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


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 mixture of very long-chain saturated fatty acids, also isolated and purified from sugar cane wax, whose main component is octacosanoic acid followed by triacontanoic, 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 (CuSO4), 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 octacosanoïque suivi par le triacontanoïque, le dotriacontanoïque et l’acide tetracontanoïque. 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 (CuSO4), 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 diènes 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 diènes 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 de lysine des aminocides et une réduction significative du contenu des 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.
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 an 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 (LDLₗₒ) exerts other effects, which may contribute to the atherogenesis. Earlier steps of LDL oxidation render an LDL minimally oxidized (LDLₘₐₓ), 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, LDLₜₐₚₚ 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 tritriacontanol 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. 1994), patients with type II hypercholesterolemia (Aneiros et al. 1995; Campilongo et al. 1997; Canetti et al. 1995; Castaño et al. 1998, 1999, 2000; Mús 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. 2000a, 2000b). Thus, oral administration of policosanol to rats over 4 weeks partially inhibited in vitro and in vivo liver micromolar 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. 2000a), patients with type II hypercholesterolemia at high coronary risk (Menéndez et al. 2000b), and elderly hypercholesterolic 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, 1995a, 1995b). 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 acids 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 (C₂₈H₅₈O₂, 40.0%), followed by triacontanoic (C₃₀H₆₀O₂, 21.7%), dotriacontanoic (C₃₂H₆₂O₂, 17.0%), and tetraccontanoic (C₃₄H₆₄O₂, 10.2%) acids. Also, hexacosanoic (C₂₆H₅₄O₂, 1.0%), heptacosanoic (C₂₇H₅₈O₂, 0.7%), nonacosanoic (C₂₉H₆₂O₂, 2.1%), hentriacontanoic (C₃₁H₆₄O₂, 2.1%), tritriacontanoic (C₃₃H₆₆O₂, 2.0%), pentatriacontanoic (C₃₅H₆₈O₂, 1.0%), and hexacotriacontanoic (C₃₆H₇₀O₂, 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), trinitrobenzolsulfonic acid (TNBS), and 2,4-dinitrophenylhydrazine (DNPH) were obtained from Sigma (Sigma Chemical Co., St. Louis, Mo.). 2,2-Azobis-2-(2-amidinopropane) hydrochloride (AAPH) was purchased from Wako Chemical (Osaka, Japan). All other chemicals were of the best available quality. The ultrasentrifuge was from Beckman (Beckman Instrument, Inc. Palo Alto, Calif.) and the Ultraspec-Plus spectrophotometer was from LKB (Pharmacia LKB Biotechnology, Uppsala, Sweden). Male Wistar rats (150–180 g) and male Balb/c 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 CuSO4 and 5 mM AAPH. In controls, 10 µM of 0.4% Tween-20 was added, whereas treated groups received 10 µM D003 emulsions in 0.4% Tween-20 to ensure concentrations of 5, 50, and 100 µg/mL. It has been previously checked that 0.4% 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 CuSO4 and 5 mM 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, measured at intervals of 10 min for a period of 5 h at 37°C. 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 × 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% NaHCO3 (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 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 (CuSO4), those having the capacity to generate free radicals.
(AAPH), and a more physiological system (resident macrophages).

Table 2 shows the effect of pretreatment with D003 on the composition of VLDL + LDL 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 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%) was 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-

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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration of lag time (min)</th>
<th>Propagation rate (Δ OD$a$ × 10$^{-3}$/min)</th>
<th>AAPH mediated (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>98.8±11.00</td>
<td>17.46±1.40</td>
<td>0.72±0.10</td>
</tr>
<tr>
<td>D003 5 μg/mL</td>
<td>105.8±13.32</td>
<td>17.30±0.83</td>
<td>0.74±0.05</td>
</tr>
<tr>
<td>D003 50 μg/mL</td>
<td>103.8±10.77</td>
<td>17.10±0.98</td>
<td>0.68±0.13</td>
</tr>
<tr>
<td>D003 100 μg/mL</td>
<td>106.4±12.77</td>
<td>16.76±1.58</td>
<td>0.72±0.21</td>
</tr>
</tbody>
</table>

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.

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

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.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration of lag phase (min)</th>
<th>Increase (%)</th>
<th>Propagation phase (Δ OD$a$ × 10$^{-3}$/min)</th>
<th>Decrease (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>103.0±10.94</td>
<td></td>
<td>16.96±0.97</td>
<td></td>
</tr>
<tr>
<td>D003 0.5 mg/kg</td>
<td>110.2±16.79</td>
<td>6.9</td>
<td>15.43±0.99</td>
<td>9</td>
</tr>
<tr>
<td>D003 5 mg/kg</td>
<td>157.8±19.91*$</td>
<td>53.1</td>
<td>14.13±0.97*$</td>
<td>16.6</td>
</tr>
<tr>
<td>D003 50 mg/kg</td>
<td>221.9±6.13*$</td>
<td>115.3</td>
<td>13.31±3.49*$</td>
<td>21.5</td>
</tr>
<tr>
<td>D003 100 mg/kg</td>
<td>226.0±16.06*$</td>
<td>119.3</td>
<td>13.63±2.12*$</td>
<td>19.6</td>
</tr>
</tbody>
</table>

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).
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 CuSO₄ 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
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
fractions 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 mM) were mixed with an equal volume of DNP (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·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 epsilon-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 antioxidative 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, 1995a, 1995b) 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 antioxidative effect of D003 is not directly related to the chemical structure of the components of D003.

An alternative explanation for the antioxidative 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 peroxy 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 macrophase-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 greater potential in preventing deleterious events involved in the pathogenesis of atherosclerosis.

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