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# INHIBITION OF CHOLESTEROL BIOSYNTHESIS IN CULTURED FIBROBLASTS BY D003, A MIXTURE OF VERY LONG CHAIN SATURATED FATTY ACIDS

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The present study was undertaken to investigate the effects of D003, a mixture of very long chain saturated fatty acids isolated and purified from sugar cane wax, on cholesterol biosynthesis in cultured fibroblasts. Cholesterol biosynthesis is regulated through feedback regulation of at least two sequentially acting enzymes, 3-hydroxy-3-methyl coenzyme A (HMG-CoA) synthase and reductase. They are up-regulated when sterol levels fall and down-regulated when sterol levels rise. The exposure of cultured fibroblasts to a lipid-depleted medium (LDM) and D003 (0.05–50 $\mu$g ml$^{-1}$) for 12 h inhibited, in a dose-dependent manner, cholesterol biosynthesis from $^{14}$C-labelled acetate (33–68%). The addition of D003 at concentrations inhibiting cholesterol biosynthesis from labelled acetate significantly decreased incorporation of radioactivity from $^{3}H_2O$ into sterols, but not from $^{14}$C-mevalonate. These data indicate that D003 inhibits cholesterol biosynthesis by interfering with early steps of cholesterol biosynthetic pathway. We reasoned that D003 acts directly on HMG-CoA reductase, the main regulatory enzyme of cholesterol biosynthetic pathway. However, when enzyme activity was measured in cell extracts in the presence of various concentrations of D003 (0.5–50 $\mu$g ml$^{-1}$), reductase activity was not inhibited. Thus, there was no evidence for a competitive or non-competitive inhibition of enzyme activity by D003. Treatment with D003 significantly suppressed (68%) the enzyme up-regulation when cells were cultured in LDM, which suggests a depression of *de novo* synthesis of HMG-CoA reductase and/or a stimulation of its degradation. However, since the suppressive action of D003 on cholesterol biosynthesis was observed in metabolic conditions under which synthase up-regulation was also enhanced, we cannot rule out a possible effect of D003 on HMG-CoA synthase. Thus, further studies are needed to clarify the precise mechanism of the inhibitory effect of D003 on cholesterol biosynthesis. © 2001 Academic Press

KEY WORDS: cholesterol biosynthesis, cultured fibroblasts, HMG-CoA reductase, D003, very long chain saturated fatty acids.

## INTRODUCTION

Elevated concentrations of plasma cholesterol, in particular low-density lipoprotein cholesterol level (LDL-C), are widely accepted as an important risk factor for the premature development of coronary atherosclerosis in humans [1–3]. Most of the cholesterol transported in plasma lipoproteins is of endogenous origin [4]; therefore, an attractive way to lower cholesterol levels is to inhibit selectively the cholesterol biosynthesis pathway. The site of inhibition should be at a step before cyclization of squalene, because accumulation of sterol intermediates causes serious adverse effects [5]. The enzyme 3-hydroxy-3-methylglutarylcoenzyme A(HMG-CoA) reductase catalyzes the rate-limiting reaction of mevalonate pathway [6, 7] and acts early in this biosynthetic pathway. Thus, inhibition of HMG-CoA reductase activity is the choice in controlling *de novo* synthesis of cholesterol. Inhibitors of cholesterol biosynthesis, particularly those that inhibit HMG-CoA reductase, are widely used to lower cholesterol levels. The effectiveness of HMG-CoA reductase inhibitors to decrease plasma cholesterol level in animals and man is well documented [8].

Policosanol is a mixture of higher primary aliphatic alcohols isolated and purified from sugar cane wax, whose main component is octacosanol, followed by

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triacontanol and hexacosanol. Policosanol has proven to be an effective cholesterol-lowering agent in experimental models [9–11], human healthy volunteers [12] and patients with type II hypercholesterolemia [13–19]. Cell cultures and *in vivo* experiments have shown that policosanol inhibits cholesterol biosynthesis. Oral administration of policosanol to endogenous hypercholesteromic rabbit inhibited cholesterol biosynthesis from $^{3}$H-labelled water [10]. In addition, exposure of human cultured fibroblasts to policosanol inhibited cholesterol biosynthesis from $^{14}$C-acetate and tritium-labelled water, but the incorporation of radioactivity from $^{14}$C-mevalonate was not reduced [20].

Previous data are consistent with the hypothesis that inhibition of cholesterol biosynthesis by policosanol is mainly caused not only by higher primary aliphatic alcohols but also by the presence of very long chain fatty acids and chain-shortened secondary metabolites. This hypothesis is in line with the fact that octacosanol, the main component of policosanol, may be oxidized and degraded *in vivo* to fatty acids through $\beta$-oxidation and sterified with sterol and phospholipids [21–23]. It is also of interest to note that Rizzo *et al.* [24, 25] have demonstrated that fatty alcohol oxidation can be produced in cultured fibroblasts via a reversible fatty alcohol cycle. Moreover, it has been shown that $\beta$-oxidative chain shortening oxidation can occur *in vivo* and *in vitro* [26–28].

D003 is a defined mixture of very long chain saturated fatty acids isolated and purified from sugar cane wax [29]. 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 ($C_{34}H_{68}O_2$, 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, this work studies the effect of D003 on cholesterol biosynthesis in cultured fibroblasts. We firstly investigated the effect of D003 (0.5–50 $\mu$g ml$^{-1}$) on cholesterol biosynthesis from $^{14}$C-acetate, $^{14}$C-mevalonate and tritium-labelled water. We also measured the effect of the addition of D003 (0.5–50 $\mu$g ml$^{-1}$) to detergent extract of fibroblasts on activity of HMG-CoA reductase and the effect of treatment of intact cells on the activity in this enzyme.

## MATERIALS AND METHODS

### *Cell culture*

Vero fibroblasts purchased by Flow Laboratory (Catalogue No. 03–230), were cultured in 75 cm$^2$ flasks (Nunc, Denmark), containing 20 ml of complete Dulbecco's modified Eagle's medium (DMEM), supplemented with 1.8 g ml$^{-1}$ $NaHCO_3$, 10% (w v$^{-1}$) fetal bovine serum (FBS), 2 mmol of glutamine, penicillin (100 units ml$^{-1}$) and streptomycin (100 mg ml$^{-1}$). Cells were maintained at 37 °C in a 5% $CO_2$ humidified atmosphere. The experiments were performed on confluent cells. Cells were dissociated with 0.25% trypsin and were seeded (day 0) at a concentration of $10^5$ cells/well in six-well plastic clusters (Nunc, Denmark). On day 2, cells were rinsed twice with $Ca^{2+}$ and $Mg^{2+}$ free PBS and the medium was changed to one containing 2% delipidated human serum (DHS) (2.5 ml). At this time, the cells were divided into five experimental groups and a further 12 h treatment was carried out. For this treatment, 25 $\mu$l of 0.4% Tween-20 were added to control wells, whereas the treated groups received 25 $\mu$l of D003 emulsions in 0.4% Tween-20 to ensure concentrations of 0.05, 0.5, 5 and 50 $\mu$g ml$^{-1}$. It has been previously checked that in this range of concentration D003 and Tween-20 has no effect on cell viability. DHS was prepared according to Mills *et al.* [30]. Briefly, human serum was submitted to sequential density-gradient ultracentrifugation in a Beckman L7 ultracentrifuge and the serum thus obtained was passed through CNBr-activated Sepharose 4B anti apoB column.

### *Incorporation of $^{14}$C-acetate $^{14}$C-mevalonate and $^{3}H_2O$*

Cholesterol biosynthesis from acetate and mevalonate, was assayed by a 6 h incorporation of sodium [$^{14}$C]-acetate (specific activity, 10 $\mu$Ci ml$^{-1}$) and [$^{14}$C]-mevalonic acid (specific activity, 5 $\mu$Ci ml$^{-1}$) (Amershan). Cells were then extensively washed with phosphate-buffered solution and collected by trypsinization (1 ml, 0.25% w v$^{-1}$). Total lipids were extracted using chloroform–methanol mixture. After evaporating organic solvents under $N_2$, saponification was performed in ethanolic (96%) 0.5 NaOH for 3 h at 95 °C. After cooling, alcoholic concentration was reduced by 50% and non-saponifiable lipids were extracted into hexane (2 × 3 ml). The combined hexane extracts were washed with 3 ml of ethanolic (48%) 0.25 M NaOH. Following evaporation under $N_2$, the radioactivity incorporated into the non-saponifiable lipids was counted a Rackbeta (LKB) scintillation counter. The specific radioactivity of the added-labelled substrates was used to calculate cholesterol synthesis. Results are given in terms of nmol of labelled precursor incorporated per mg of cell protein. To determine cholesterol biosynthesis from tritiated water, the medium was supplemented with $^{3}H_2O$ (200 $\mu$Ci ml$^{-1}$). After being washed with ethanolic NaOH, the hexane extract was saponified in alcoholic NaOH. The non-saponifiables were concentrated under nitrogen and applied to silica gel plates. The plates were developed in n-hexane : diethyl ether : acetic acid (85 : 15 : 2, v/v/v) and the spots corresponding to cholesterol were scraped off and counted for radioactivity. Protein was determined by a

modification of the Lowry's procedure [31]. The data on the disintegration per minute were corrected for losses based on the recovery of the [$^3$H]-cholesterol internal standard, which averaged 90%. The rate of cholesterol synthesis was calculated by assuming that 23 g-atoms of $^3$H are incorporated per mol of cholesterol. Data are expressed in terms of nmol of cholesterol formed per mg of cell protein [32].

### *Effect on HMG-CoA reductase activity*

Assays were performed essentially as described by Berkhout *et al.* [33]. Cells were washed twice with $Ca^{2+}$- and $Mg^{2+}$-free PBS. The medium was replaced by fresh growth medium containing 2% LDM (2.5 ml) and the cultures incubated for 12 h. Cell extracts were then obtained using Brij 96. The 100 $\mu$l assay mixture contained 0.1 M sucrose, 40 mM $KH_2PO_4$, 30 mM EDTA, 50 mM KCl, 5 mM dithiothreitol and NADPH-generating system (2.5 mM NADP, 20 mM glucose 6-phosphate and 15 U ml$^{-1}$ glucose 6-phosphate dehydrogenase) and 100–200 $\mu$g ml$^{-1}$ cell extract. The enzyme reaction was performed in the presence of Tween-20 (0.04% final concentration) and D003 (final concentration 0.5, 5 and 50 $\mu$g ml$^{-1}$). The reaction was started by the addition of substrate ($^{14}$C-HMG-CoA, 0.01 $\mu$Ci, 30 $\mu$mol l$^{-1}$) and stopped after 60 min by the addition of 10 $\mu$l of 5 M HCl. After an additional incubation for 30 min to allow complete lactonization of the product, the mixture was centrifuged. The product was isolated by thin-layer chromatography on silica gel plates (Merck) using benzene : acetone (1 : 1, v v$^{-1}$) as developing solvent. HMG-CoA reductase activity was expressed as nmol of mevalonate formed per minute per mg of protein. In parallel, [$^3$H]-mevalonolactone was added as recovery marker. Recoveries ranged between 85% and 95%. To study the effect of D003 treatment on enzyme activity, cells were rinsed twice with $Ca^{2+}$- and $Mg^{2+}$-free PBS. The medium was changed by growth medium supplemented with 2% LDM (2.5 ml). At this time, the cells were divided into three experimental groups and treated with Tween-20 (0.04%) and D003 at 5 and 50 $\mu$g ml$^{-1}$ for 12 h. After treatment, cell extracts were obtained using Brij 96 and enzyme activity was determined as described previously. In order to check the up-regulation of HMG-CoA reductase by incubation with LDM, a parallel group of cells were grown in growth medium supplemented with normal serum (NS) (2%).

### *Calculation*

The data are presented as mean ± SD. Comparisons between groups were done using the Mann–Whitney *U*-test, statistical significance being established for $\alpha = 0.05$.

## RESULTS

D003 (0.5–50 $\mu$g ml$^{-1}$) significantly and dose-dependently inhibits cholesterol biosynthesis from [1-$^{14}$C]-acetate (Table I). Maximal inhibition of about 70% was reached with (5 $\mu$g ml$^{-1}$), since no additional effects were observed after incubation with higher concentrations.

**Table I**
**Effects of D003 on cholesterol biosynthesis from $^{14}$C-acetate in cultured fibroblasts**

| *Treatment ($\mu g\ ml^{-1}$)* | *Cholesterol biosynthesis (nmol of precursor incorporated mg protein$^{-1}$)* | *Inhibition (%)* |
|---|---|---|
| Control | 3.54 ± 0.60 | — |
| D003 (0.05) | 1.99 ± 0.22 | 33.7 |
| D003 (0.5) | 1.30 ± 0.15* | 53.2 |
| D003 (5) | 1.09 ± 0.38* | 69.2 |
| D003 (50) | 1.13 ± 0.14* | 68.0 |

*$P < 0.05$. Comparison with control (Mann–Whitney *U*-test).

**Table II**
**Effects of D003 on cholesterol biosynthesis from $^{14}$C-mevalonate and $^3$H-$H_2O$**

| *Treatment* | *$^{14}$C-mevalonate (nmol precursor incorporated mg protein$^{-1}$)* | *$^3H$-$H_2O$ (nmol cholesterol mg protein$^{-1}$)* |
|---|---|---|
| Control | 81.61 ± 9.26 | 7.92 ± 0.82 |
| D003 (5 $\mu$g ml$^{-1}$) | 73.97 ± 8.01 | 4.23 ± 0.34* |

*$P < 0.05$. Comparison with control (Mann–Whitney *U*-test).

The effect of D003 on cholesterol biosynthesis using [2-$^{14}$C]-mevalonate and $^3H_2O$ as precursors can be observed in Table II. When D003 was added at the same concentration that inhibited cholesterol biosynthesis from labelled acetate, no reduction of radioactivity was observed using labelled mevalonate as precursor. On the contrary, tritiated water incorporation into sterol was significantly depressed.

In order to evaluate the effect of D003 on HMG-CoA reductase activity, the generation of [$^{14}$C]-mevalonate was measured in cell homogenates in the presence of various concentrations of policosanol. As can be seen in Table III, D003 did not suppress HMG-CoA reductase when added to cell extracts at concentrations ranging from 0.5 to 50 $\mu$g ml$^{-1}$.

Treatment of intact cells with D003 resulted in HMG-CoA reductase activity depression (Table IV). When fibroblasts were cultured for 12 h in lipoprotein-free medium, enzyme activity significantly rose threefold. However, in the presence of D003 (0.5 and 5 $\mu$g ml$^{-1}$) the enzyme up-regulation was significantly suppressed.

## DISCUSSION

In culture fibroblasts as in other mammalian cells, the rate of cholesterol synthesis is determined by the activity of HMG-CoA reductase, which catalyzes the reduction of HMG-CoA to mevalonate [34]. Low-density

**Table III**
**Lack of effect of D003 on HMG-CoA reductase activity when added to reductase mixture assay**

| *Treatment* ($\mu g\ ml^{-1}$) | *Enzyme activity* ($pmoles\ min^{-1}\ mg\ protein^{-1}$) |
|---|---|
| Control | $239.84 \pm 70.00$ |
| D003(0.5) | $231.45 \pm 29.30$ |
| D003 (5) | $215.04 \pm 32.00$ |
| D003 (50) | $241.11 \pm 56.00$ |

Enzyme activity was measured in cell extracts in the presence (0.5, 5 and 50 $\mu$g ml$^{-1}$) and absence of D003 (Control) during the reaction time. Each value represents the mean ± SD of triplicate samples of two separately performed experiments. Comparisons were not significantly different.

**Table IV**
**Inhibition of up-regulation of HMG-CoA reductase activity by the treatment of intact fibroblasts with D003**

| *Treatment* | *Enzyme activity* ($pmoles\ min^{-1}\ mg\ protein^{-1}$) | *Inhibition* (%) |
|---|---|---|
| Control | $90.54 \pm 19.34$ | — |
| LDM | $269.84 \pm 70.00$* | — |
| LDM + D003 (0.5 $\mu$g ml$^{-1}$) | $94.90 \pm 20.40$** | 54 |
| LDL + D003 (5 $\mu$g ml$^{-1}$) | $75.40 \pm 17.60$** | 65 |

Enzyme activity was measured in cell extracts after fibroblasts were grown in the presence of normal serum (Control) and after treatment either with lipid-depleted medium (LDM) or LDM + D003. Each value represent the mean ± SD of triplicate samples of two separately performed experiments. *Significant increment of reductase activity as a result of incubation in LDM. **Significant decrease in reductase activity as a result of treatment with D003 ($P < 0.05$. Mann–Whitney $U$-test).

lipoprotein (LDL) and very low-density lipoprotein (VLDL) suppress the activity of this enzyme by delivering cholesterol to the cell [35]. When normal cells are cultured in the absence of lipoprotein, the depletion of cholesterol up-regulates the enzyme levels and increases enzyme activity which in turn is followed by an accelerated biosynthesis of cholesterol [7, 36]. However, incubation of cultured fibroblasts in lipoprotein-deficient medium in the presence of D003 inhibits cholesterol biosynthesis. D003 exerts a dose-related inhibition of cholesterol biosynthesis using [1-$^{14}$C]-acetate. At first glance, it could be possible that D003 may compete with acetate during cholesterol synthesis and dilute the radiolabel rather than inhibit biosynthesis. However, D003 inhibits radioactivity incorporation from tritiated water. Hence, the observed inhibition of cholesterogenesis of cultured fibroblasts in the presence of D003 can be explained by an effect of the very long chain fatty acid components of D003 on this metabolic process.

We used labelled mevalonate as a substrate for cholesterol biosynthesis. This process occurs distal to HMG-CoA reductase and is a measure of enzyme activities beyond the main rate-limiting enzyme in the cholesterol biosynthesis pathway [5]. As observed, incorporation of radioactivity into sterols was not suppressed by D003, which indicates that the inhibition of cholesterol biosynthesis occurs at some step before mevalonate generation. We reasoned that D003 could act directly on HMG-CoA reductase. To test this hypothesis we studied the effect of D003 on the activity on this enzyme. However, in the current study no suppression of enzyme activity was observed when D003 was added to the HMG-CoA reductase assay mixture. Thus, there was no evidence for a competitive or non-competitive inhibition of enzyme activity by D003, excluding the possibility that the compound could be itself a direct HMG-CoA reductase inhibitor.

Experiments performed in order to investigate the activity of HMG-CoA after treatment with D003 showed an inhibition of enzyme activity after incubation of the cells. This fact suggests that D003 treatment of intact fibroblasts led to a down-regulation of enzyme activity. Exposure of cultured cells to cholesterol-depleted medium causes an increased transcription of the HMG-CoA reductase gene followed by enhanced formation of the enzyme protein [36]. Also, the intracellular cholesterol pool regulates enzyme activity by modifying the rate of degradation of reductase protein [38–40]. Therefore, the avoidance of enzyme up-regulation in LDM may suggest a depression of *de novo* synthesis of the enzyme as well as a stimulation of its degradation. However, it should be noted that in the current work the enzyme was not measured directly. Thus, it is not possible from the present data to ascertain whether the loss of reductase activity is due to decreased synthesis and/or increased degradation of the enzyme.

Two sequentially acting enzymes, cytosolic HMG-CoA synthase and mitochondrial HMG-CoA reductase, catalyze the synthesis of mevalonate. Cytosolic HMG-CoA synthase is responsible for the generation of HMG-CoA from acetyl-CoA and acetoacetyl-CoA. This reaction is also a regulatory step in the cholesterol biosynthesis pathway [36] and, as occurred for HMG-CoA reductase, is subjected to feedback suppression by cholesterol and other end products. In response to cellular cholesterol deprivation HMG-CoA synthase activity is up-regulated and when sterol levels rise the enzyme is down-regulated. Thus, when cells are cultured in the absence of sterols a co-ordinated induction of both HMG-CoA synthase and reductase is produced. This control is exerted primarily at the transcription level throughout the action of the sterol regulatory element-binding proteins [37]. Thus, since D003 inhibited cholesterol biosynthesis after incubation of cultured cells in lipid-free medium, we can hypothesize that the inhibition of cholesterol biosynthesis could be produced not only by the observed modulation of HMG-CoA reductase, but also by an effect on HMG-CoA synthase up-regulation. It should be noted however, that the inhibition of HMG-CoA reductase activity appears to account per se for the inhibition of cholesterol biosynthesis. Thus the ability of D003 to inhibit cholesterol seems to be related more to its effect

on HMG-CoA up-regulation rather than to an effect on both enzymes. However, the possibility of a simultaneous action of D003 on both regulatory enzymes remains open, since the effect of D003 on HMG-CoA synthase was not investigated.

The effect of D003 on the up-regulation of HMG-CoA reductase could be related with the physical status of the membrane after D003 treatment. Several studies have suggested that when cultured cells are incubated in the presence of different fatty acids, they can be incorporated into cells affecting membrane composition and fluidity [41–44]. Also, it must be noted that the activity of HMG-CoA reductase can be modulated by the physico-chemical properties of cell membrane, especially by membrane microviscosity [45]. Therefore, there exists the possibility that the observed effect of D003 on cholesterol biosynthesis might be related to a modulation on HMG-CoA reductase activity through its effect on the physico-chemical characteristics of the endoplasmatic reticulum.

In conclusion, when fibroblasts were cultured in presence of LDM and D003 (0.05–50 $\mu$g ml$^{-1}$) for 12 h an inhibition of cholesterol biosynthesis from $^{14}$C-acetate and $^{3}H_2O$ was observed. However, the synthesis from $^{14}$C-mevalonate was not suppressed. The present results provide strong evidence that the inhibitory effect of D003 on cholesterol biosynthesis is produced by interfering with the earliest steps of the cholesterol biosynthetic pathway. In agreement with this fact, D003 modulates HMG-CoA reductase activity. Treatment with D003 significantly suppressed the enzyme up-regulation when cells were cultured in LDM which suggests a depression of *de novo* synthesis of HMG-CoA reductase and/or a simulation of its degradation. It should be noted, however, that HMG-CoA synthase is also a key regulatory enzyme of cholesterol biosynthesis, which is also up-regulated when cells are cultured in LDM. Therefore, a possible effect on HMG-CoA synthase cannot be ruled out. However, whether the mechanism for the inhibition of cholesterol biosynthesis involves an action of D003 on both enzymes remains to be determined. Thus, further studies are needed to clarify the precise mechanism of action of the inhibitory of D003 on cholesterol biosynthesis.

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