment	Duration of lag phase (min)	Increase (%)	Propagation phase (Δ OD[a] $\times 10^{3}$/min)	Decrease (%)
Control	103.03±10.94		16.96±0.97	
D003 0.5 mg/kg	110.23±16.79	6.9	15.43±0.99	9
D003 5 mg/kg	157.83±19.91*	53.1	14.13±0.97*	16.6
D003 50 mg/kg	221.90±6.13*	115.3	13.31±3.49*	21.5
D003 100 mg/kg	226.04±16.06*	119.3	13.63±2.12*	19.6

Note: Lipoprotein fractions (VLDL + LDL) were obtained 4 weeks after D003 and vehicle (control) administration and were oxidized as described in Fig. 1. Results are means ± SD of three independently performed experiments, each corresponding to a pool of 3 rat plasmas.
[a]OD, optical density.
*$p < 0.05$, compared with control (Mann Whitney U test).

(AAPH), and a more physiological system (resident macrophages).

Table 2 shows the effect of pretreatment with D003 on the composition of VLDL + LDL rat lipoprotein fractions. Pretreatment with D003 did not affect the cholesterol, phospholipid, or triglyceride content in VLDL + LDL fraction.

In the copper ion oxidative system, oral administration of D003 inhibited conjugated-diene generation in a concentration-dependent manner (Table 3). Thus, the combined fraction VLDL + LDL isolated from the treated groups (5, 50, and 100 mg/kg) were resistant to lipid peroxidation as judged by the significant increase in the length of the lag phase and the significant decrease in the rate of conjugated-diene generation (Fig. 1). Thus, oral administration of D003 at doses of 5, 50, and 100 mg/kg significantly increased lag phase by 53.1, 115.3, and 119.3%, respectively, and significantly decreased the rate of conjugate-diene generation by 16.6, 21.5, and 19.6%, respectively. Even when a slight increase in the lag time (6.9%) and a decrease in propagation rate (9.0%) were observed when administered at 0.5 mg/kg, these effects were not statistically significant.

To determine whether or not the inhibitory effect of D003 on conjugated-diene generation is limited to the oxidation induced by metal ions, the effects on the oxidation of VLDL + LDL by the nonmetal and aqueous radical initiator AAPH were investigated. As shown in Fig. 2, oral administration of D003 also inhibited azo-compound initiated lipid peroxidation, since the generation of TBARS was significantly inhibited. As occurred for copper-initiated oxidation, the lowest dose assayed did not significantly inhibit the generation of TBARS. It should be also observed that in both systems, copper- and AAPH-initiated oxidation, the maximum effect was attained at 50 mg/kg, since no additional effects were observed after the administration of 100 mg/kg.

It is interesting to note that D003 reduced the susceptibility of VLDL + LDL fractions to macrophage-mediated oxi-

Fig. 1. Representative kinetics of conjugated-diene formation of VLDL + LDL lipoprotein fraction. Rate of diene generation was measured in a pool of freshly prepared lipoprotein fraction of each experimental group. Lipoproteins (0.5 mg/mL) were incubated in PBS in the presence of 5 μM $CuSO_4$. The absorbance at 234 nm was continuously monitored at 10 min intervals. Each point represents the mean of triplicate determinations for each experimental group.

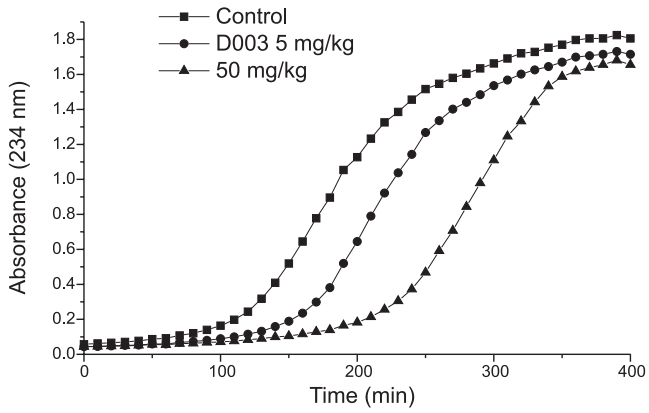
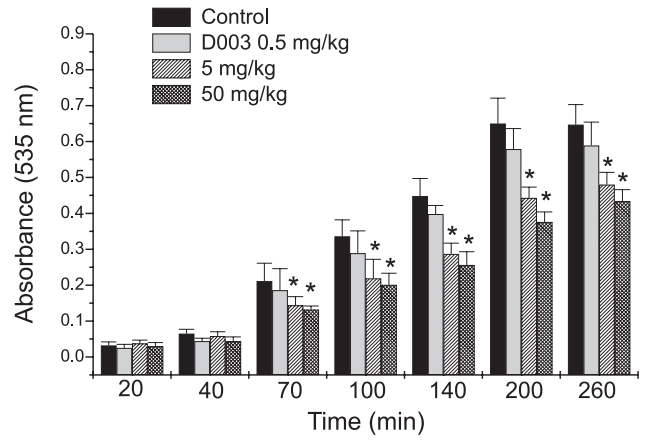


Fig. 2. Effect of pretreatment with D003 on the susceptibility of VLDL + LDL fraction to azo compound-initiated oxidation as measured by the formation of TBARS. Lipoprotein (100 μg) from control and treated rats was incubated with 5 mM AAPH. Oxidation was terminated by the addition of 10 μM butylated hydroxytoluene (BHT) and refrigeration at 4°C. TBARS were measured in lipoprotein samples. Bars show mean absorbance values ± SD of three independently performed experiments, each corresponding to a pool of 3 rats. *$p < 0.05$, significant difference from control (Mann Whitney U test).



dation in a dose-dependent manner, as assessed by determination of TBARS generation (Fig. 3). Thus, mean TBARS accumulation significantly decreased in the groups treated with 5, 50, and 100 mg/kg. As occurred for copper- and azo-compound inhibition, the lowest dose assayed did not protect lipoprotein fractions against lipid peroxidation. Besides, the maximal effect also occurred at 50 mg/kg.

Treatment of VLDL + LDL fraction with $CuSO_4$ reduced the TNBS reactivity (Fig. 4). However, this response was qualitatively different in controls than in treated rats. Thus, lysine reactivity of oxidized lipoproteins from treated groups

Fig. 3. Effect of pretreatment with D003 on macrophage-induced lipid peroxidation. After dialysis, EDTA-free VLDL + LDL samples isolated from control and treated rats (100 μg/mL) were subjected (20 h) to a cell-mediated oxidation by resident macrophages freshly isolated from male BalC mice. After incubation, EDTA and butylated hydroxytoluene (BHT) were added (final concentration of 20 μM and 2 mM, respectively) to avoid any further oxidation and the medium was removed and centrifuged (250 g for 10 min at 4°C). Then TBARS were determined. Bars show mean values ± SD of triplicate experiments performed with a pool of 3 rats. *$p < 0.05$, significant difference from control (Mann Whitney U test).

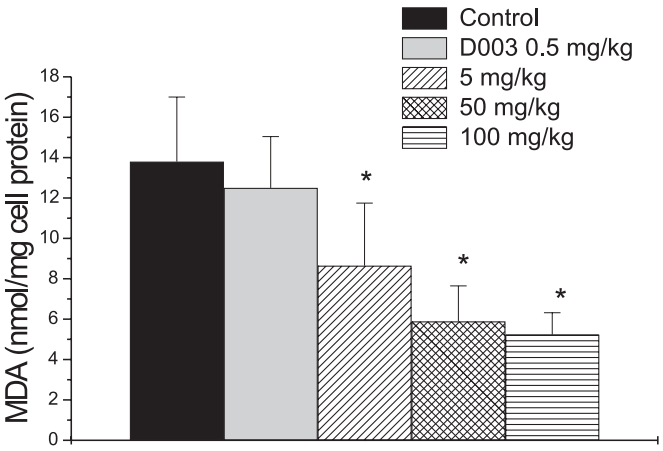
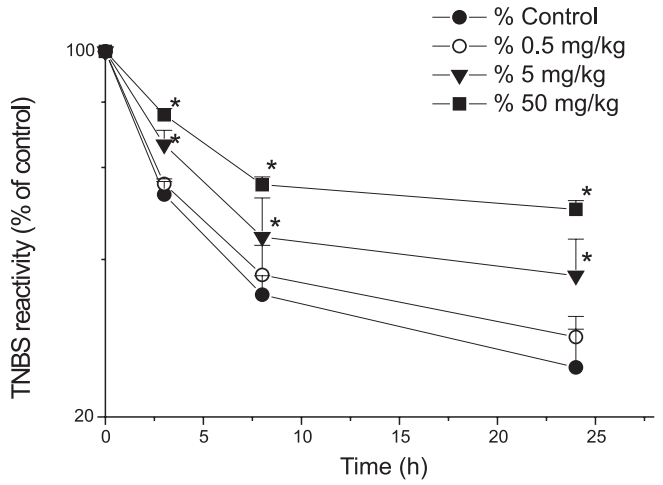
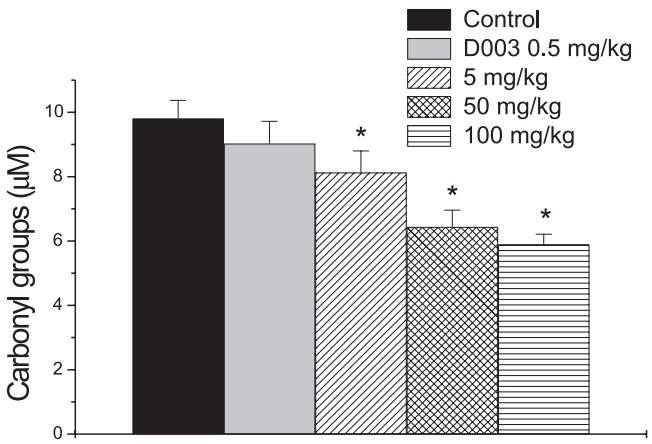


Fig. 4. Effect of D003 pretreatment on reactive lysine groups in copper-mediated oxidized VLDL + LDL fractions. Lipoproteins were oxidized as described in Fig. 1 and, samples were taken at various time points and analyzed for TNBS reactivity (expressed as a percentage of the value obtained for nonoxidized lipoprotein fractions). Results represent the mean ± SD of three experiments. *$p < 0.05$, significant difference from control (Mann Whitney U test).



was reduced less than it was in the control. Also, formation of carbonyl residues is diminished by D003 (5, 50, and 100 mg/kg) in a dose-dependent manner, since the content of carbonyl groups was significantly reduced in lipoprotein isolated from the treated group than from the control group (Fig. 5).

Fig. 5. Effect of D003 pretreatment on carbonyl groups in oxidized VLDL + LDL fractions. Lipoprotein fractions were oxidized in the presence of AAPH as described in Fig. 2. After 3 h, lipoprotein fractions (final concentration 1.6 μM) were mixed with an equal volume of DNPH (1 mM in HCl). After incubation for 30 min at 50°C, 1.5 volume of 1 M NaOH was added and the absorbance at 430 nm was measured. An avera ge molar absorption coefficient of 22 000 $M^{-1} \cdot cm^{-1}$ was used to estimate the carbonyl content. Results represent the mean ± SD of three experiments.



Discussion

The present study demonstrated that oral administration of D003 (5, 50, and 100 mg/kg) for 4 weeks inhibited the in vitro oxidation of VLDL + LDL rat lipoprotein fractions. Oral administration of D003 prolonged the stability of rat lipoprotein fractions against free radicals generated by the thermal decomposition of AAPH and in the presence of copper ions. It is interesting to note that oral administration of D003 inhibited macrophage-induced lipid peroxidation. Oxidative modification of LDL has been demonstrated to occur in vivo (Haberland et al. 1988; Rosenfeld et al. 1990; Hoff and Gaubatz 1982) and therefore the oxidative modification of LDL in the arterial wall is currently believed to be central in the generation of the atherosclerotic plaque. Arterial wall cells, including macrophages can mediate lipid peroxidation of LDL (Leake and Rankin 1990). The oxidized LDL possesses several atherogenic properties, including its cytotoxicity to arterial cells, its proinflammatory and prothrombotic characteristics, and its enhanced uptake by macrophages via scavenger receptors (Krieger 1997). Thus, the finding that D003 protected VLDL + LDL against cell-mediated oxidation suggests that D003 might also protect lipoproteins in vivo. There was also a parallel attenuation in the reduction of lysine amino groups and the carbonyl groups of the VLDL + LDL fraction after D003 administration. It has been shown previously that oxidation of LDL is accompanied by a marked decrease in amino group reactivity (Steinbrecher 1987). Derivatization of lysine epsilon-amino group residues of apolipoprotein B are produced by aldehydes derived from peroxidation of lipids, causing a decrease in TNBS reactivity. Also, it is known that the attack of free radicals can transform side chain amine groups of several amino acids, such as lysine, to carbonyl derivatives (Reznick and Packer 1994). Therefore, the present results suggest that D003 could also protect the protein moiety of the lipoprotein.

As we mentioned above, policosanol, a mixture of higher aliphatic alcohols, inhibited rat lipoprotein lipid peroxidation (Menéndez et al. 1999). Therefore, the effect of D003 on VLDL + LDL rat lipoprotein fractions is reasonable, bearing in mind that higher aliphatic acids are structurally similar to their respective alcohols. However, the effects of D003 are more pronounced since the antioxidant effects of policosanol on the combined rat lipoprotein fraction VLDL + LDL were observed after administration during a similar period of time but at higher doses (Menéndez et al. 1999). This fact seems to be reasonable considering not only that the major component of policosanol could be possibly metabolized to its corresponding acid (Kabir and Kimura 1993, 1995*a*, 1995*b*) but also that it is an active metabolite of policosanol (Más 2000). Thus, since the major component of D003 is octacosanoic acid, the metabolic transformation from alcohol to acid form is not necessary and the effect of D003 can be exerted more directly.

The mechanism of action whereby pretreatment with D003 prevents the oxidation of the combined fraction VLDL + LDL is beyond the objective of the present work. However, as mentioned above, D003 protects the combined fraction VLDL + LDL from metal and nonmetal lipid peroxidation. Geiseg and Esterbauer (1994) have established that the sensitivity of LDL to copper-initiated oxidation was closely related to the occupation of pro-oxidant binding sites by copper. On the other hand, AAPH is a water-soluble azo-compound that generates free radicals in the aqueous phase, and radicals that diffuse into LDL from the medium can initiate oxidation (Thomas et al. 1997). Therefore, the effects of D003 on the oxidative modification of rat lipoprotein fractions are not only related to a chelating action on copper ions.

However, it should be considered that when added in vitro, D003 did not prevent copper- and AAPH-mediated oxidation of the combined fraction VLDL + LDL, even at high concentrations. It suggests that D003 is not an antioxidant itself. As seen in the present work, the effect of D003 is detected after prolonged treatment because, as occurred when added in vitro, a single dose does not give the same result (results not shown). Therefore, we believe that the antioxidant effect of D003 is not directly related to the chemical structure of the components of D003.

An alternative explanation for the antioxidant effects of D003 may be related to the lipid composition of the lipoprotein particles. Frei and Gaziano (1993) have demonstrated that the increase in cholesterol content in lipoprotein fraction was associated with an increased susceptibility to lipid peroxidation to copper ions and aqueous peroxyl radicals. However, no modifications in cholesterol, triglyceride, and phospholipid content in lipoprotein fractions were observed after administration of D003 in this concentration range. Nevertheless, esterified unsaturated fatty acyl side chains of lipids are the true substrates for lipid peroxidation and thus they are directly related to the oxidative susceptibility of LDL. It has been demonstrated that the reduction of polyun-

saturated fatty-acid content of lipoprotein can reduce the susceptibility of these particles to oxidative modification by reducing the number of fatty acids as targets for lipid peroxidation (Parthasarathy et al. 1990; Scaccini et al. 1992). Therefore, further studies should be conducted to investigate the composition of fatty acids transported by VLDL and LDL after D003 administration.

An important factor that should be further considered is the antioxidant content of lipoprotein in control and D003-treated groups. Lipoprotein contains a number of antioxidants that can inhibit lipid peroxidation. However, vitamin E is by far the most abundant antioxidant in LDL and the capacity of lipoprotein particles to resist oxidation can be increased by increasing the amount of endogenous antioxidants in the particles (Dieber-Rotheneder et al. 1991; Harats et al. 1991; Jailal et al. 1991; Princen et al. 1992). Therefore, measurements of vitamin E content of the combined lipoprotein fraction in control and treated groups would more definitely show by which mechanism D003 inhibited lipoprotein oxidation.

In summary, the present study demonstrates that oral administration of D003 (5, 50, and 100 mg/kg) over 4 weeks protected combined rat lipoprotein fraction containing VLDL + LDL against lipid peroxidation. D003 inhibited, in a dose-dependent manner, copper-mediated conjugated-diene generation as judged by the significant increase in the length of the lag phase and the decrease in the propagation rate. Also, oral administration of D003 at the same concentration range inhibited AAPH-initiated lipid peroxidation, since TBARS generation was significantly inhibited in the treated groups. In addition, D003 inhibited macrophage-mediated TBARS generation. There was also a parallel attenuation in the reduction of lysine amino groups and a significant reduction of carbonyl content after oxidation of lipoprotein samples. Taken together, the present results indicate that oral administration of D003 protects lipoprotein fractions in the lipid as well in the protein moiety against lipid peroxidation. Previous experiments have shown that D003 also reduces serum cholesterol LDL-C levels in normocholesterolemic rabbits (Mendoza et al. 2001) and exhibits antithrombotic and platelet effects in experimental models (Molina et al. 2001). These effects together with D003's antioxidant effects may have great potential in preventing deleterious events involved in the pathogenesis of atherosclerosis.

References

Aneiros, E., Más, R., Calderón, B., Illnait, J., and Fernández, L. 1995. Effects of policosanol in lowering cholesterol levels in patients with type II hypercholesterolemia. Curr. Ther. Res. **56**: 176–181.

Arruzazabala, M.L., Carbajal, D., Más, R., Molina, V., Valdés, S., and Laguna, A. 1994. Cholesterol lowering effect of policosanol on normocholesterolemic rabbits. Biol. Res. **27**: 205–210.

Aviram, M. 1993. Modified forms of low-density lipoprotein and atherosclerosis. Atherosclerosis, **98**: 1–9.

Brown, M.S., and Goldstein, J.L. 1983. Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. Annu. Rev. Biochem. **52**: 223–261.

Campilongo, R., Sardini, P., and Fieldman, R. 1997. Efficacia, seguridad y tolerabilidad del policosanol en pacientse argentinos con hipercolsterolemia tipo II. Estudio abierto. La prensa Médica Argentina, **83**: 665–672.

Canetti, M., Morera, M.S., Illnait, J., Más, R., Fernández, J., Castaño, G., and Fernández, J.C. 1995. One-year study on the effect of policosanol (5 mg-twice-a-day) on lipid profile in patients with type II hypercholesterolemia. Adv. Therapy, **12**: 245–251.

Castaño, G., Más, R., Fernández, L., Fernández, J.C., Illnait, J., and Sellman, E. 1998. A double-blind placebo controlled study of the effect of policosanol in patients with intermittent claudication. Angiology J. Vasc. Dis. **50**: 123–130.

Castaño, G., Más, R., Fernández, J.C., López, L.E., and Fernández, L. 1999. A long-term open study of the efficacy and tolerability of policosanol in patients high global coronary risk. Curr. Ther. Res. **60**: 379–391.

Castaño, G., Más, R., Fernández, L., Fernández, J.C., Illanit, J., López, L.E., and Alvarez, E. 2000. Effect of policosanol on postmenopausal women with type II hypercholesterolemia. Gynecol. Endocrinol. **13**: 1–9.

Chapman, M.J. 1980. Animal lipoprotein: chemistry, structure and comparative aspects. J. Lipid Res. **21**: 789–853.

Cowan, C.L., and Steffen, R.P. 1995. Lysophosphatidylcholine inhibits relaxation of rabbit abdominal aorta mediated by endothelium-derived nitric oxide and endothelium-derived hyperpolarizing factor independent of protein kinase C activation. Arteriorcler. Thromb. Vasc. Biol. **15**: 2290–2297.

de Whalley, C.V., Ramkin, S.M., Hoult, J.R., Jessup, W., and Leaker, D.S. 1987. Flavonoids inhibit the oxidative modification of low-density lipoprotein by macrophages. Biochem. Pharmacol. **39**: 1743–1750.

Dieber-Rotheneder, M., Ouhl, H., Waeg, G., Striegl, G., and Esterbauer, H. 1991. Effect of oral supplmentation with d-alpha-tocopherol on the vitamin content of human low density lipoprotein and its oxidation resistance. J. Lipid. Res. **32**: 1325–1332.

Fernández, J.C., Más, R., Castaño, G., Menéndez, R., Amor, A.M., González, R.M., and Alvarez, E. 2001. Comparison of the efficacy and tolerability of policosanol Vs fluvastatine in elderly hypercholesterolemic women. Clin. Invest. **21**: 103–113.

Fraga, V., Menéndez, R., Amor, A.M., González, R.M., Jiménez, S., and Más, R. 1997. Effect of policosanol on in vitro and in vivo rat liver microsomal lipid peroxidation. Arch. Med. Res. **28**: 355–360.

Frei, B., and Gaziano, J.M. 1993. Content of antioxidant, preformed lipid hydroperoxides and cholesterol as predictors of susceptibility of human LDL to metal ion-dependent and independent oxidation. J. Lipid Res. **34**: 2135–2145.

Frei, B., Stocker, R., and Ames, B.N. 1988. Antioxidant defenses and lipid peroxidation in human blood plasma. Proc. Natl. Acad. Sci. U.S.A. **85**: 9748–9752.

Frick, M.E., Elo, O., and Haapa, K. 1987. Helsinki heart study: Primary intervention trial with gemfibrozil in middle age men with dislypidemia. N. Engl. J. Med. **317**: 1237–1245.

Geiseg, S.P., and Esterbauer, H. 1994. Low-density lipoprotein is saturable by pro-oxidant copper. FEBS Lett. **343**: 188–194.

González, L., Marrero, D., Laguna, A., Más, R., Arruzazabala, M.L. Carbajal, D., Cora, M., Menéndez, R. 1998. A mixture of primary fatty acids obtained from sugar cane wax. WO98/43631/Octb1998.

Haberland, M.E., Fong, D., and Cheng, L. 1988. Malondialdehyde-altered protein occurs in the atheroma of Watanabe heritable hyperlipidemic rabbits. Science, **241**: 215–218.

Harats, D., Ben-Naim, ?., Dabach, Y., Hollaander, G., Havivi, E., Sten, O., and Sten, Y. 1991. β-carotene inhibits the oxidative

modification of low density lipoprotein. Biochem. Biophys. Acta, **1086**: 134–138.

Hazell, L.J., van den Berg, J.J.M., and Stocker, R. 1994. Oxidation of low-density lipoprotein by hypochlorite causes aggregation that is mediated by modification of lysine residues rather that lipid oxidation. Biochem. J. **302**: 297–304.

Hernández, F. Innait, J. Más, R., Castano, G., Fernández, L., González, M., Cordov, N., Fernández, J.C. 1992. Effects of policosanol on serum lipids and lipoproteins in healthy volunteers. Curr. Ther. Res. **51**: 568–575.

Hoff, H.F., and Gaubatz, J.W. 1982. Isolation, purification and characterization of a lipoprotein containing apo B from human aorta. Atherosclerosis, **42**: 273–297.

Jailal, I., Norkus, E.P., Cristol, L., and Grundy, S.M. 1991. β-carotene inhibits the oxidative modification of low density lipoprotein. Biochim. Biophys. Acta, **1086**: 134–138.

Kabir, Y., and Kimura, S. 1993. Biodistribution and metabolism of orally administered octacosanol in rats. Ann. Nutr. Metab. **37**: 33–38.

Kabir, Y., and Kimura, S. 1995*a*. Tissue distribution of (8-^{14}C)-octacosanol in liver and muscle of rats after serial administration. Ann. Nutr. Met. **39**: 279–284.

Kabir, Y., and Kimura, S. 1995*b*. Metabolism of octacosanol in liver and muscle of rats. Acta Alimentaria, **24**: 39–46.

Krieger, M. 1997. Then other side of scavenger receptor: pattern recognition for host defense. Curr. Opin. Lipidol. **8**: 275–280.

Lasser, N.L., Rohein, P.S., Edelsein, D., and Eder, H.A. 1973. Serum lipoprotein of normal and cholesterol-fed rats. J. Lipid Res. **14**: 1–8.

Leake, D.S., and Rankin, S. 1990. The modification of LDL by macrophages. Biochem. J. **270**: 741–744.

Lipid Research Clinical Program. 1984. The lipid research clinics primary prevention trial result. II the relationship of reduction in the incidence coronary heart disease to cholesterol-lowering. JAMA, **251**: 365–374.

Markwell, M.A., Hass, S.M., Bieber, L.L., and Tolbert, N.R. 1987. A modification of the Lowry procedure to simply protein determination in membrane and lipoprotein samples. Anal. Biochem. **87**: 206–210.

Más, R. 2000. Policosanol. Drugs of the future, **25**(6): 569–586.

Más, R., Castaño, G., Illnait, J., Fernández, J.C., Alemán, C., Pontigás, V., and Lescay, M. 1998. Effects of policosanol in patients with type II hypercholesterolemia and additional coronary risk factors. Clin. Pharmacol. Ther. **65**: 439–447.

Más, R., Rivas, P., Izquierdo, J.E., Hernández, R., Fernández, L., Ferenández, J.C., Orta, S.D., Illanit, J., and Ricardo, Y. 1999. Pharmacoepidemiologic study of policosanol. Curr. Ther. Res. **60**: 458–467.

Mendoza, S., Gámez, R., Noa, M., Más, R., Castaño, G., Mesa, R., Mesa, M., and de Armas, M. 2001. The effects of D003 and policosanol on the lipid profile and endothelemia in normocholesterolemic rabbits: a head to head comparison. Curr. Ther. Res. **62**: 209–220.

Menéndez, R., Arruzazabala, M.L., Más, R., del Rio, A., Amor, A.M., González, R.M., Carbajal, D., Fraga, V., Molina, V., and Illnait, J. 1997. Cholesterol-lowering effect of policosanol on rabbits with hypercholesteroemia induced by wheat starch-casein diet. Br. J. Nutr. **77**: 923–32.

Menéndez, R., Fraga, V., Amor, A.M., González, R.M., and Más, R. 1999. Oral administration of policosanol inhibits in vitro copper ion-induced rat lipoprotein peroxidation. Physiol. & Behav. **67**: 1–7.

Menéndez, R., Más, R., Amor, A.M., Fernández, J.C., and González, R.M. 2000*a*. Effects of policosanol on the susceptibility of low-density lipoprotein isolated from hypercholesterolemic patients at high coronary risk to *in vitro* copper-mediated ;lipid peroxidation. A randomized, double-blind pilot study. Curr. Ther. Res. **61**: 609–620.

Menéndez, R., Más, R., Amor, A.M., González, R.M., Fernández, J.C., Rodeiro, I., Zayas, M., and Jiménez, S. 2000*b*. Effects of policosanol treatment on the susceptibility of low-density lipoprotein (LDL) isolated from healthy volunteers to oxidative modification in vitro. Br. J. Clin. Pharmacol. **50**: 255–262.

Molina, V., Arruzazabala, M.L., Carbajal, D., Más, R., and Valdés, S. 2001. Antiplatelet and antithrombotic effect of D003. Pharmacol. Res. **42**: 137–143.

Ohkawa, H., Ohishi, N., and Yagi, K. 1978. Assay for lipid peroxides in animal tissue by TBA. Anal. Biochem. **95**: 351–358.

Parthasarathy, S., Khoo, J.C., Elizanbeth, M., Barnett, J., Witztum, J.L., and Steinberg, D. 1990. Low-density lipoprotein rich in oleic acid is protected against oxidative modification: implication for dietary prevention of atherosclerosis. Proc. Natl. Acad. Sci. U.S.A. **87**: 3894–3898.

Princen, H.M., vam Poppel, G., Vogelezang, C., Buytenhek, R., and Kok, F.J. 1992. Supplementation with vitamin E but not beta-carotene in vivo protects low-density lipoprotein from lipid peroxidation in vitro. Effect of cigarette smoking. Arteriosc. Thromb. **12**: 554–562.

Puhl, H., Waeg, G., and Esterbauer, H. 1994. Methods to determine oxidation of low-density lipoprotein. *In* Methods of Enzymology, Vol. 233, ed. Lester Parker, New York, Academic Press. pp. P425–P441.

Reznick, A.Z., and Packer, L. 1994. Oxidative damage to proteins. Spectrophotometric method for carbonyl assay. *In* Methods in enzymology. Vol. 233. Academic Press, New York. pp. P357–P363.

Rizzo, E., Dammann, A., and Craft, D. 1988. Sjögre-Laarsson syndrome. Impaired fatty alcohol oxidation in cultured fibroblasts due to deficient fatty alcohol:nicotinamide adenine nucleoside oxidoreductase activity. J. Clin. Invst. **81**: 738–744.

Rizzo, E., Craft, D., Dammann, A., and Philip, M. 1990. Fatty alcohol metabolism in cultured fibroblasts. Evidence for a fatty alcohol cycle. J. Clin. Chem. **262**: 17 412 – 17 419.

Rodríguez-Echenique, C., Mesa, R., Más, R., Noa, M., Menéndez, R., González, R.M., Amor, A.M., Fraga, V., Sotolongo, V., and Laguna, A. 1994. Effects of policosanol chronically administered in male monkeys. Food. Chem. Toxicol. **11**: 74–83.

Rosenfeld, M.E., Palinski, W., Ylä-Herttuala, S., Butler, S., and Witztum, J.L. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. Atherosclerosis, **10**: 336–349.

Scaccini, C., Nardini, M., D'Aquino, M., Gentili, V., Di Felice, M., and Tomassi, G. 1992. Effect of dietary oils on lipid peroxidation and antioxidant parameters of rat plasma and lipoprotein fractions. J. Lipid Res. **33**: 627–633.

Scandinavian Simvastatin Survival Study Group. 1994. Randomized trial of cholesterol-lowering in 4444 patients with coronary heart disease. The Scandinavian Simvastatin survival study (4S). Lancet, **344**: 1383–1389.

Singh, H., and Poulos, A.A. 1986. A comparative study of stearic acid and lignoceric acid oxidation by human skin fibroblasts. Arch. Biochem. Biophys. **250**: 171–179.

Singh, H., Derwas, N., and Poulos, A.A. 1987. Very long chain acid β-oxidation by the rat liver mitochondria and peroxisomes. Arch. Biochem. Biophys. **359**: 382–3909.

Steinberg, D., Parthasaranthy, S., Carew, T.E., Khoo, J.C., Witztum, J.L. 1989. Beyond cholesterol. Modification of low-

density lipoprotein that increases its atherogenicity. N. Engl. J. Med. **320**: 915–924.

Steinbrecher, U.P. 1987. Oxidation of human low-density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxides. J. Biol. Chem. **262**: 3603–3608.

Tanner, F.C., Noll, G., Boulanger, C.M., and Luscher, T.F. 1991. Oxidized low-density lipoprotein inhibits relaxation of porcine coronary arteries. Role of scavenger receptor and endothelium-derived nitric oxide. Circulation, **83**: 2012–2020.

Thomas, M.J., Chen, Q., Franklin, C., and Rudel, L. 1997. A comparison of the kinetics of low-density lipoprotein oxidation initiated by copper or by azobis (2 amidinopropane). Free Rad. Biol. Med. **23**: 927–935.

Wanders, R.J.A., Van Roermund, C.W.T., Van Wijland, M.J.A., Schutgens, R.B.H., Scham, A.W., Van den Bosch, H., and Tager, J.M. 1987. Studies of peroxisomal oxidation of palmitate and lignocerate in rat liver. Biochim. et Biophys. Acta, **919**: 21–25.