ACTION OF HMG-CoA REDUCTASE INHIBITORS ON THE MEVALONATE PATHWAY IN CULTURED HUMAN CELLS

Proefschrift

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STELLINGEN

Behorende bij het proefschrift

ACTION OF HMG-CoA REDUCTASE INHIBITORS ON THE MEVALONATE PATHWAY IN CULTURED HUMAN CELLS

1. De humane hepatoma cellijn Hep G2 als model systeem voor het testen van de leverspecifieke werking van galzuur gemodificeerde HMG-CoA reductase remmers is ongeschikt.
Kramer et al. Biochim Biophys Acta 1994;1227:137-154.
Dit proefschrift (Hoofdstuk 2).

2. De conclusie van Keidar et al. dat de selectieve opname van pravastatine in hepatocyten ook geldt voor de macrophagen cellijn J-774 A.1 is niet bewezen.
Keidar et al. Br J Clin Pharmacol 1994;38:513-519.
Dit proefschrift (Hoofdstuk 3).

3. In het artikel van Corsini et al. is het onduidelijk of bij hoge vastatine concentratie de celafname veroorzaakt wordt door remming van de celdeling of dat deze afname te wijten is aan celtoxiciteit.
Corsini et al. Atherosclerosis 1993;101:117-125.
Dit proefschrift (Hoofdstuk 5).

4. Het ontstaan van resistente HIV, reeds na twee weken in patienten behandeld met verschillende antiretrovirale geneesmiddelen, maakt een effectieve therapie voorlopig ondenkbaar.
Wei et al. Nature 1995;373:117-122.
Condra et al. Nature 1995;374:569-571.

5. Het verdient aanbeveling voor α-aminozuren *N*-gesubstitueerde glycine-derivaten de benaming "peptoïden" te gebruiken in plaats van "peptiden".
Heizmann et al. Peptide Res 1994;7:328-332.
Simon et al. Proc Natl Acad Sci USA 1992;89:9367-9371.

6. Geen orgaandonor, dan ook geen acceptor.

7. De term *wachtgeld* heeft alles te maken met de tijd die voorbij gaat voordat het geld op de bankrekening wordt bijgeschreven.

8. De aanwezigheid van een 'sissy-bar' op een motorfiets, hoeft niet te betekenen dat er een 'sissy' achterop zit.

9. Liever franse wijn proeven, dan franse kernproeven.

10. Aangezien de meeste klinische studies worden verricht met mannelijke proefpersonen, kan men zich afvragen wie het werkelijke proefkonijn is.

11. "More women will enter the scientific field, only when there are more women in it"

    Marguerite Holloway:
    A lab of her own.
    Scientific American,
    November 1993:68-77.

Leiden, 30 november 1995.

Arlène van Vliet

'Don't take your organs to heaven,
heaven knows we need them here'

Stichting Donorvoorlichting

Aan Nejohajomaliar

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1. INTRODUCTION

The general introduction covers the introduction to the pathogenesis of atherosclerosis and major risk factors for coronary heart disease, followed by emphasis on cholesterol metabolism and targets for treatment of hypercholesterolemia, cholesterol synthetic pathway and its regulation, inhibitors (vastatins) of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (the rate limiting enzyme of cholesterol synthesis), some aspects of drug transport in the liver, and ends with an outline of the thesis. This thesis focusses on the effects of one particular class of lipid lowering drugs on the mevalonate pathway in cultured human cells: the HMG-CoA reductase inhibitors lovastatin, simvastatin and pravastatin.

1.1 *Pathogenesis of atherosclerosis*

There is a strong direct correlation between incidence for coronary heart disease (CHD) in men, and serum-concentrations of total or low density lipoprotein (LDL) cholesterol. The causal role of LDL, or its oxidized form, in atherogenesis is discussed below (1). LDL accumulates at the site of endothelial injury in the blood vessel, an event which attracts blood monocytes to this site. These monocytes penetrate the intima of the vessel wall to become macrophages, and ingest the denaturated or oxidized LDL-particle together with its accompanying cholesteryl esters, giving rise to foam cells. Platelets adhere and release growth factors. Smooth muscle cells migrate from the medial layer to the intimal layer of the vessel wall. These processes result in the formation of a fibrous cap of proliferated smooth muscle cells and the extracellular matrix that they have produced. Eventual necrosis of foam cells results in the formation of cholesteryl ester crystals within the core of the mature plaque. The characteristic lesion of atherosclerosis is the fatty fibrous plaque. This plaque narrows the lumen of the vessel, which can lead to a complete inhibition of blood flow. This situation may ultimately lead to a heart attack or a stroke.

1.2 *Major risk factors for CHD*

Atherosclerosis leading to CHD is currently the most common cause of death in the United States of America and Western Europe. Several studies have shown that, with increasing concentrations of serum cholesterol, there is an increase in risk for CHD, appreciably so when serum-cholesterol levels exceed 6.46 mMol/L and even more steeply above 7.24 mM/L (2,3). Hyperlipidemia can be attributed to a number of factors such as diet, hormonal disturbances, drugs, or co-existing diseases and genetic abnormalities. In particular, hypercholesterolemia is the most important risk factor, as shown in epidemiological studies and occurs especially in patients with homozygous familial hypercholesterolemia. These patients have an inherited defect in the two genes encoding the LDL receptor, leading to defective clearance of cholesterol-carrying LDL from the circulation (4). Hypercholesterolemia, along with hypertension and smoking,

has become one the major risk factors for the development of atherosclerosis, the primary cause of CHD.

2. *CHOLESTEROL METABOLISM AND TARGETS FOR TREATMENT OF HYPERCHOLESTEROLEMIA*

2.1 Lipoprotein metabolism

Cholesterol and triglycerides have important functions in the body: triglycerides provide a source of energy, and cholesterol plays a role as an essential structural component of cellular membranes and is a precursor molecule for the synthesis of bile acids, steroid hormones and vitamins. Cholesterol can be synthesized *de novo* in almost every mammalian cell.

Since cholesterol, in its unesterified form, and triglycerides are not polar, they are transported to their destination via lipoproteins. Lipoproteins can be classified according to their buoyant density: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) (5,6). In general, these lipoproteins consist of a core of hydrophobic lipids and cholesteryl esters (CE), surrounded by a shell of polar lipids, harbouring different apolipoproteins (7). Eight types of apolipoproteins (apo) have been isolated and characterized thus far: apo A-I, A-II, B, C-I, C-II, C-III, D and E (6). Furthermore, their specific apo-identity determines and regulates the entry and exit of particular lipoproteins at specific sites (8-10).

The plasma lipoproteins are synthesized and secreted by the liver and intestine. Chylomicrons transport dietary triglycerides, cholesterol and other lipids from the intestine to adipose tissue and liver. The triglycerides are hydrolyzed within a few minutes by a lipase located in the capillaries of adipose and peripheral tissue (11). The free fatty acids released are taken up by the tissues, for oxidation or storage. Free cholesterol (FC) and phospholipids (PL), surface components of the chylomicron particle released during hydrolysis, are incorporated into HDL_3 (12). Lecithin:cholesterol acyltransferase (LCAT), a plasma enzyme, esterifies the cholesterol in HDL_3 in the conversion of this particle in HDL_2. Cholesterol in HDL_2 is transferred back to the liver. By the action of the cholesteryl ester transfer protein (CETP), CE are exchanged for triglycerides of VLDL, IDL and LDL and/or by other mechanisms. HDL_2 can be converted back to HDL_3 by depletion of triglycerides by action of hepatic lipase (HPL). The cholesterol-rich residue, known as chylomicron remnant particles, is taken up by a liver-located receptor that binds their apo E moiety (13-16). From the liver, VLDL delivers endogenously synthesized cholesterol and triglycerides to adipose tissue (17-19). VLDL, like chylomicrons, are also hydrolyzed by lipoprotein lipase; the resultant VLDL remnants are called IDL. The IDL residue, which is partially removed from the circulation by the B/E or LDL receptor, is transformed into LDL upon tri-

glyceride-hydrolysis, resulting in a CE-rich particle (18,20,21). The role of LDL is to transport cholesterol to peripheral tissues and regulate the *de novo* cholesterol synthesis at these sites, as will be discussed later on, but the majority is removed by LDL receptors in the liver (22). Cholesterol fluxes to- and from the liver are schematically presented in Figure 1.

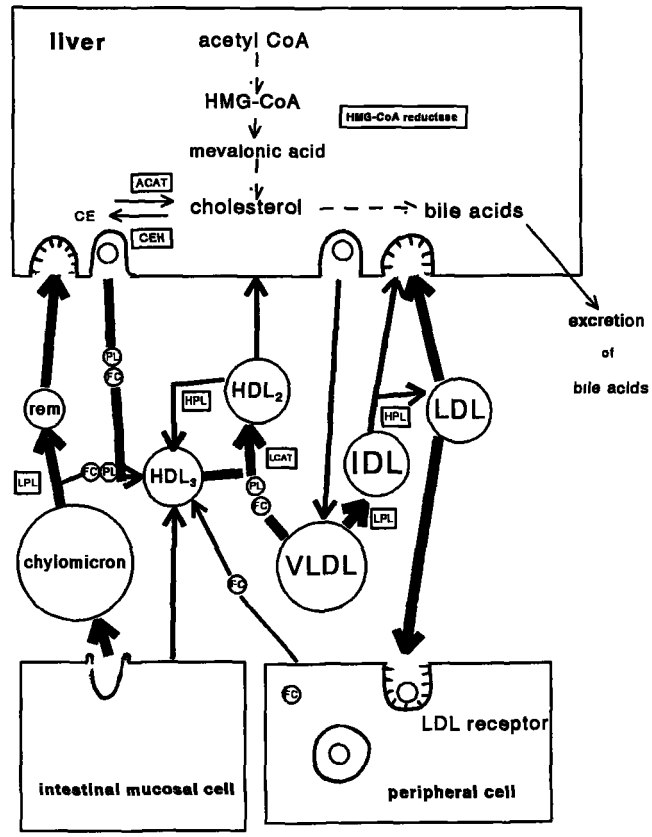

Figure 1: Tissue sites of origin and degradation, and intra-vascular metabolism of lipoproteins
Rem, chylomicron remnant; LPL, lipoprotein lipase; HPL, hepatic lipase; FC, free cholesterol; PL, phospholipids: CE, cholesteryl ester; ACAT, acyl CoA-cholesterol acyltransferase; LCAT, lecithin:cholesterol acyltransferase; CEH, cholesteryl ester hydrolase.

2.2 The LDL receptor pathway of cholesterol uptake

Cholesterol can be obtained from the diet or it can be synthesized *de novo*. The major site of cholesterol synthesis in mammals is the liver. The synthesis of mevalonate is the committed step in cholesterol formation. The enzyme catalyzing this irreversible step, 3-hydroxy-3-methylglutaryl coenzyme A reductase, is an important control site in the cholesterol biosynthesis. Studies by Brown and Goldstein using cultured human

fibroblasts gave insight into the control of cholesterol metabolism in nonhepatic cells. In general, the primary source of cholesterol for extrahepatic cells is LDL. The uptake of LDL by cultured human fibroblasts is discussed below. LDL binds to a specific receptor, after which the receptor-LDL complex is internalized by endocytosis. These vesicles fuse with lysosomes, hydrolysing the LDL derived proteins and CE. The hydrolysis of CE is catalyzed by cholesteryl ester hydrolase (CEH), whereas the conversion of free cholesterol to CE to allow storage, is catalyzed by the enzyme acyl CoA-cholesterol acyltransferase (ACAT).

The accumulation of unesterified cholesterol within the cells regulates three events in cellular metabolism: suppression of HMG-CoA reductase with reduction of cholesterol synthesis: activation of ACAT, which facilitates re-esterification and storage of esterified cholesterol; and feedback suppression of synthesis of the LDL receptor - a step that prevents excessive cellular accumulation in the presence of the lipoprotein (23).

2.3 Cholesterol as a precursor

Cholesterol is a precursor molecule for bile acids, steroid hormones and vitamins. Bile acids are synthesized in the liver, stored and concentrated in the gall bladder, and then released into the small intestine. Bile acids also solubilize and facilitate uptake of dietary lipids. The resulting increase in the surface area of these lipids has two consequences: it promotes their hydrolysis by lipases, and facilitates their absorption. Bile acids are the major break-down products of cholesterol (24).

Cholesterol is also the precursor molecule of five major classes of steroid hormones: progestagens, glucocorticoids, mineralocorticoids, androgens, and estrogens. Progesterone, one of the progestagens, prepares the lining of the uterus for implantation of the ovum. Progesterone is also essential in the maintenance of pregnancy. Androgens, such as testosterone, are responsible for the development of male secondary sex characteristics, whereas estrogens are required for the development of female secondary sex characteristics. Estrogens also participate in the ovarian cycle. Glucocorticoids (such as cortisol) promote gluconeogenesis and the formation of glycogen, and enhance the degradation of fat and protein. Mineralocorticoids, such as aldosterone, increase reabsorption of Na^+, Cl^-, and HCO_3^- by the kidney, which leads to an increase in blood volume and blood pressure. The major sites of synthesis of these class of hormones are: corpus luteum (progestagens), ovary (estrogens), testis (androgens), and adrenal cortex (glucocorticoids and mineralocorticoids) (25). Cholesterol is also the precursor of vitamin D, which plays an essential role in the control of calcium and phosphorus metabolism (26).

2.4 Targets for treatment of hypercholesterolemia

The primary goal in treatment is the reduction of total and LDL cholesterol, and, consequently, the risk of atherosclerosis and CHD. Although an accent is put on

cholesterol, one should not be distracted from the fact that the atherogenic potential of this molecule is a function of the lipoprotein within which it is carried, and that the risk equation has components other than plasma-lipids and lipoproteins, such as fibrinogen (27-29) and Factor VII (29).

The National Cholesterol Education Program has categorized a patient's CHD risk on the basis of her or his total and LDL-cholesterol levels, and criteria for tri-glyceride-values have been established (30). These values are presented in Table I.

Table I: Lipid levels in mmol/L and CHD risk.

	desirable	borderline-high	high
Total cholesterol	<5.17 (<5.0)	5.17-6.18 (5.0-6.5)	≥6.20 (≥6.5)
LDL cholesterol	<3.36	3.36-4.11	≥4.13
Triglycerides	<2.84 (<2.0)	2.84-5.68	>5.68

The concensus criteria in the Netherlands is stated within parentheses.

Dietary advice is the first step in the treatment of all forms of hyperlipidemia, and is often successful. If diet, weight reduction, and exercise have not lowered total or LDL cholesterol levels to the desired ranges within 3 to 6 months, drug therapy should be considered. Lipid lowering drugs should be used when: 1. an accurate diagnosis of the hyperlipidemia is first established, 2. associated cardiovascular risk factors are identified and controlled, 3. several baseline lipid measurements are obtained, 4. a lipid lowering diet is applied, 5. drugs are added only if the dietary regimen fails, 6. the chosen drug (most potent with the least adverse side-effects), indicated for a specific lipid-transport disorder, is substituted by another or combined with another depending on response and tolerance, and 7. changes in plasma lipid-levels and clinical manifestations (e.g. xanthoma's), side-effects and drug interactions, body weight and compliance with diet and drugs are closely monitored (31).

Five classes of lipid-lowering agents have been approved by the U.S. Food and Drug Adminstration: bile acid sequestrants, nicotinic acid, fibric acid derivatives, probucol and HMG-CoA reductase inhibitors.

3. *CHOLESTEROL SYNTHETIC PATHWAY AND ITS REGULATION*

3.1 The mevalonate pathway

In both the liver and in extrahepatic tissues, cholesterol is obtained, by two distinct but coordinately regulated processes. Cholesterol is either derived from

blood-borne lipoproteins, which enter the cell by receptor mediated endocytosis. Upon internalization the particle enters a lysosomal pathway, in which LDL-derived CE are hydrolyzed. Cells can also synthesize cholesterol *de novo* as presented in Figure 2 (32).

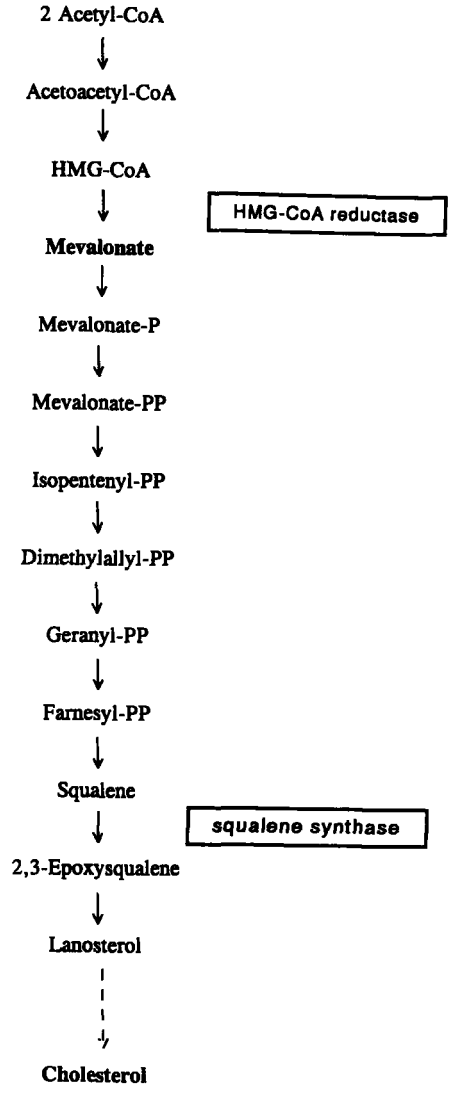

Figure 2: The de novo cholesterol synthesis (32).

The rate-limiting enzyme in the cholesterol biosynthesis, HMG-CoA reductase, converts HMG-CoA to mevalonate. Mevalonate is converted to six known end products: cholesterol; haem a, an electron transferring protein in the inner mitochondrial membrane; dolichol, required for glycosylation of proteins; ubiquinone, which takes part in electron transport of the oxidative phosphorylation; isopentenyl tRNA, and isoprenylated proteins (Figure 3) (33).

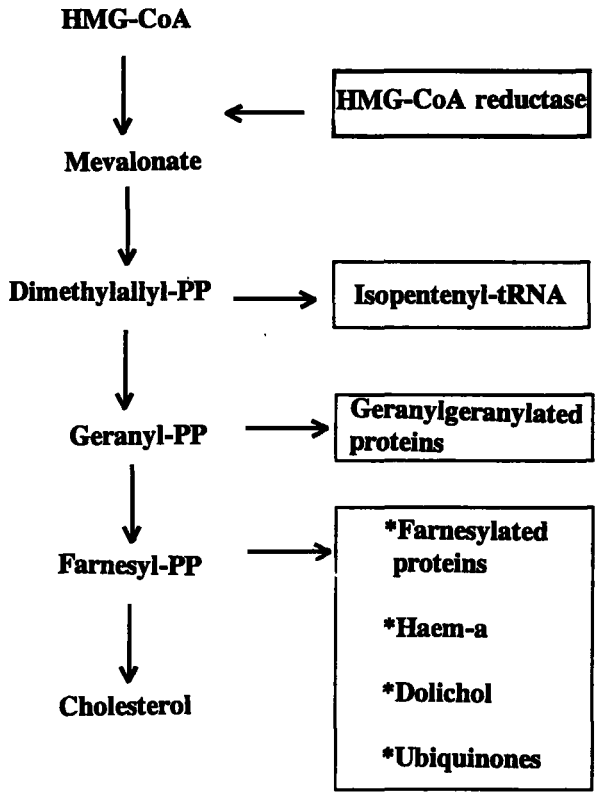

Figure 3: Isoprenoid synthesis in mammalian cells (33).

Studies in the late 1970s and early 1980s have shown that treatment of cells with high concentrations of vastatin result in an arrest of cell growth which could not be reversed by the addition of an exogenous cholesterol source, or other sterols (34,35), and suppresses tumour growth *in vivo* (36). However, small amounts of mevalonic acid (MVA) could reverse the effect of the vastatin, suggesting that a non-sterol metabolites of MVA or MVA itself was important in this recovery from cell growth arrest. When this type of experiment was performed using [^{3}H]-MVA to follow the metabolic fate of the labelled compound, it was observed that the label was incorporated into a subset of cellular proteins (37).

Nuclear lamins (38,39) which play a fundamental role in the assembly of the nucleus and ras-proteins (40-42), involved in the process of cell proliferation, represent one of the first characterized isoprenylated mammalian proteins. Isoprenylated proteins are modified by a C15 farnesyl isoprenoid moiety, or by C20 geranylgeranyl isoprenoid group. The latter one is the major isoprenoid group in mammalian proteins. Lamins A and B, and ras, are modified by farnesylation of the cysteine contained within the so called "CAAX-motif", at their carboxyl terminus, where the cysteine (C) is followed by two aliphatic (A) amino acids (valine, leucine or isoleucine), and then by any amino acid (X) at the carboxy terminus. Hereafter, the three carboxy terminal amino acids are proteolytically removed and the resulting terminal cysteine is carboxy-methylated (32,43). It seems that other proteins that undergo this lipid modification, represent critical components involved in the regulation of a range of diverse cellular functions. The majority of the identified isoprenylated proteins are additional members of the ras protein superfamily, whose specific members have been implicated in the regulation of intracellular vesicular transport (rab/YPT1) (44,45), cytoskeletal organization (rho) (46), the oxidative burst of phagocytic cells (rac) (47,48), and negative growth (rap/Krev-1) (49). A number of isoprenylated proteins, other than ras, also represent critical elements within signal transduction pathways. Isoprenylation is a critical modification for the γ-subunits of the heterotrimeric G proteins, and this modification is important for their function in receptor-mediated signal transductions in mammalians (50) and yeast cells (51). Isoprenoid groups may function as lipid anchors and increase the avidity of the protein for the membrane (52). Our knowledge of the processes involved in isoprenylation of proteins is far from complete, and rapid and exciting developments in this area are guaranteed over the next few years.

3.2 Regulation of de novo cholesterol synthesis

In tissue culture cells, the LDL receptor and HMG-CoA reductase are both subject to end-product feedback regulation by cholesterol. When cellular cholesterol levels rise, the synthesis of HMG-CoA reductase and the synthesis of LDL receptors are suppressed. On the other hand, when cells have lower regulatory cholesterol pools, LDL receptors are induced and HMG-CoA reductase is increased (Figure 4) (34,53). A similar type of feedback regulation of these two proteins was observed in the livers of several animal species. Under certain conditions, the level of LDL in plasma is directed by the balance between the activities of HMG-CoA reductase and LDL receptors in the liver. If this balance is not preserved, hypercholesterolemia and atherosclerosis can result.

In the case of HMG-CoA reductase, sterols exercise both transcriptional control and post-translational control (54), promoting the increased degradation of the enzyme in the endoplasmatic reticulum. A 10bp sequence within the 5'flanking region of the LDL receptor gene, designated sterol regulatory element 1 (SRE-1), controls LDL receptor transcription so that when cellular sterol pools are depleted,

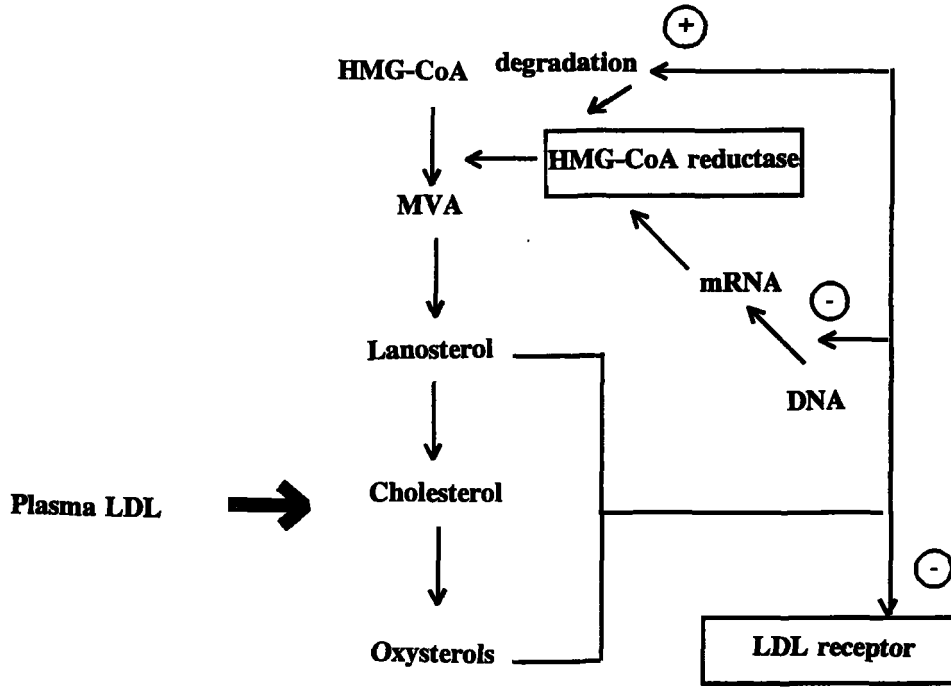

Figure 4: Regulation of de novo cholesterol synthesis by LDL, sterols and oxysterols (34,53).

the gene is transcribed, receptor biosynthesis increases, and LDL-uptake through receptor mediated endocytosis increases (33). Likewise, when cellular sterol-stores are replenished, interaction with the SRE-1 leads to a decrease in transcription of the LDL receptor gene. SRE-1 is a conditional positive element that enhances expression of the LDL receptor-, and HMG-CoA synthase gene, the latter of which is a consecutive enzyme within mevalonate synthesis, in the absence but not in the presence of sterols. In tissue culture, the most potent inactivators of SRE-1 are oxysterols, which are derivatives of cholesterol with additional hydroxyl or keto groups.

Recently, proteins which bind to the SRE-1 region of the LDL receptor promotor region were purified (55-57) and named "sterol regulatory element binding proteins" (SREBP). Kawabe et al. observed that HMG-CoA reductase mRNA was highly induced by sterols in Hep G2 cells, but the rate of induction was found to be lower than expected, as judged from the results obtained on expression of the LDL receptor mRNA (58). This suggests that HMG-CoA reductase is not induced directly by the SREBP-1 mediated pathway, which was supported by data showing that
SREBP-1 binds to SRE-1 on the LDL receptor gene but does not bind to the similar element on the HMG-CoA reductase gene (57).

HMG-CoA reductase activity may also be regulated via a number of other

mechanisms (59), including conversion of the enzyme between active and inactive forms by way of a phosphorylation (inactive form)/dephosphorylation (active form)-event, by virtue of specific protein kinases (60). Others include changes in the fluidity of the endoplasmatic reticulum (ER) membrane, brought about by increasing (less fluid) or decreasing (more fluid) cholesterol levels in the organelle (61), or by isoprenylated proteins (33). As, mevalonate is required for isoprenylation, decreased mevalonate production will result in accumulation of unmodified proteins. These proteins may have a role in upregulation or downregulation of translation or degradation of HMG-CoA reductase, e.g. degradation of HMG-CoA reductase, mediated by its membrane attached domain, anchoring the enzyme to the ER. Farnesylated proteins could affect this degradation by regulation of the rate at which the reductase is incorporated into vesicles that transport it from the ER to a degradative site (33).

4. HMG-CoA REDUCTASE INHIBITORS

4.1 History and development of HMG-CoA reductase inhibitors

In 1971, Endo et al. (62) isolated a HMG-CoA reductase inhibitor of microbial origin. Compactin (ML-236B) (Figure 5a), synthesized by a strain of *Penicillium citrinum*, inhibited sterol synthesis, from both [^{14}C]-acetate and [^{14}C]-HMG-CoA at nM concentrations, but showed no effect on the conversion of [^{3}H]-mevalonate into sterols. The compound appeared to inhibit selectively the conversion of HMG-CoA, catalyzed by HMG-CoA reductase (62). Subsequently, pravastatin sodium (pravastatin) (Figure 5b), was discovered as the active metabolite of compactin in dog urine (63). The compound inhibited cholesterol biosynthesis primarily in the liver, and small intestine. Another HMG-CoA reductase inhibitor, lovastatin (Figure 5c), was isolated by Merck Sharp & Dohme from a different species of fungus (64). Simvastatin, also an HMG-CoA reductase inhibitor, is a semi-synthetic compound, having an additional methyl group in its side chain as compared with lovastatin (Figure 5d) (65). Lovastatin and simvastatin are prodrugs; the lactone ring is hydrolyzed *in vivo* to form the corresponding active hydroxy acid. Pravastatin, however, is administered as the sodium salt of the active hydroxy acid. Fluvastatin is the first fully synthetic HMG-CoA reductase inhibitor, which differs from the original structure in its lipophilic moiety (66) (Figure 5e). Several other HMG-CoA reductase inhibitors, e.g. atorvastatin (Figure 5f) and rivastatin (Figure 5g) have been reported some of which are now at different stages between development and clinical trials (67-71). The continuous growth of this class of compounds has prompted a classification under the common name of vastatins. The K_i-values for the acid forms of the vastatins, currently in clinical use, determined using rat microsomal preparations for the inhibition of HMG-CoA reductase, are reported in Table II.

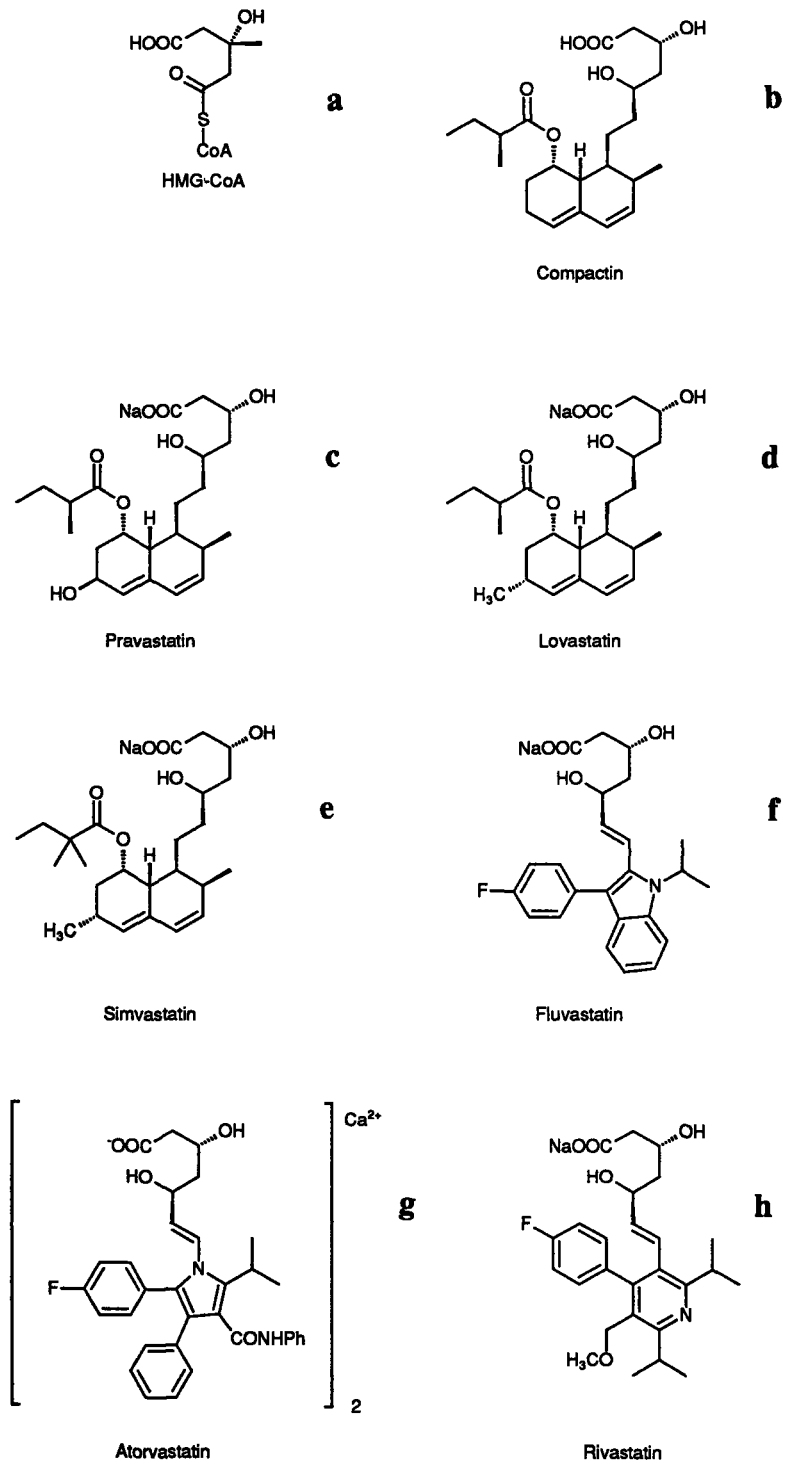

Figure 5: Structures of HMG-CoA and different HMG-CoA reductase inhibitors.

Table II: Inhibition of microsomal HMG-CoA reductase activity (72).

Inhibitor	*Source*	K_i (M)
HMG-CoA		K_m $3x10^{-6}$
Compactin	*Penicillum citrinum*	$1.4x10^{-9}$
Lovastatin	*Aspergillus terreus*	$0.6x10^{-9}$
Simvastatin	*Aspergillus terreus* + *Methylation*	$0.12x10^{-9}$
Pravastatin	*Penicillum citrinum* + *Streptomyces carbophilus*	$2.3x10^{-9}$
Fluvastatin	Synthetic	$0.3x10^{-9}$

In the mid 80s, clinical trials with compactin were suspended, because it produced toxic effects in some dogs at higher doses in a long-term toxicity study.

4.2 Effects of vastatins on sterol synthesis in vitro and in animal studies

The objectives of ascertaining the relative effectiveness of different vastatins on plasma cholesterol levels, and on selected tissues has been approached in a number of ways. Since the liver is the major cholesterol-producing tisssue, one way can be to determine the relative effect of the different drugs on liver, versus other tissues, in both *in vitro* and *in vivo* situations. Our interest is focussed on the vastatins: lovastatin, simvastatin and pravastatin.

In a cell-free system of rat liver microsomes, all three drugs inhibited sterol synthesis to a similar extent, with IC_{50}-values (the drug concentration at which 50% inhibition of the sterol synthesis occurs), in the range of 1-2 nM (63,73). In freshly isolated rat hepatocytes, all three drugs strongly inhibited sterol synthesis to a similar extent (63,73). In extrahepatic cells, however, the inhibitory effect of pravastatin on sterol synthesis was distinct from that of lovastatin or simvastatin. Lovastatin and simvastatin showed a similar strong inhibition of sterol synthesis in extrahepatic cells, while the inhibitory effect of pravastatin was much weaker. These *in vitro* data were followed by *ex vivo* and *in vivo* experiments using animals.

Tsujita et al. have observed that, in rats dosed with 25 mg/kg pravastatin or lovastatin, sterol synthesis in the liver and small intestine of these animals was strongly inhibited by pravastatin within 2 hours. The inhibitory effect on sterol synthesis in other organs was minimal (63). In contrast, lovastatin inhibited sterol synthesis in all the organs examined. Cholesterol synthesis in rat lenses was measured *ex vivo,* in the absence or presence of various concentrations of lovastatin, simvastatin or pravastatin (74). A strong inhibitory effect on sterol synthesis was observed for lovastatin and simvastatin. Pravastatin, however, was a less potent inhibitor of sterol synthesis. This difference was also observed in *ex vivo* experiments, using human lenses (75). Koga et al. investigated the tissue- selectivity of several HMG-CoA reductase inhibitors in mice *in vivo* (73). In each case, the drugs used produced 70-90% inhibition of sterol synthesis in liver, at doses of 5 and 20 mg/kg. In other organs tested, lovastatin and simvastatin inhibited sterol synthesis significantly, while no inhibition was observed with

Table III: ***Effects of lovastatin, simvastatin and pravastatin on cholesterol synthesis in cultured cells (IC_{50} in nM).***

cell type	lovastatin	simvastatin	pravastatin
Mouse			
L cells	2 [73]	3.6 [73]	1354 [63]
Lens epithelial cells	16 [78]	9 [78]	8200 [78]
Peritoneal macrophages	61 [78]		14000 [78]
Rat			
Adrenal cells	<50 [79]		
Spleen cells	3.5-4.5 [73,79]	5 [73]	158 [32]
Hepatocytes	3-146 [71,73,78-80]	3-50 [73,78,82]	5-500 [63,73,78,81]
Testis cells	50 [79]		
Myotubes	120 [83]	90 [83]	5900 [83]
Smooth muscle cells		240 [84]	195000 [84]
Hamster			
CHO	30 [78]		26000 [78]
Rabbit			
Lens epithelial cells	11 [78]	11 [78]	8200 [78]
Aortic fibroblasts			1693 [63]
Bovine			
Lens epithelial cells	61 [79]		
Human			
Skin fibroblasts	4-188 [71,73]	2.7-10 [73,86]	452-9000 [63,71,78,85]
ML-60	33 [78]		13000 [78]
Monocyte-macrophages	1 [85]		
Smooth muscle cells		30 [84]	15000 [84]
CaCo-2	<1 [87]		
Hep G2	43-50 [71,88]	150 [89]	700-2650 [88,89]

Corresponding literature cited is stated in superscript. This table is modified from reference 72.

pravastatin. In a distribution study using orally rats, Germerhausen et al. reprorted that the pravastatin concentration in the liver was approximately 50% lower than lovastatin or simvastatin concentration, and 3-6 times higher than both compounds in extrahepatic tissues (76). These results are contradictory to what had been observed previously by other investigators. The reason for this difference might be due to the method used for in the study of Germershausen, no distinction was made between peripherally distributed and intracellular drug concentrations. Koga et al. (77) demonstrated that, in microautoradiographic and *in vitro* cellular-uptake studies, pravastatin remained in the extracellular space within the spleen, whereas other drugs (lovastatin and simvastatin) entered the cell. The inhibition of cholesterol synthesis by vastatins in a given tissue should therefore be determined by the actual intracellular drug concentrations at the site of HMG-CoA reductase. The IC_{50}-values for lovastatin, simvastatin and pravastatin for inhibition on cholesterol synthesis in various cultured cells from different species, is presented in Table III (72). In all the extrahepatic cells investigated, both lovastatin and simvastatin are by far more potent inhibitors of sterol synthesis than pravastatin. In rat hepatocytes, however, there is no difference between the three drugs, in terms of potency to inhibit the sterol synthesis pathway.

4.3 Mechanism of hypocholesterolemic action and hydrophobicity of vastatins

The liver is the major producer of plasma cholesterol derived from *de novo* cholesterol synthesis, and takes up approximately 70% of circulating LDL-cholesterol, and is therefore the main target organ for cholesterol lowering drugs. HMG-CoA reductase inhibitors are competitive inhibitors of the rate-limiting enzyme in the cholesterol biosynthesis: HMG-CoA reductase. This enzyme catalyses the conversion of HMG-CoA into mevalonic acid. This inhibition, in turn, leads to an increased expression of hepatic LDL receptors, which is followed by increased removal of LDL-cholesterol from the blood by the liver, thus reducing the risk of CHD (Figure 6).

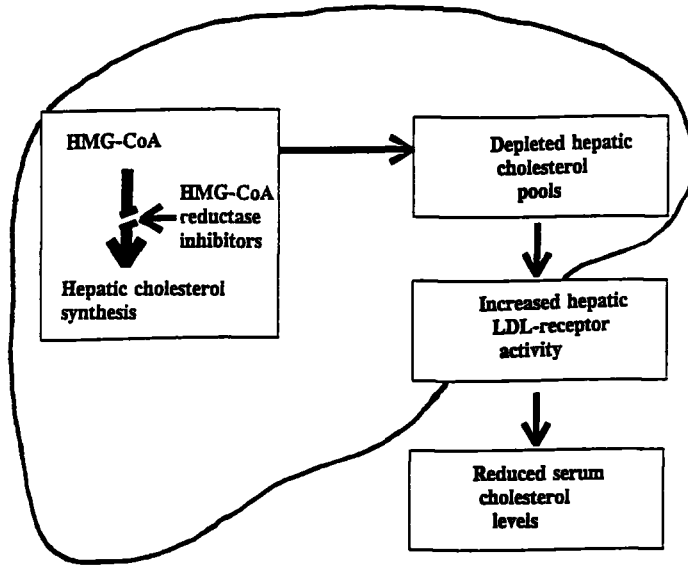

Figure 6: Mechanism of action of HMG-CoA reductase inhibitors.

Observation of the chemical structures of the three vastatins, shows that pravastatin is more hydrophilic compared with lovastatin or simvastatin (78,90). This major physicochemical parameter has been evaluated *in vitro* by determining the octanol-water partition coefficient ($P_{o/w}$) of both the hydroxy acids, and the lactones of the three agents (90). Both the hydroxy acid form, and the lactones of the three drugs, have a sequence of $P_{o/w}$ values in the order: pravastatin < lovastatin < simvastatin, at each evaluated pH, with approximate ratios of 1 : 75 : 200, respectively (Table IV).

Table IV: Apparent octanol-water partition coefficient of hydroxy acid and lactone forms of various HMG-CoA reductase inhibitors.

HMG-CoA reductase inhibitor	Apparent partition coefficient				
	Hydroxy acid			Lactone	
	pH 2	pH 5	pH 7	pH 5	pH 7
lovastatin	11000	1560	50	18400	18700
simvastatin	29500	4200	115	53800	48400
pravastatin	152	20.9	0.59	263	280

This table is modified from Serajuddin et al. (90), by Sirtori et al. (91).

Lovastatin and simvastatin are virtually insoluble in water. In comparison, pravastatin is hydrophilic of character.

4.4 Absorption and metabolic fate of the vastatins

The HMG-CoA reductase inhibitors currently in clinical use vary considerably in the extent of their absorption after oral dosing (92-98). Absorption ranges from approximately 33% of an oral dose for lovastatin and pravastatin to 98% for fluvastatin. Once absorbed, all these drugs are targeted to the liver, their primary site of action, by first pass hepatic extraction. Hepatic extraction of an oral dose is substantial for each of the HMG-CoA reductase inhibitors, estimated to be greatest for simvastatin (>79%) and least for pravastatin (46%) (98). In theory, the high degree of hepatic extraction and protein binding of these drugs, may protect peripheral tissues from possible adverse effects. The lipophilicity or hydrophilicity of these compounds also plays a very important role in the distribution to the peripheral tissues. Pravastatin, the more hydrophilic compound, hardly partitions in extrahepatic tissues, whereas simvastatin was distributed, to practically all extrahepatic tissues (73,99).

4.5 Therapeutic efficacy

HMG-CoA reductase inhibitors, or vastatins, are administered primarily to reduce total and LDL-cholesterol levels in patients with familial hypercholesterolemia, familial combined hyperlipidemia or polygenic hypercholesterolemia. Reductions in total cholesterol range from 15-30% and in LDL-cholesterol from 20-40%, have been observed using doses of 20-80 mg vastatin/day (100-104). The dose response in terms

of percentage reduction in total- and LDL-cholesterol is similar for the different forms of primary and secondary hypercholesterolemia (98,105-107). Vastatins, in combination with a bile acid sequestrant, such as colestipol or cholestyramine, reduce total- and LDL-cholesterol levels by 50-60%. HMG-CoA reductase inhibitors also modestly increase serum HDL-cholesterol levels, generally in the range of 5-10% (108-111), and lead to a decrease in plasma triglycerides of 2-30% (112).

4.6 Side-effects reported with vastatins

4.6.1 Side-effects of vastatins related to their mechanism of action

Apart from the differences described above, distinct effects with respect to other processes, related to the cholesterol biosynthesis pathway, have been assessed using these drugs. Inhibition of cell proliferation was observed in human smooth muscle cells in culture, by both lovastatin and simvastatin (84,113). Lovastatin inhibited proliferation in human and bovine endothelial cells, fibroblasts, and smooth muscle cells *in vitro,* at concentrations in the range of 0.1-2 μM (114). Pravastatin, however, inhibited sterol synthesis up to 20%, with no effect on cell proliferation of either rat or human arterial myocytes. It was also observed that simvastatin-sodium delayed cell death of anoxic cardiomyocytes, by inhibition of the Na^+/Ca^{2+} exchanger (115). Inhibition of fusion of L6-myoblasts in the presence of 0.25 μM of lovastatin was reported by Belo et al. (116). In *in vitro* experiments, using neonatal rat skeletal myocytes, pravastatin, lovastatin, and simvastatin caused myotoxicity. In the system used, pravastatin was less myotoxic than either lovastatin or simvastatin (83). Smith et al. observed myopathy in rats, dosed with high concentrations of lovastatin, simvastatin, or pravastatin (117). However, pravastatin did not induce skeletal muscle degeneration at a dose of 200 mg/kg, whereas both simvastatin and lovastatin did at this dose. Simvastatin also induced myopathy in rabbits (118). Ubiquinone levels were decreased in lovastatin-treated rats (119). Dogs treated with lovastatin or simvastatin developed cataract in a dose-dependent manner (120). This was not observed with pravastatin (121). In summary, differences in action of the three vastatins, with respect to extrahepatic cells, have been reported by several investigators.

4.6.2 Side-effects of vastatins reported in humans

The effects of vastatins has been extensively studied in humans, in several large clinical trials. A number of adverse side-effects have been reported for these cholesterol synthesis inhibitors. Results from controlled clinical trials, and clinical use of these compounds, have indicated that changes in liver function and, occasionally, the development of myopathy, represent the most common side effects observed in hypercholesterolemic patients using HMG-CoA reductase inhibitors (106,107,112). The development of myopathy has been reported in patients treated with either lovastatin (122-127) or simvastatin (128-131). Until now, only a few cases of pravastatin-induced myopathy have been reported (132-134). This small number may be related to its more recent introduction, as compared with lovastatin or simvastatin.
The underlying mechanism has not been elucidated. However, the risk of myopathy,

which may be associated with rhabdomyolysis and acute renal failure, is increased in patients treated with cyclosporine, gemfibrozil, nicotinic acid and erythromycin, in addition to one of the vastatins (135-138). In humans treated with lovastatin, lower ubiquinone levels have been measured (139). It has also been reported that lovastatin, simvastatin, and, in some cases, pravastatin influenced the central nervous system, causing sleep disturbances (140) and changing daytime performances (141-144) in man. Occasionally, lovastatin therapy has been associated with jaundice (145). However, in the many clinical studies performed, these side effects have only rarely been reported (109,110,146,147). Extensive evaluation of the lens in clinical studies with all three HMG-CoA reductase inhibitors revealed no evidence for increased rate of cataract formation or progression of other ophthalmologic disorders (110).

Since cholesterol is the precursor molecule for all steroid hormones, one might expect adverse effects of HMG-CoA reductase inhibitors on steroidogenesis. Testicular and adrenal steroidogenesis have been examined in several studies using lovastatin, simvastatin, and pravastatin (109,110,146,148). None of these studies showed any evidence of a drug-related effect. At present, no comparative data are available on the potential difference of the agents on gonadal steroid synthesis, neither *in vitro* nor *in vivo*. Symptoms of depression have been reported in patients treated with pravastatin and simvastatin (149,150). There seems to be an association between increased mortality from suicide, and low total cholesterol levels (151).

Apart for adverse side effects, some beneficial effects have been reported besides a cholesterol-lowering effect. Two clinical studies with lovastatin treatment (152,153) reported a decrease in the rate of progression of atherosclerosis. The development of new coronary lessions was also inhibited. Pravastatin produced beneficial effects on serum lipids, and was also associated with a reduction in the incidence of adverse cardiovascular events in a placebo-controlled, multicentered study including 1,062 patients with hypercholesterolemia (154). From the Pravastatin Limitation of Atherosclerosis in the Coronary arteries (PLAC I) study, it was observed that within a patient population, exhibiting moderately elevated LDL-C level, and manifest coronary artery disease, treatment with pravastatin monotherapy was associated with a significant reduction in cardiovascular morbidity (155). In the PLAC II study, a significant slowing-down of progression of early atherosclerosis in the common carotid artery was observed (156). A study with simvastatin was conceived in April 1987, to test the hypothesis that lowering of cholesterol would improve survival of patients with CHD (the Scandinavian Simvastatin Survival Study; 4S). It showed that long-term treatment with simvastatin is a safe means to improve survival in patients with CHD (157). In the above mentioned trials, no increased incidence of suicidal tendencies was reported.

5. *SOME ASPECTS OF DRUG TRANSPORT IN THE LIVER*

Differences in IC_{50}-values, as observed for pravastatin on sterol synthesis, in hepatocytes versus extrahepatic cells, was suggested to be due to the presence of a specific hepatic transporter (63,73).

5.1 Liver structure

The mammalian liver is located centrally in the body, between the gastrointestinal tract and the general circulation. Nutrients, drugs and other potential toxic xenobiotics, that are absorbed in the gastrointestinal tract, have to pass through this chemically active organ, before reaching other tissues in the body. The liver is a highly compartimentalized organ, and consists of several cell types. The highly branched capillary system (the hepatic sinosoides) enables efficient exposure to the sinusoidal cell types. Hepatocytes, which are parenchymal cells, represent about 80% of the liver volume with minor number of Kupffer cells, and endothelial cells (2 and 3 per cent of the total liver volume, respectively) (158). The sinusoidal cells form a protective cell-layer between blood and hepatocytes. The fenestrated endothelial lining permits direct contact of blood constituents, with limited molecular size, with the villous plasma membranes of the liver volume. Each hepatocyte is exposed to the blood plasma of at least two sinusoids, representing up to 40% of the surface area (basolateral membrane), whereas the canalicular membrane constitutes 13 % of the cell-surface area (158). The various cell types in the organ do not only have to deal with nutrients and endogenous metabolic products from other parts of the body, but also with drugs, toxins, immune complexes, and denaturated proteins, as well as LDL and HDL particles. After primary uptake of compounds by the liver, organelles such as lysosomes and endoplasmatic reticulum are able to degrade and biotransform these substances (159). Subsequently, products may be secreted via at least three ways: the systemic circulation, the biliary system, and the lymphatic system. Hepatocytes possess the ability to take-up various compounds from the circulation, such as bile acids (160), fatty acids (160), various types of amino acids (161), sugars (162), nucleosides (163), organic cations (164), as well as organic anions (165), as depicted in Figure 7.

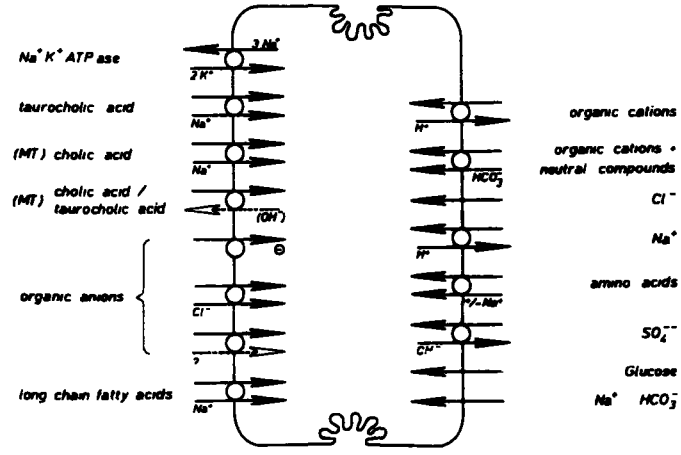

Figure 7: Transport systems for physiologicol substrates, drugs and xenobiotics described in the basolateral plasma membrane of rat liver cells (178).

5.2 Uptake of various compounds by hepatocytes

The uptake of organic solutes by hepatocytes can be accomplished via seven, entirely different mechanisms: 1. passive diffusion of uncharged lipophilic drugs through the lipid phase of the membrane; 2. passive diffusion of relatively small, charged drugs through aqueous pores in the membrane (166); 3. aspecific fluid-phase endocytosis (167); 4. adsorptive endocytosis; 5. receptor-mediated endocytosis (168); 6. carrier-mediated primary passive transport (166), and 7. carrier-mediated active or secondary transport (169). Only the last three processes are saturable, and display concentration dependency (159).

It is clear that the uptake mechanisms, mentioned above, can contribute to some extent to the internalization of a ligand, leading to hepatic uptake. However, the physicochemical features of the molecule, such as charge, number of charges per molecule, distribution of charge, as well as the size and the hydrophilicity-lipophilicity balance of the drug molecule, determines which process will be favoured (170-174). Pravastatin is anionic under physiological conditions, and therefore it is expected to be transported with known transporters for other anionic compounds, such as bile acids and bilirubin.

5.3 Organic anion transport and transport of vastatins

For organic anions, two major transport systems are described: a sodium-dependent bile salt transport system, and carrier-mediated diffusion of organic anions like bilirubin or bromosulfophthalein (165,175,176). These two systems are likely to have overlapping substrate specificity (170).

The inhibitory effect of pravastatin in hepatocytes might be due to (a) liver specific transporter(s), not present in extrahepatic cells. Therefore, the uptake of [^{14}C]-labelled lovastatin, simvastatin or pravastatin in freshly isolated rat hepatocytes was compared with that in spleen cells. Lovastatin and simvastatin were taken up into hepatocytes and spleen cells to a similar degree. In contrast, the uptake of pravastatin in hepatocytes was high, but very low in spleen cells (63,73). This led to the assumption that the more hydrophilic compound, pravastatin, is able to penetrate the plasma membrane of hepatocytes by other means. Uptake studies were performed with [^{14}C]-labelled pravastatin in adherent rat hepatocytes in primary culture (177). Indeed, pravastatin was taken up concentration- and temperature-dependently. This temperature-dependent uptake as a function of [^{14}C]-pravastatin concentration showed saturation kinetics with a K_m-value of 32.2 μM, and a maximal uptake rate of 68 pmol per mg of cellular protein per minute (177).

The uptake was inhibited by metabolic inhibitors, such as rotenon and potassium cyanide (inhibitors of electron transport within the process of oxidative phosphorylation). Unlabelled pravastatin, as well as structural analogues of pravastatin, competed for the hepatic uptake of [^{14}C]-pravastatin. So, in rat hepatocytes, pravastatin is taken up by a form of active transport. Ziegler et al., and Yamazaki et al. demonstrated carrier-mediated uptake of [^{14}C]-pravastatin in rat hepatocytes in suspension (178-180). Ziegler et al. observed that pravastatin competitively inhibits the sodium-independent hepatocellular uptake of cholate, taurocholate and ouabain, whereas the total uptake of cholate is non-competitively blocked (178). The affinity of pravastatin to the sodium-dependent taurocholate transporter is very low. Pravastatin had no affinity

for the other transport systems in liver cells, such as those for long-chain fatty acids, amino acids, rifampicin or bivalent cations. Uptake studies with [^{14}C]-pravastatin in fibroblasts (179) and bovine endothelial cells (181) demonstrated that pravastatin is not taken up by a carrier-mediated mechanism into these particular cells. This low extrahepatic uptake of pravastatin might be mediated by passive diffusion. It has been mentioned that [^{14}C]-simvastatin and [^{14}C]-lovastatin are also taken up in rat hepatocytes via a carrier-mediated mechanism (78,177). Tsuji et al. have performed uptake studies with [^{14}C]-simvastatin in bovine endothelial cells (181). At a pH of 6.5, simvastatin is taken up via a carrier. Whether this carrier may play a significant role in the uptake of [^{14}C]-simvastatin under physiological conditions (pH 7.4) is not yet clear.

6. OUTLINE OF THIS THESIS

The HMG-CoA reductase inhibitors have proved useful as cholesterol lowering drugs in many *in vivo* studies. The inhibitory action on sterol synthesis of the three drugs differed in extrahepatic cells compared with hepatocytes, the latter being the main target of lipid lowering drugs. Related processes in the cholesterol biosynthesis pathway, such as steroid synthesis, and cell proliferation, might be affected by inhibition of cholesterol synthesis, and may thus lead to adverse side-effects. At the beginning of this project, not much was known about the action of these drugs in human tissues or human cells in culture.

Therefore, the effect of lovastatin, simvastatin, and pravastatin on cholesterol synthesis, and related processes was investigated in this thesis using various types of human cells in culture. In Chapter 2, inhibition of sterol synthesis by lovastatin, simvastatin and pravastatin was studied in the human hepatoma cell-line Hep G2, often used as a representative model of the human hepatocyte. The sterol inhibitory effect of pravastatin and simvastatin was also investigated in cultured human hepatocytes to compare the outcome with the human hepatoma cell-line. In the latter cell-type, a possible effect on the feedback regulation mechanism was investigated, by measuring the mRNA levels of HMG-CoA reductase and the enzyme activity of squalene synthase in the absence or presence of various concentrations of the drugs.

The inhibitory action of the three drugs on sterol synthesis in human hepatocytes was further investigated and compared with that in several extrahepatic cells in culture (Chapter 3). The extrahepatic cells studied were human umbilical endothelial cells, retinal epithelial pigment cells, corneal fibroblasts and granulosa cells. The endothelial cells are of interest because the inner lining of blood vessels consists of these cells, and therefore they are in direct contact with any drug present in the circulation. Retinal pigment epithelium cells play an active role in the normal functioning of the photoreceptor cells. Corneal fibroblasts and granulosa cells are both located in an avascular surrounding, which makes these cells solely dependent on the *de novo* cholesterol synthesis for their cholesterol demand. Additionally, granulosa cells, are able to produce steroid hormones, e.g. progesterone, for which cholesterol is the precursor. Messenger RNA levels of HMG-CoA reductase were measured in human endothelial cells incubated with the three drugs for 3.5 or 20 hours, to investigate a possible effect of HMG-CoA reductase via a feedback mechanism. The involvement of a possible

carrier in the uptake of simvastatin and pravastatin, in human hepatocytes versus endothelial cells, was investigated in pilot studies, using ^{14}C-labelled vastatins.

Chapter 4 deals with the effects of the three vastatins on sterol synthesis in relation to progesterone secretion in human granulosa cells, both under cholesterol- limiting conditions, and in the presence of sufficient amounts of exogenous cholesterol.

Human smooth muscle cell proliferation plays a very important role in atherosclerotic plaque formation as well as restenosis after percutaneous transluminal coronary angioplasty. IC_{50}-values of the three drugs were determined, together with their effect on cell proliferation, DNA synthesis and cell viability. Additionally, the last three parameters were also determined in corneal fibroblasts and endothelial cells, for all vastatins used (Chapter 5).

Cell proliferation of myoblasts is a very important repair mechanism for damaged skeletal muscle tissue. In myoblasts, IC_{50}-values were obtained for the different drugs (Chapter 6). Accordingly, uptake experiments were performed with [^{14}C]-pravastatin and [^{14}C]-simvastatin to identify the possible involvement of a specific carrier present in myoblasts. Again, cell proliferation, DNA synthesis, and cell viability markers were measured, and related to the effects of the vastatins on sterol synthesis.

Finally, in Chapter 7, possible uptake mechanism(s) for pravastatin and simvastatin in human hepatocytes are described, with the aid of radiolabelled vastatins.

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CHAPTER 2

PRAVASTATIN INHIBITED THE CHOLESTEROL SYNTHESIS IN HUMAN HEPATOMA CELL LINE Hep G2 LESS THAN SIMVASTATIN AND LOVASTATIN, WHICH IS REFLECTED IN THE UPREGULATION OF 3-HYDROXY-3-METHYLGLUTARYL COENZYME A REDUCTASE AND SQUALENE SYNTHASE

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SUMMARY

The possible differences between lovastatin (mevinolin, MK-803), simvastatin (MK-733) and pravastatin (CS-514), all chemically-related competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, were tested in the human hepatoma cell line Hep G2, which is often used as a model for the human hepatocyte. After a 18-hr incubation of the cells with the drugs pravastatin (IC_{50} = 1900 nM) was less potent than simvastatin and lovastatin (IC_{50} = 34 nM and 24 nM, respectively) in inhibiting the sterol synthesis. As a consequence of this inhibition, the HMG-CoA reductase mRNA levels and squalene synthase activity, both negatively-regulated by sterols, were increased equally by simvastatin and lovastatin, whereas the induction by pravastatin was much less. In contrast, there were fewer differences between the compounds in inhibiting HMG-CoA reductase activity, when assayed directly in Hep G2 cell homogenates (IC_{50} values = 18, 61 and 95 nM for simvastatin, lovastatin and pravastatin, respectively). Moreover, in experiments with human hepatocytes in primary culture the IC_{50} values for inhibition of the cholesterol synthesis by simvastatin and pravastatin were of the same order of magnitude (23 and 105 nM, respectively). The results are therefore explained as follows: the three drugs act in the same way within the Hep G2 cell in terms of inhibiting HMG-CoA reductase and their subsequent effect on the feedback regulation of the cholesterol synthesis, i.e. increasing squalene synthase and HMG-CoA reductase mRNA. However, pravastatin seems to be less able to enter the cells compared with simvastatin and lovastatin, possibly because of the higher hydrophobicity of the latter compounds. The observation with human hepatocytes suggests that in Hep G2 cells a specific hepatic transporter is missing. On one hand the human hepatoma cell line Hep G2 has proven to be a good model for the study of the feedback regulation of enzymes of the cholesterol biosynthetic pathway such as HMG-CoA reductase and squalene synthase, but on the other hand seems to be less suitable as a model for the study of specific uptake of drugs, e.g. the vastatins, in human hepatocytes.

INTRODUCTION

The search for new cholesterol-lowering drugs induced the development of different analogues of compactin (ML-23B, CS-500) [1], a competitive inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), which is the major rate limiting enzyme of sterol synthesis in eukaryotic cells. During the last years these analogues such as lovastatin (MK-803, mevinolin) [2], simvastatin (MK-733) [3] and pravastatin (CS-514) [4], have been taken by many people in order to lower their serum cholesterol levels [5].

Although their efficacy in lowering serum cholesterol levels in men is of the same order of magnitude (simvastatin is about twice as potent as pravastatin and lovastatin; see Ref. 5) several authors have reported a large difference in the potency of the three drugs to inhibit the cholesterol synthesis in animal extrahepatic tissue *in vivo* and cells in culture compared to that in hepatic tissue and cells [4,6,7]. Pravastatin, the more hydrophilic one, compared to simvastatin and lovastatin [8] had a reduced ability to inhibit cholesterol synthesis in extrahepatic tissue, whereas similar inhibition was

observed for the three drugs in hepatic cells. These differences have also been observed in human fibroblasts [4] and human lens [9] *in vitro* but no data have been published on this matter concerning human liver tissue.

The human hepatoma cell line Hep G2 still possesses a number of human hepatocyte characteristics [10] and therefore has been frequently used as a model for the human hepatocyte. We have been using this cell line as such a model system in order to study the feedback regulation of the cholesterol synthesis and of low density lipoprotein (LDL)-receptor activity. In these cells compactin, inhibiting the incorporation of [^{14}C]-acetate into cholesterol, induced in a concentration-dependently manner, the LDL-receptor and the HMG-CoA reductase activity [11], HMG-CoA reductase mRNA levels [12] and the squalene synthase (EC 2.5.1.21) activity [13]. From these results we concluded that the negative feedback regulation of these processes by mevalonate-derived products takes place in Hep G2 cells as well.

In the present study we investigated the inhibitory potency of the analogues of compactin, i.e. lovastatin, simvastatin and pravastatin on the cholesterol synthesis in intact Hep G2 cells in culture and directly on the HMG-CoA reductase activity in homogenates of this human hepatocyte-derived cell line. Further we checked whether the differences observed were sustained by comparable effects on the co-ordinate regulation of HMG-CoA reductase and squalene synthase. Some data on the potency of the vastatins in inhibiting the cholesterol synthesis in human hepatocytes in primary culture are presented as well.

MATERIALS AND METHODS

Materials

Lovastatin (mevinolin, MK-803) and simvastatin (synvinolin, MK-703) were a kind gift from Merck, Sharp and Dohme, Rahway NJ, USA and pravastatin (CS-514) was donated by Sankyo Co. (Tokyo, Japan). Stock solutions were made in ethanol. The open acid forms of lovastatin and simvastatin were freshly prepared before use as described previously [9].

cDNA probes coding for human HMG-CoA reductase and for human serum albumin were isolated from plasmids obtained from Dr. K.L. Luskey (Dept. of Molecular Genetics and Internal Medicine, University of Texas, Dallas, TX, USA) and from Dr. H. Pannekoek (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) respectively, as described previously [12].

Human hepatoma cell line Hep G2

The cells were cultured in 10 cm^2 multiwell dishes in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (w/v) foetal bovine serum. At 18 hr before harvesting, the medium was replaced with DMEM, supplemented with 1% (w/v) palmitate-loaded (10 μmol/g) albumin [14] and the open acid forms of the different inhibitors at concentrations as indicated in the Results. Ethanol was added to all

incubations upto 0.05% (v/v), which was present at the highest drug concentration used. At this concentration ethanol did not influence the parameters determined. Cells were incubated for 18 hr at 37°C in a 5% CO_2/ 95% air atmosphere.

Human hepatocytes

The procedure and conditions for the isolation and culture of human hepatocytes have been described by Kooistra *et al.* [15]. Twenty-four hours after seeding of 1-1.5 x 10^6 of viable cells in 10 cm^2-wells the incubations were performed in Williams E medium supplemented with 10% heat-inactivated foetal bovine serum, 135 nM insulin, 50 nM dexamethasone, 100 IU/mL penicillin and 0.1 mg/mL of streptomycin, at 37°C in a 5% CO_2/95% air atmosphere.

Determination of the cholesterol synthesis

Measurements of the sterol synthesis ([^{14}C]-acetate incorporation into non-saponifiable lipids) in Hep G2 cells and in human hepatocytes in the absence or presence of different drug-concentrations was performed according to a modification of a previously described method [14]. After the cells had been incubated for 1 hr with the medium containing the inhibitors [^{14}C]-acetate (Amersham, U.K.; sp. radioact. 56.2 mCi/mmol) was added (0.4 or 2 μCi/well containing 1 mL of medium for Hep G2 or hepatocytes, respectively). The incubation was continued for 17 hr and then the medium was removed, the cells were lysed in 300 μL of 0.2 M NaOH and subsequently neutralized with 30 μL 2 M HCl. Media and cell lysates were stored at -20°C. After thawing samples were taken for protein determination. Thereafter, total lipids were extracted from cell lysate and medium together, according to Bligh & Dyer [16], in the presence of 0.008% (w/v) butylated hydroxytoluene (Sigma Chemical Co. Poole, U.K.) as an antioxidant and 0.01 μCi of [^{3}H]-cholesterol (New England Nuclear, Stevenage, U.K.; sp. radioact. 24 Ci/mmol) as a recovery standard. After evaporating the chloroform from the lipid extract under N_2, saponification was conducted in 0.2 mL of ethanolic (96%) 0.5 M NaOH for 1.5 hr at 60°C. After cooling, 0.2 mL of water was added and the non-saponifiable lipids were extracted with 2 x 0.5 mL of hexane. The combined hexane extracts were washed with 0.8 ml of ethanolic (48%) 0.25 M NaOH, followed by evaporation of the hexane under nitrogen. The ^{14}C-radioactivity incorporated into the non-saponifiable lipids were determined in a Tri-carb liquid scintillation analyzer (Packard), corrected for the recovery of [^{3}H]-cholesterol and expressed as ^{14}C-dpm/mg of cellular protein. Values are the average of duplicate cell incubations. The data presented in the figures are expressed as percentages of the control values, as means ± SEM, obtained from four to five separately performed Hep G2 cell-experiments and from three experiments using human hepatocytes.

Isolation of RNA from Hep G2 cells

RNA was isolated from Hep G2 cells according to the procedure described by Chomczynski and Sacchi [17]. In brief, cells from two 10 cm^2-wells were washed with phosphate buffered saline (PBS) and harvested in 0.5 mL of 4 M guanidinium isothiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% (w/v) sarkosyl and 0.1 M-β-mercaptoetha-

nol. RNA was extracted from the cell lysates with phenol/chloroform/isoamyl alcohol and precipitated subsequently with isopropanol. After washing the pellets with 70% ethanol they were solved in 40 μL of 10 mM Tris-HCl (pH 7.0). The RNA concentration of each sample was determined spectrophotometrically. The yield of RNA was usually 100-150 μg.

Determination of HMG-CoA reductase mRNA levels

Using the cDNA probes as described above, the HMG-CoA reductase mRNA levels were determined by Northern blot hybridization. The albumin probe was used as an internal mRNA standard, while the albumin mRNA levels were not significantly influenced by the treatments of the cells (compare with Ref. 12). Five micrograms (which was within the range of linearity between mRNA concentration and hybridization signal) of total RNA from the different dishes were incubated with formamide in gel running buffer at 55°C for 15 minutes to denature RNA and then subjected to gel electrophoresis in formaldehyde agarose gels. After electrophoresis, RNA was transferred to Hybond N filters according to the instructions of the manufacturer. Prehybridization and hybridization were performed at 60°C (1 mM EDTA; 7% sodium dodecyl sulfate; 0.25 M NaCl, 0.25 M NaH_2PO_4/Na_2HPO_4; pH 7.2) essentially as described elsewhere [18]. Hybridization was usually performed with 1 ng/mL probe labelled by the random-primer method (Multi-prime, Amersham, Houten, The Netherlands) with [^{32}P] dCTP to approximately 10^9 cpm/μg DNA. The filters were washed at a stringency of 0.1 x standard saline citrate and 1% sodium dodecyl sulfate twice for 15 minutes each at 65°C [19]. The membranes were subsequently exposed to Amersham Hyperfilm-MP with an intensifying screen at -80°C. For quantification of the relative amounts of mRNA on the autoradiograph a scan of the bands was made on a CS 910 Shimadzu scanner. The areas under the peaks were integrated and plotted with the aid of a United Technology Packard data processor. The values for the HMG-CoA reductase mRNA contents are expressed as HMG-CoA-reductase-probe-derived blackening (arbitrary) units divided by albumin-probe-derived blackening units.

Assay of HMG-CoA reductase activity

HMG-CoA reductase activity was determined in the Hep G2 cell homogenate essentially as described previously [11]. Cells were washed three times with cold PBS (0.15 M NaCl/10 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.4) and once with 0.1 M potassium phosphate/0.1 M NaCl (pH 7.4), and were harvested by scraping them into assay buffer (0.1 M potassium phosphate/0.1 M NaCl/10 mM EDTA, pH 7.4). The cell suspension was frozen in liquid N_2 before storage at -80°C. After thawing and rupture of the cells by sonication (Branson sonifier B-12, 70 W output, for 5 sec at 0°C), 50 μL samples (160-230 μg of protein) were preincubated for 25 min at 37°C. The enzyme reaction, which was performed in the presence of various drug concentrations (as indicated in the Results) for 40 min at 37°C, was started by the addition of cofactors and substrate. The 100 μL assay mixture contained, besides the vastatins, 0.5 mM [^{14}C]-HMG-CoA (sp. radioact. 3000-4500 dpm/nmol), 5 mM $NADP^+$, 50 mM glucose 6-phosphate, 0.7 unit of glucose-6-phosphate dehydrogenase, 50 mM potassium phosphate (pH 7.4), 50 mM NaCl, 10 mM EDTA, 5 mM dithiothreitol and 1.6-2.3 mg of Hep G2 cell protein/mL.

The incubation was stopped by addition of 25 μl 1.2 M HCl, which contained 2-3 x 10^4 dpm.[^{3}H]-mevalonic acid (New England Nuclear, sp. radioact. 5 Ci/mmol) as recovery standard. Mevalonic acid was converted into the lactone form by incubation for 30 min at 37°C and isolated by TLC on silica plates (Merck DC 60; developed in acetone/ toluene = 1:1). Silica in between R_f-values 0.3 and 0.6 was scraped and ^{3}H-/^{14}C-radioactivity counted. The [^{14}C]-mevalonate formed was corrected for the recovery of [^{3}H]-mevalonate (70-80%) and the HMG-CoA reductase activity was expressed in pmol of mevalonic acid formed/min/mg of cellular protein. The values are the averages of duplicate determinations and given as percentage of control.

Assay of squalene synthase activity

The determination of squalene synthase activity in Hep G2 cell homogenates after incubation was performed as described previously [13]. Values, expressed as nmol of squalene formed/min/mg of cellular protein, were obtained from duplicate determinations in two homogenates of identically-treated cells. The average value for the individual homogenates agreed within 10%. Protein concentrations were determined according to Lowry *et al.* [20]. Values resulting from the experiments, which were separately performed at least three times (designated as N in the legends of the figures), are depicted as the mean percentages of the control values ± SEM. Statistical differences ($P < 0.05$) from control values, as determined by a paired *t*-test, are indicated in the figures.

RESULTS

Inhibition of cholesterol synthesis by vastatins in Hep G2 cells

Hep G2 cells were incubated with different concentrations of the drugs in the presence of [^{14}C]-acetate for 18 hr and the incorporation of ^{14}C-label into non-saponifiable lipids was determined. As is shown in Fig.1 simvastatin inhibited the cholesterol synthesis at lower concentrations (IC_{50} = 34 nM) than pravastatin (IC_{50} = 1900 nM). The values for lovastatin were of the same order of magnitude as those for simvastatin (IC_{50} = 24nM).

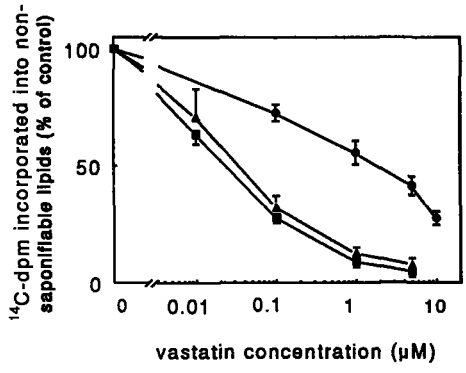

Figure 1: Inhibition of sterol synthesis by vastatins in Hep G2 cells

Hep G2 cells were incubated with the indicated concentrations of either pravastatin (●), lovastatin (■) or simvastatin (▲) for 18 hr at 37°C in the presence of [^{14}C]-acetate (0.4 μCi/mL). The incorporation of label into non-saponifiable lipids was determined as described in Materials and Methods. Values are expressed as percentages of control (34,330 ± 4325 dpm/mg of cellular protein); means ± SEM (N = 4-5).

Effect of vastatins on HMG-CoA reductase mRNA levels in Hep G2 cells
In order to investigate the effect of the inhibitors on the feedback regulation of HMG-CoA reductase, after the 18 hr-incubation the HMG-CoA reductase mRNA levels were investigated by Northern blot analysis. The levels of the albumin mRNA, which were not influenced by the reductase inhibitors, were used as an internal standard in the assay. The results are depicted in Fig. 2. The same concentration-dependent increase of the reductase mRNA levels by simvastatin and lovastatin was observed, whereas only a smaller increase at a high pravastatin concentration was observed. These data corresponded with the concentration-dependent inhibition of the cholesterol synthesis by the drugs (Fig. 1). The values obtained with lovastatin are in good agreement with the data published by Molowa and Cimis [21], using actin mRNA as an internal standard.

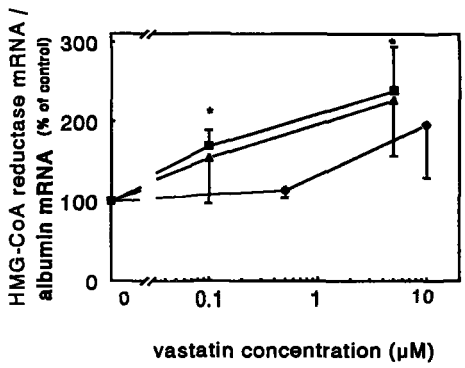

Figure 2: Effect of vastatins on HMG-CoA reductase mRNA levels in Hep G2 cells
Cells were incubated for 18 hr at 37°C with DMEM supplemented with 1% albumin and the indicated concentrations of either pravastatin (●), simvastatin (▲) or lovastatin (■). RNA was isolated from cells grown in two 10 cm^2 wells and the HMG-CoA reductase mRNA concentration was determined by Northern blot hybridization as described under Materials and Methods. Values for the reductase mRNA levels, the ratio of the reductase- and the albumin-probe-derived blackening on the autoradiograph, are expressed as mean percentages of control ± SEM (N = 3). (*) Values are significantly different ($P < 0.05$) from control values using a paired *t*-test.

Effect of vastatins on squalene synthase activity in Hep G2 cells
Previously we have reported that in Hep G2 cells squalene synthase activity is regulated by sterols in the same way as the HMG-CoA reductase mRNA levels [13]. In order to extend those observations and to support the data depicted in Fig. 2 the squalene synthase activity in the Hep G2 cell homogenates were determined after incubation with the different reductase inhibitors for 18 hr. As shown in Fig. 3, the squalene synthase activity was increased in the same way as was observed for the reductase mRNA.

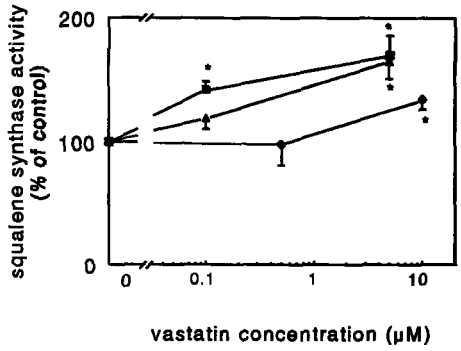

Figure 3: Effect of vastatins on squalene synthase activity in Hep G2 cells
Cells were incubated with the different vastatins as described in the legend of Fig. 2. Squalene synthase activity was determined in the cell homogenate as described in Materials and Methods. Values are expressed as percentages of control (2.00 ± 0.10 nmol/min/mg of cellular protein); means ± SEM (N = 3). (*) Values are significantly different (P < 0.05) from control values.

Inhibition of HMG-CoA reductase activity by vastatins directly in Hep G2 cell homogenates

In order to exclude that the differences between the vastatins observed in intact cells were the result of differences in the potency of the drugs to inhibit the Hep G2 HMG-CoA reductase, the inhibition of the enzyme activity was directly measured in the Hep G2 cell homogenate. Therefore, the HMG-CoA reductase assay was performed in Hep G2 cell homogenate in the presence of various concentrations of the three compounds. As can be seen in Fig. 4 simvastatin, lovastatin and pravastatin inhibited the enzyme to the same order of magnitude (IC_{50} values of 18, 61 and 95 nM respectively). The difference in inhibitory potency of maximal five times cannot explain the difference in inhibition of the cholesterol synthesis in intact cells (compare Fig. 1).

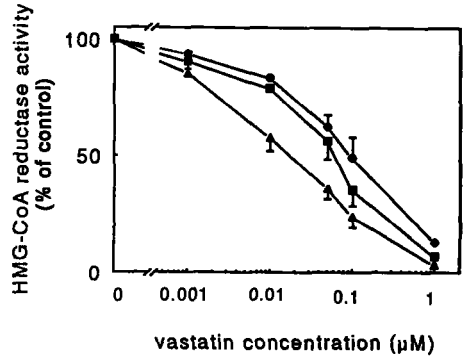

Figure 4: Inhibition of HMG-CoA reductase activity in Hep G2 cell homogenate by vastatins
The HMG-CoA reductase assay was performed as described in the Materials and Methods in 50 μL-samples of the same batch of Hep G2 cell homogenate in the absence or presence of the indicated concentrations of either pravastatin (●), lovastatin (■) or simvastatin (▲). The en-zyme activity was expressed as percentage of controls (90.6 ± 10.4 pmol/min/mg of cellular protein) and depicted as the average value of three separate performed experiments ± SEM.

Inhibition of cholesterol synthesis by vastatins in human hepatocytes

As discussed below the results obtained with the Hep G2 cells can be explained by an impaired uptake of pravastatin by the cells, possibly by missing a specific hepatic

transporter. In order to support this explanation the inhibition of the sterol synthesis in human hepatocytes in primary culture by simvastatin and pravastatin was investigated. The results are shown in Fig. 5. In contrast with the large difference in inhibitory potency of simvastatin and pravastatin in Hep G2 cells, in human hepatocytes pravastatin is much more potent and its IC_{50} value (105 nM) was much closer to that of simvastatin (23 nM).

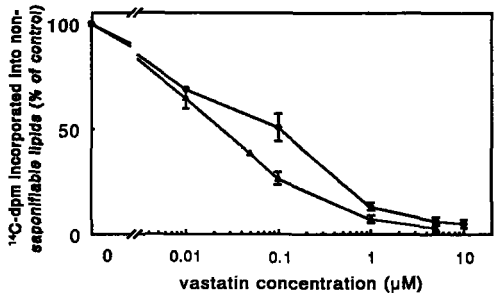

Figure 5: Inhibition of sterol synthesis by vastatins in human hepatocytes
Human hepatocytes were incubated for 18 hr at 37°C in Williams E medium/10% foetal bovine serum/135 nM insulin/50 nM dexamethasone with the indicated concentrations of either pravastatin (●) or simvastatin (▲). After 1 hr, 2 μCi/mL of [^{14}C]-acetate was added and the incubation was proceeded for 17 hr. The [^{14}C]non-saponifiable lipids have been determined as described in Materials and Methods. Values are expressed as percentages of control (28,830 ± 6040 dpm/mg of cellular protein); means ± SEM (N=3); for values with N < 3 no SEM has been given).

DISCUSSION

The HMG-CoA reductase inhibitors simvastatin, lovastatin and pravastatin, presently in use as cholesterol-lowering drugs, inhibited the cholesterol synthesis in the human hepatoma cell line Hep G2 to a different extent. Under the conditions used, pravastatin was about 60-80 times less potent than simvastatin and lovastatin. This was also reflected in their influence on HMG-CoA reductase and squalene synthase mediated by the co-ordinate feedback regulation of these enzymes by inhibiting the synthesis of sterol suppressors. HMG-CoA reductase mRNA levels (Fig. 2) and squalene synthase activity (Fig. 3) were increased to the same extent by simvastatin and lovastatin and to a lesser extent by pravastatin.

The three drugs have comparable IC_{50} values for the inhibition of HMG-CoA reductase activity in rat liver microsomal preparations (unpublished results: and Ref. 22) and inhibit the reductase activity at the same order of magnitude in Hep G2 cell homogenates (Fig. 4). Therefore, we explain the observed difference between the effects of simvastatin and lovastatin, on one hand, and of pravastatin, on the other, by a limited transport of pravastatin over the Hep G2 cell membrane compared with the other two compounds. There is evidence [4,7] that differences in uptake of the drugs play a role in the difference in inhibitory potency of pravastatin compared to lovastatin

and simvastatin in hepatic cells versus non-hepatic cells. Very recently it was shown that the uptake of pravastatin in rat hepatocytes was carrier-mediated [23] and that a specific hepatic sodium-independent bile acid transporter was responsible for that action [24]. Hep G2 cells may have lost this transporter to a certain extent, limiting the uptake of pravastatin, while the two more lipophilic compounds are still able to enter the cells possibly by diffusion or via other transporters. This suggestion is supported by the observation that Hep G2 cells have lost the ability to transport taurocholate (Dr. H.M.G. Princen, personal communication) in contrast with freshly isolated human hepatocytes [25] and in which taurocholate and pravastatin are competitors for the same transporter [24]. Also the bilirubin transporter has been found to be strongly reduced in Hep G2 cells [26].

The presence of a transporter may possibly be influenced by the growth conditions of the Hep G2 cells, because we observed a different IC_{50} value for pravastatin (18-hr incubation 1900 nM) than was very recently published by Nagata *et al.* [27] (IC_{50} value pravastatin 18-hr incubation > 10,000 nM) and by Shaw *et al.* [22], (IC_{50} value pravastatin 8-hr incubation 2650 nM). The IC_{50} values for the other vastatins were of the same order of magnitude (24-80 nM) at the three independent observations.

Further support for a missing hepatic pravastatin-transporter in Hep G2 cells is given by our observation that in human hepatocytes in primary culture pravastatin is a much stronger inhibitor of cholesterol synthesis, comparable to simvastatin (Fig. 5). While the taurocholate transporter is present in these cells [25] this observation suggests that a specific transporter was involved in the action of pravastatin. Further investigations, using radiolabelled drugs, will give more insight into this mechanism.

From the results discussed above we conclude that the human hepatoma cell line Hep G2 is a good model for the study of the feedback regulation of the cholesterol synthesis. However, this cell line is less suitable as a human hepatocyte model for the study of specific uptake of drugs.

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CHAPTER 3

DIFFERENT EFFECTS OF 3-HYDROXY-3-METHYLGLUTARYL-COENZYME A REDUCTASE INHIBITORS ON STEROL SYNTHESIS IN VARIOUS HUMAN CELL TYPES

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SUMMARY

The three vastatins examined, lovastatin, simvastatin and pravastatin, are equally strong inhibitors of the sterol synthesis in human hepatocytes in culture with IC_{50}-values of 4.1, 8.0 and 2.0 nM, respectively. However, in the human extrahepatic cells: umbilical vascular endothelial cells, retinal pigment epithelial cells, cornea fibroblasts and granulosa cells, pravastatin was much less inhibiting the sterol synthesis than lovastatin or simvastatin. It was observed as well that longer incubation with the vastatins resulted in higher IC_{50}-values. In order to show that the feedback regulation mechanism for 3-hydroxy-3-methylglutaryl-coenzyme A reductase was involved in this phenomena mRNA levels were measured in human vascular endothelial cells after incubation with the vastatins for 3.5 h and for 20 h. Indeed, lovastatin and simvastatin gave rise to higher levels of HMG-CoA reductase mRNA after 20 h than after 3.5 h of incubation.

The differences observed in different human cell types can be explained by supposing that pravastatin is transported into the human hepatocyte via a liver-specific transporter. This was supported by the results of uptake experiments with ^{14}C-labelled pravastatin and ^{14}C-labelled simvastatin into human hepatocytes compared to that into human umbilical endothelial cells (as an example of an extrahepatic cell type). [^{14}C]-Simvastatin was associated with both cell types, whereas [^{14}C]-pravastatin was hardly associated with human endothelial cells, but to a similar extent as [^{14}C]-simvastatin with human hepatocytes.

INTRODUCTION

Coronary heart disease is the main cause of death in Western countries. A high level of low density lipoprotein cholesterol (LDL-C) in the blood plasma is a major risk factor in the development of atherosclerosis. To lower LDL-C, dietary restrictions and in some cases drug intervention can be used. Lovastatin [1], simvastatin [2] and pravastatin [3], inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis, are currently in use as cholesterol-lowering drugs. Since the liver plays a major role in the metabolism of cholesterol in the body, it is the target for these HMG-CoA reductase inhibitors [4]. The reduction in the de novo synthesis of cholesterol leads to an induction of LDL-receptors in the liver, lowering the LDL-C in the blood.

Despite similarities in chemical structure of the vastatins, pharmacological differences have been observed. Pravastatin is more hydrophilic than simvastatin or lovastatin [5,6]. Probably due to this property they have different inhibitory effects on different cell types as shown in animal studies. Tsujita et al. [3] showed in freshly isolated rat hepatocytes that compactin and pravastatin are equally potent inhibitors of HMG-CoA reductase. Additionally, Koga et al. [7,8] observed the same inhibitory action of lovastatin, simvastatin and pravastatin on the rat liver sterol synthesis in *in vivo* and *ex vivo* experiments. In contrast with that, it was observed in freshly isolated rat spleen cells, mouse L-cells and rabbit aortic fibroblasts in culture that pravastatin was much less potent than compactin in inhibiting sterol synthesis [3]. Furthermore,

Koga et al. [7,8] and Mosley et al. [9] showed in *in vivo* and *ex vivo* experiments that in several extrahepatic tissues inhibition of [^{14}C]-acetate incorporation into cholesterol was equally inhibited by simvastatin and lovastatin, but much less by pravastatin.

Little is known about whether these differences in relative inhibitory potency also exist in different tissues in humans. To obtain such information, cells isolated from different human tissues were brought into culture and used as model systems. The differences between simvastatin and lovastatin on one hand and pravastatin on the other, as observed in rat lens [9,10], was confirmed in experiments performed with human lens in tissue culture by De Vries et al. [11,12]. The same difference was observed in cultured human fibroblasts [3]. Corsini et al. [13] isolated smooth muscle cells from the human femoral artery and reported that in these cells in culture simvastatin and pravastatin had IC_{50}-values of 0.03 μM and of 15 μM, respectively. So, in these human extrahepatic cells pravastatin inhibits the sterol synthesis less than the other vastatins examined. In a pilot experiment with human hepatocytes in culture, we observed the same inhibitory potency of simvastatin and pravastatin on sterol synthesis [14].

In order to gain more information on human cells, the influence of the three vastatins on sterol synthesis was investigated further in human hepatocytes and compared with that in several human extrahepatic cells in culture. The cells investigated were human umbilical vascular endothelial cells (HUVEC), which are *in vivo* in direct contact with the drugs in the circulation, human retinal pigment epithelial cells (HRPEC), human cornea fibroblasts (HCF) and human granulosa cells (HGC). In the body, the latter two kinds of cells are not in direct contact with the circulation and therefore largely dependent on *de novo* synthesis for their cholesterol demand. Furthermore, in granulosa cells, cholesterol is converted into steroid hormones (e.g. progesterone) which may be influenced by inhibition of cholesterol synthesis.

Further, the uptake of [^{14}C]-simvastatin and [^{14}C]-pravastatin into human hepatocytes and into human umbilical vein endothelial cells, as a model for an extrahepatic cell type, was studied.

MATERIALS AND METHODS

Lovastatin, simvastatin, pravastatin (all sodium salts), [^{14}C]-simvastatin (spec. radioactivity 17.1 μCi/mg) and [^{14}C]-pravastatin (spec. radioactivity 23.5 μCi/mg) (labelled compounds in lactone form) were kindly donated by Sankyo Co. (Tokyo, Japan). Stock solutions were made in ethanol. Before use, the lactone ring of the [^{14}C]-labelled drugs was hydrolysed by a 30-min incubation in 0.1 M NaOH at 37°C, followed by neutralisation with HCl.

A c-DNA probe coding for human HMG-CoA reductase was isolated from plasmid pHred-102 obtained from Dr. K.L. Lusky (Dept. of Molecular Genetics and Internal Medicine, University of Texas, Dallas, TX, USA) as described previously [15]. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA [16] was used as an internal standard to correct for loss of mRNA due to the procedure used.

Isolation and culture of human primary cells

Part of livers were obtained from donors, which could not be used for transplantation due to technical problems. Human hepatocytes were isolated and cultured as described by Rijntjes et al. [17], except that during culture fungizone, gentamycin, tetracyclin and vancomycin were replaced by 100 IU of penicillin per ml, and 100 μg of kanamycin and of streptomycin per ml.

HUVEC were isolated as described by Jaffe et al. [18] and cultured as described by Van Hinsbergh [19]. Cells were cultured in RPMI 1640 instead of M199 and supplemented with 0.1% heparin, 10% filter-sterilized human serum, 10% newborn calf serum (heat-inactivated at 56°C during 30 minutes), 1% glutamine, 1% endothelial cell growth factor [20], 100 IU/ml penicillin and 0.1 mg/ml streptomycin.

HRPEC and HCF were obtained from the Cornea Bank, Amsterdam. These cells were isolated as described by Baumgartner et al. [21] and Hoppenreijs et al. [22], respectively. HRPEC were grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 1% glutamine. The culture conditions for HCF were the same, except that RPMI 1640 was replaced by MEM.

HGC were harvested from pre-ovulatory follicles of hormone-treated women, undergoing ovum retrieval for in vitro fertilisation (IVF, Academic Hospital Leiden, Leiden). Cells from individual patients were pooled and resuspended in RPMI 1640 medium containing 4 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 10% human serum and 10% newborn calf serum. Cells were layered onto Ficoll paque (Pharmacia) and centrifuged at room temperature for 20 minutes at 1200 x g. The HGC were collected from the interface and resupplemented with medium. The cells were washed and the pellet was resuspended in medium. Viability was measured by the trypan blue exclusion method.

All cells used were cultured in a humidified environment with 5% CO_2/95% air at 37°C. All experiments were performed with cells at near confluence.

Determination of inhibition of sterol synthesis

Cells were preincubated for a period of 30 min in the absence or presence of 0.001, 0.01, 0.1, 1 or 10 μM of lovastatin or simvastatin; for pravastatin the concentrations of 0.01, 0.1, 1, 5 or 10 μM, respectively, were used. After this preincubation [2-^{14}C]-acetate (spec. radioactivity of 56.2 mCi/mmol, Amersham) was added in different amounts of radioactivity depending on the cell types used (see legends of figure or table in Results section). [^{14}C]-acetate incorporation into sterols was measured after 3 h. according to a previously described method [14]. Experiments with HUVEC were also performed with a preincubation and incubation time of 2 h. and 18 h., respectively. Non-saponifiable lipids were separated by thin layer chromatography according to Boogaard et al. [23]. Curve fitting through all datapoints using a dose-response equation was performed to calculate the IC_{50}-values and these values are expressed as mean $\pm$ S.E. (n = 3).

Isolation of RNA from human umbilical vein endothelial cells and determination of HMG-CoA reductase mRNA levels

RNA was extracted from HUVEC according to the procedure as described by Chom-

czynski and Sacchi [24].

HMG-CoA reductase mRNA levels were determined by Northern blot hybridization. Equal amounts (5 μg) of RNA from different incubations were separated by electrophoresis on a 0.8% agarose gel containing 1 M formaldehyde, and transferred to Hybond-N filter (Amersham) in accordance with the instructions given by the manufacturer. RNA was cross-linked to the filter with UV light for 5 minutes and hybridized with cDNA probes coding for HMG-CoA reductase and GAPDH in 0.5 M sodium phosphate buffer (pH 7.2), containing 7% (w/v) SDS and 1 mM EDTA at 65°C for 18 h. Hybridization was performed with 25 ng of probe, labelled by the random primer method (Multi-Prime, Amersham), to approximately 10^9 cpm/μg DNA. After hybridization blots were washed twice with 2 x SSC/1% SDS (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.8) for 15 min at 65°C and twice with 1 x SSC/1% SDS (15 min at 65°C). The relative amounts of mRNA were quantified with the use of a Phosphor Imager (Molecular Dynamics). The areas under the peak were integrated using image quant software. HMG-CoA reductase mRNA levels are expressed as HMG-CoA reductase-probe-derived units divided by GAPDH-probe-derived units (arbitrary).

Determination of uptake of ^{14}C-labelled vastatins in human hepatocytes and endothelial cells

Ten cm^2 of monolayer cultures of human hepatocytes or HUVEC were incubated for 1-30 min with ^{14}C-labelled vastatins (sodium salts) in Williams E medium (WE, Gibco) or Krebs-Ringer solution (KR, Sigma), respectively. HUVEC were preincubated for 30 min with KR at 37°C under 5% CO_2/95% air. In an additional experiment, HUVEC were incubated for 7.5 min with 2 to 200 μM of [^{14}C]-simvastatin or [^{14}C]-pravastatin at 37°C. The incubation was stopped by addition of 1 ml of ice-cold 0.5% bovine serum albumin in KR. This medium was promptly removed and the cells were subsequently washed with 2 ml of the same solution, and three times with 1 ml cold KR. Cells were lysed with 300 μl of 0.2 N NaOH per well. The lysate was collected and neutralized by adding 30 μl of 2 N HCl. Samples were taken for the determination of protein [25]. To 250 μl of cell lysate 10 ml scintillation fluid (Ultima Gold, Packard) was added and radioactivity was counted in a Packard 1900 CA liquid scintillation counter. Values are expressed as pmol of [^{14}C]-vastatin associated/mg of cellular protein.

RESULTS

Inhibition of sterol synthesis by vastatins in human hepatocytes and human umbilical vein endothelial cells

After an incubation of 3 h of human hepatocytes or human umbilical vein endothelial cells with different concentrations of the three HMG-CoA reductase inhibitors the incorporation of ^{14}C-labelled acetate into sterols was measured. The inhibitor curves are shown in Fig. 1A and 1B. The IC_{50}-values, the drug concentration at which 50% inhi-

bition of sterol synthesis occurs, were calculated from these curves and presented in Table I. In human hepatocytes the three vastatins inhibited the sterol synthesis to a similar extent. However, in human umbilical vein endothelial cells pravastatin inhibited the sterol synthesis about 300 times less than lovastatin or simvastatin (Fig. 1B, Table I).

Previously, we showed the results of an 18-h incubation of human hepatocytes with simvastatin and pravastatin [14]. Higher IC_{50}-values for both drugs were obtained (Table I). Also 20-h incubations of human umbilical vein endothelial cells with the three vastatins led to higher IC_{50}-values (Fig. 1C, Table I). However, the different inhibitory potencies between pravastatin on one hand and lovastatin and simvastatin on the other hand remained.

TABLE I: Inhibition by lovastatin, simvastatin and pravastatin of the sterol synthesis in human hepatocytes and human umbilical vein endothelial cells in culture.

Cells were incubated for 3.5 h and 18 h or 20 h with 0.001-10 μM of vastatin and [^{14}C]-acetate incorporation into sterols was measured as mentioned in Materials and Methods. The drug concentration (nM) at which 50% inhibition of the sterol synthesis occurs was calculated from three separately obtained inhibitory curves (mean ± S.E.). The composite curves are depicted in Fig. 1.

Cell type	incubation time	IC_{50}-values (nM)		
		lovastatin	simvastatin	pravastatin
hepatocytes	3.5 h	4.1 ± 1.3	8.0 ± 4.2	2.0 ± 0.07
	18 h	n.d.	23.0 ± 10	105 ± 30
endothelial cells	3.5 h	2.4 ± 0.5	5.5 ± 2.5	1172 ± 247
	20 h	79.3 ± 12.8	56.1 ± 35.4	8520 ± 3673

n.d. = not determined.

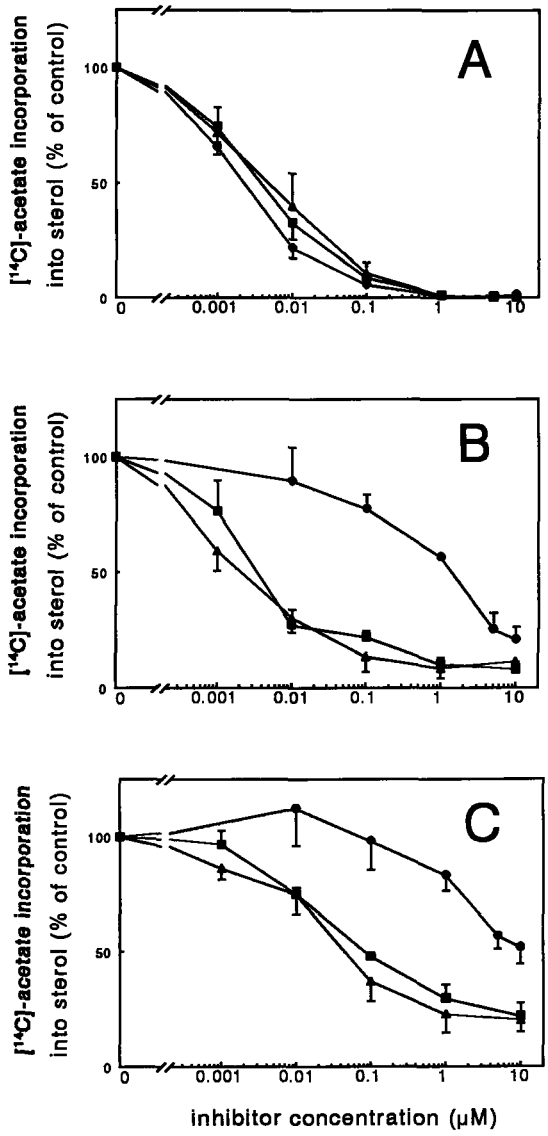

Figure 1.
Inhibition of sterol synthesis by vastatins in human hepatocytes (A) and human umbilical vein endothelial cells (B,C).
Synthesis was measured by incorporation of [^{14}C]-acetate into sterols and expressed as percentage of control (see Materials and Methods). The experimental conditions used were: a 30-min preincubation with the indicated drug concentrations, addition of 5 μCi/ml [^{14}C]-acetate and subsequent incubation of 3 h (A,B) or a 2-h preincubation, followed by addition of 0.8 μCi/ml [^{14}C]-acetate and an incubation of 18 h (C). The acid forms were used of lovastatin (■), simvastatin (▲) and pravastatin (●). The bars represent S.E. of 3 separately performed experiments (if not shown, the bars coincide with the symbols).

Increase of mRNA levels of HMG-CoA reductase by vastatins in human umbilical vein endothelial cells at prolonged incubation time

Higher IC_{50}-values at longer incubation times can be explained by assuming that at prolonged inhibition of sterol synthesis, less regulatory sterols, which suppress HMG-CoA reductase, will be present in the cells. As a consequence, more HMG-CoA reductase mRNA and enzyme will be present and more inhibitor is needed to reach the same level of inhibition. In order to support this explanation, the effect of 1 μM of the

three vastatins on mRNA levels of HMG-CoA reductase was measured in human umbilical vein endothelial cells after an incubation of 3.5 h and of 20 h. The levels of GAPDH mRNA were used as an internal standard, because they were not influenced by the vastatins. The results of a typical experiment are shown in Table II. In all cases the mRNA levels of HMG-CoA reductase were slightly upregulated by the vastatins at 3.5 h of incubation, but more than 2 times induced after 20 h of incubation with 1 μM of lovastatin or 1 μM of simvastatin. There was only a small increase in the mRNA levels of HMG-CoA reductase after 20 h of incubation with 1 μM of pravastatin (Table II).

TABLE II: Effect on HMG-CoA reductase mRNA levels by incubation with lovastatin, simvastatin and pravastatin for different times.

Human endothelial cells were incubated with 1 μM of vastatin for 3.5 or 20 h, followed by RNA isolation and mRNA determination by blotting and hybridization as described under Methods. Reductase mRNA levels were corrected using the GAPDH mRNA values measured. The control value of reductase mRNA after 20 h-incubation was 170% of the 3 h-control values.

	HMG-CoA reductase mRNA/GAPDH mRNA (% of control)	
incubation time	3.5 h	20 h
control	100	100
lovastatin	122	246
simvastatin	103	218
pravastatin	114	109

Inhibition of sterol synthesis by HMG-CoA reductase inhibitors in other human extrahepatic cells in culture

Three other human extrahepatic cells i.e. cornea fibroblasts, retinal pigment epithelial, cells and granulosa cells were incubated with 0.001, 0.01, 0.1, 1 or 10 μM of lovastatin and simvastatin and with 0.01, 0.1, 1, 5 or 10 μM of pravastatin. [^{14}C]-acetate incorporation into sterols was measured (preincubation and incubation period of 30 min and 3 h, respectively). IC_{50}-values of lovastatin, simvastatin and pravastatin for these cell types were calculated from the inhibitor curves obtained from 3 separately performed experiments (curves not shown) and presented in Table III. As for human umbilical vein endothelial cells, it was observed again that pravastatin was inhibiting the sterol synthesis to a much lesser extent in these extrahepatic cell types than in human hepatocytes. Lovastatin and simvastatin IC_{50}-values were in the same order of magnitude.

TABLE III: Inhibition of sterol synthesis by lovastatin, simvastatin and pravastatin in several human extrahepatic cells in culture.

Cells were incubated for 3.5 h with different concentrations of vastatins and [^{14}C]-acetate (5 μCi/well) incorporation into sterols was measured, as described in Materials and Methods. The inhibitory potencies of the three vastatins on the sterol synthesis are expressed as IC_{50}-values (mean ± S.E.). These IC_{50}-values (nM) were calculated from three separately performed experiments.

	IC_{50}-values (nM)		
Cell type	lovastatin	simvastatin	pravastatin
cornea fibroblasts	15.0 ± 4.8	4.6 ± 2.0	1340 ± 595
retinal pigment epithelial cells	17.5 ± 9.1	8.0 ± 2.4	4119 ± 2173
granulosa cells	27.0 ± 15.5	16.3 ± 10.3	1539 ± 779

Association of ^{14}C-labelled vastatins with human hepatocytes and human umbilical vein endothelial cells

The difference in inhibitory potency of the vastatins in human hepatocytes and in extrahepatic cells seems to be dependent on the uptake mechanism present in that particular cell type and on the drug used. Experiments were performed with human umbilical vein endothelial cells (as an example for an extrahepatic cell type) and with human hepatocytes in KR and WE-medium, respectively, to investigate the optimal experimental conditions for uptake of [^{14}C]-simvastatin and [^{14}C]-pravastatin. Cells were incubated for a period of 0-30 min. at 37°C under 5% CO_2/95% air. [^{14}C]-Simvastatin was equally associated to both cell types (Fig. 2A and B). Pravastatin, however, showed hardly any association with endothelial cells (Fig. 2B). In human hepatocytes pravastatin and simvastatin associated to a similar degree (Fig. 2A).

As shown in Fig. 2 association of [^{14}C]-simvastatin was linear up to 7.5 min. in human umbilical vein endothelial cells at the concentration investigated. In the hepatocytes the association of 15 μM of both drugs was linear up to 2 min. An additional experiment with human umbilical vein endothelial cells performed with [^{14}C]-simvastatin and [^{14}C]-pravastatin in a concentration range of 2-200 μM at 37°C showed a linear relationship between [^{14}C]-simvastatin concentration and association (Fig. 3). However, [^{14}C]-pravastatin showed only very little association even at high concentrations.

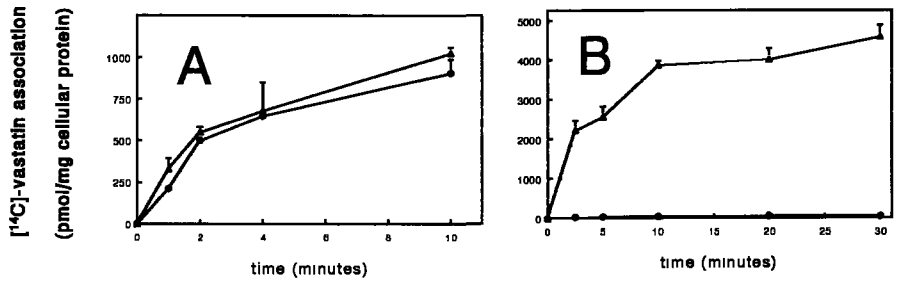

Figure 2: Association of [^{14}C]-simvastatin and [^{14}C]-pravastatin to human hepatocytes and endothelial cells with time.
Human hepatocytes (A) were incubated with 15 μM of [^{14}C]-simvastatin (▲) or [^{14}C]-pravastatin (●) for the indicated times in WE at 37°C. Incubation of endothelial cells (B) was performed for the indicated times at 37°C with 14 μM of [^{14}C]-simvastatin or 19 μM of [^{14}C]-pravastatin in Krebs-Ringers solution. The values are expressed as [^{14}C]-vastatin associated in pmol/mg cellular protein. The bars indicate the range of duplicate measurements.

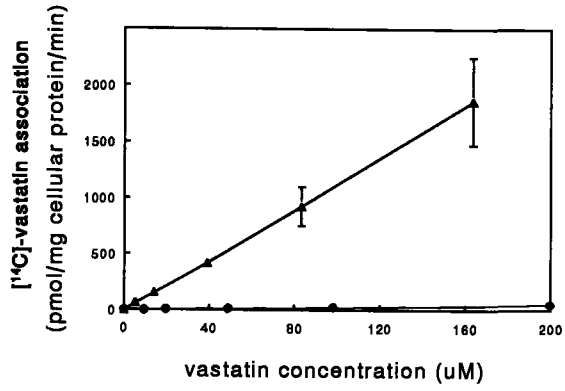

Figure 3: Concentration dependency of [^{14}C]-simvastatin and [^{14}C]-pravastatin association with human endothelial cells in culture.
Human endothelial cells were preincubated for 30 min at 37°C and incubated with the indicated concentrations of [^{14}C]-simvastatin (▲) and [^{14}C]-pravastatin (●) for 7.5 min in KR solution. The bars depict the range of duplicate measurements.

DISCUSSION

In human hepatocytes in primary culture similar inhibition of the sterol synthesis was observed for lovastatin, simvastatin and pravastatin. The sterol synthesis in the human extrahepatic cells investigated was strongly inhibited by lovastatin and simvastatin but to a much lesser extent by pravastatin. This difference between pravastatin on one hand and lovastatin and simvastatin on the other was also observed in human lens in organ culture [11,12]. Corsini et al. [13] also reported distinct inhibitory effects of simvastatin

and pravastatin on the sterol synthesis of human smooth muscle cells in culture. So, these data together confirm the conclusion drawn from animal cell studies that also in human cells pravastatin is more specific in the inhibition of the sterol synthesis in hepatocytes than the other vastatins examined.

IC_{50}-Values increased with longer incubation times. This was previously observed in experiments with Hep G2 cells by Nagata et al. [26]. This phenomena is consistent with the observed increase of mRNA levels of HMG-CoA reductase in endothelial cells at prolonged incubation with lovastatin or simvastatin. At prolonged inhibition of sterol synthesis less regulatory sterols, which suppress HMG-CoA reductase, will be present in the cells. Subsequently, more HMG-CoA reductase mRNA and enzyme will be present and so, higher inhibitor concentrations are necessary in order to reach 50 % of inhibition of sterol synthesis. In agreement with our results Choi et al. showed in baby hamster kidney cells that 25 μM of lovastatin increased HMG-CoA reductase mRNA levels after an incubation of 20 h but not after 3 h [27]. One μM of pravastatin only slightly increased the mRNA levels of HMG-CoA reductase after 20 h. As can be seen in Fig. 1C pravastatin at a concentration of 1 μM was scarcely inhibiting the sterol synthesis after 20 h of incubation. Therefore, in agreement with the above mentioned feedback regulation mechanism hardly any induction of reductase mRNA levels was detected. The difference in upregulation of reductase mRNA levels by lovastatin/simvastatin and pravastatin was also observed in Hep G2 cells incubated for 18 h [14] which supports our observation with human endothelial cells.

The differences between the inhibitors observed in extrahepatic cells compared to hepatocytes can be explained by assuming that a specific carrier for pravastatin, the more hydrophilic one, is present in human hepatocytes. The more lipophilic drugs lovastatin and simvastatin may probably enter the extrahepatic cells by other means. This is supported by the observation that under the conditions used [^{14}C]-pravastatin is hardly associated with human umbilical vein endothelial cells, used as an example of extrahepatic cells. Uptake experiments with another extrahepatic cell type, i.e. human cornea fibroblasts gave the same results (unpublished results). In human hepatocytes [^{14}C]-pravastatin was taken up as well as [^{14}C]-simvastatin. In rat liver, there is evidence that the sodium-independent bile acid uptake and pravastatin transport are possibly mediated by the same transporter [28-31]. Experiments are currently in progress to investigate whether this is also the case for human hepatocytes.

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CHAPTER 4

VASTATINS HAVE A DISTINCT EFFECT ON STEROL SYNTHESIS AND PROGESTERONE SECRETION IN HUMAN GRANULOSA CELLS *IN VITRO*

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SUMMARY

Lovastatin and simvastatin are strong inhibitors of cholesterol synthesis in cultured human granulosa cells, as measured within 6 days after isolation, with IC_{50}-values of respectively 27.0 and 18.2 nM obtained after 3.5 hours of incubation with the drugs. Pravastatin is a much weaker inhibitor of the cholesterol synthesis (IC_{50}-value of 977.8 nM) in these cells. Under these conditions inhibition of cholesterol synthesis had no influence on progesterone secretion into the medium which was probably due to the presence of large cholesterol pools in the cells. To decrease these pools, granulosa cells were cultured for 7 days after which the culture medium was changed to a medium supplemented with 20% lipoprotein depleted serum to deprive the cells of exogenous cholesterol. Additionally, 30 mIU of follicle stimulating hormone and luteinizing hormone per ml were added to increase the progesterone production and secretion, thereby decreasing the cholesteryl ester pools. After 48 hours of incubation, culture was continued without hormones for another two days. Thereafter, the cells were preincubated for 24 hours without or with 1 μM of lovastatin, simvastatin or pravastatin in medium containing lipoprotein deficient serum and the above-mentioned hormones and incubated for another 24 hours in the presence of [^{14}C]-acetate after which cells and media were collected for determination of [^{14}C]-sterols synthesized and progesterone secreted into the media. Now, lovastatin and simvastatin, which strongly inhibited sterol synthesis, had a significantly decreasing effect on the secretion of progesterone. One μM of pravastatin had no significant effect on sterol synthesis nor on progesterone secretion. When the latter experiment was performed under conditions where exogenous cholesterol was provided in the form of human low density lipoproteins no influence on progesterone secretion was observed. So under conditions where the cholesterol pools were decreased, lovastatin and simvastatin had a decreasing influence on the progesterone secretion, while pravastatin had not. When pools were filled by exogenous cholesterol no effect on progesterone secretion by either of the drugs was observed.

INTRODUCTION

Human granulosa cells are located in the Graafian follicle in the ovary. These cells surround the ovum (corona radiata) and the inner lining of the follicle. Before ovulation, cells are in an avascular environment. That means that for their cholesterol demand these cells are deprived of circulating low density lipoproteins (LDL) and are dependent on *de novo* synthesis of cholesterol (1). Besides that, high density lipoprotein in the follicular fluid might be an exogenous cholesterol source (2,3). Large amounts of progesterone, a steroid hormone of which cholesterol is the precursor, have been detected in the follicular fluid prior to ovulation (1). It might be that under these avascular circumstances the *de novo* cholesterol synthesis plays an essential role in the production of progesterone. After ovulation, vascularization of the corpus luteum provides the means by which LDL is delivered to luteinized granulosa cells, thereby allowing progesterone synthesis from exogenous cholesterol (4).

Lovastatin (5), simvastatin (6) and pravastatin (7) are hypocholesterolaemic drugs. They are competitive inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A

(HMG-CoA) reductase, a crucial enzyme in the cholesterol metabolism. By inhibiting this enzyme in the liver, less cholesterol is synthesized and accordingly more LDL-receptors are induced, resulting in the withdrawal of LDL-cholesterol from the circulation. These three drugs are equally strong inhibitors of the cholesterol synthesis in the liver. However, in extrahepatic cells the more lipophilic compounds lovastatin and simvastatin are much stronger inhibitors of the sterol synthesis than pravastatin, the more hydrophilic one (7-12).
Their differences in inhibition of sterol synthesis in human granulosa cells as observed *in vitro* (12), may lead to a difference in progesterone secretion. Therefore, the influence of the inhibition of *de novo* cholesterol synthesis by the three vastatins on the secretion of progesterone in human granulosa cells in culture was investigated.

MATERIALS AND METHODS

Hormones and drugs

Luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were purchased at Sigma. Lovastatin, simvastatin and pravastatin were kindly provided by Sankyo Co. (Tokyo, Japan). Sodium salts of the vastatins were used and stock solutions were made in ethanol.

Preparation of serum and lipoproteins

Human serum was prepared from pooled blood, collected from healthy donors. Human lipoprotein depleted serum (LPDS) and LDL were prepared as described by Havekes et al. (13) and Redgrave et al. (14), respectively.

Isolation and culture of human granulosa cells from Graafian follicles

Granulosa cells were harvested from pre-ovulatory follicles of hormone-treated women, undergoing ovum retrieval for in vitro fertilisation and embryo transfer. Cells were isolated and cultured as previously described (12). Viability was measured by the trypan blue exclusion method. Cells were cultured in RPMI 1640 medium containing 4 mM L-glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 10% human serum and 10% newborn calf serum (Gibco, heat inactivated) at 37°C in an atmosphere of humidified air (5% CO_2/95% air). Initial experiments were performed within 3-6 days after isolation. After 7 days additional experiments were performed. Cells were cultured in the same medium supplemented with serum without lipoproteins (LPDS) and with the addition of 30 mIU of luteinizing hormone (LH) and 30 mIU of follicle stimulating hormone (FSH) per ml to lower the endogenous cholesterol pools. Cells were cultured with this medium for a 48 hours period. Subsequently, this medium was replaced by the same medium without LH and FSH and incubation proceeded for 48 hours.

Hereafter cells were preincubated for 24 hours with or without 1 μM of the vastatins in the presence of 30 mIU of LH/FSH and 20% LPDS in RPMI 1640 medium. Then the medium was replaced by the same medium containing 5 μCi of [^{14}C]-acetate

per well and the cells were further incubated for a period of 24 hours. Cells and media were collected for determining cholesterol synthesis and progesterone secretion respectively. Additional experiments were performed with cells treated similarly as described above, but this time in the presence of human LDL (100 μg of protein/ml) from day 7 onwards.

Determination of inhibition of sterol synthesis

After culturing for 3-6 days, granulosa cells at near confluence were incubated for 3.5 hours in RPMI medium containing 20% of LPDS, in the absence or presence of 0.001, 0.01, 0.1, 1, or 10 μM of lovastatin or simvastatin; for pravastatin the concentrations of 0.01, 0.1, 1, 5 and 10 μM were used. After a 30 minutes preincubation, 5 μCi [2-^{14}C]-acetate (spec. radioactivity of 56.2 mCi/mmol, Amersham) was added to each well (2.5 cm^2) containing 0.5 ml medium. [^{14}C]-acetate incorporation into sterols was measured after 3 hours as previously described (15). Non-saponifiable lipids were separated by thin layer chromatography according to Boogaard et al. (16) using TLC system I. Sterol bands were scraped and quantified. IC_{50}-values were calculated by curve fitting through all datapoints using a dose-response equation and expressed as mean $\pm$ S.E.M. (n=3).

Sterol synthesis in granulosa cells was also measured after a 24 hours incubation period with [^{14}C]-acetate after pretreatment with LPDS and LH/FSH as described above. Additionally, in a similar experiment, but this time in the absence or presence of 100 μg of LDL/ml, incorporation of [^{14}C]-acetate into non-saponifiable lipids was determined as well. Media were stored at -20°C, for further analysis of the progesterone secreted. Protein was determined in cell lysate samples according to Lowry et al. (17).

Progesterone measurement

Total amounts of secreted progesterone in the culture media were measured with the aid of a "coat a count" kit (RIA) from the Diagnostic Products Corporation (DPC) according to the instructions of the manufacturer.

Measurements of intracellular triglycerides (TG), free cholesterol (FC) and cholesteryl ester (CE) content

Granulosa cells cultured as described above in the presence of LPDS and LH/FSH were harvested on the last day of incubation with or without 1 μM of the three vastatins and in the absence or presence of LDL (100 μg of protein/ml). Media were aspirated and stored at -20°C for progesterone determination. Cells were washed three times with phosphate-buffered saline, harvested by scraping with a rubber policeman and resuspended by trituration with a syringe. Samples were taken for determining the protein content (17). Lipids were extracted and TG, FC and CE were quantified as descibed by Havekes et al. (18).

Statistical analysis
The Mann-Whitney test was used to determine statistical significance of the values obtained.

RESULTS

Effect of vastatins on sterol synthesis and progesterone secretion shortly after isolation
The first experiments were performed using cells within 3-6 days after isolation. Human granulosa cells were preincubated for 30 minutes with different concentrations of lovastatin, simvastatin and pravastatin. After this preincubation cells were further incubated for 3 hours in the presence of [^{14}C]-acetate and subsequently incorporation of ^{14}C-label into sterols was measured. The inhibitor concentration dependent curves are presented in Fig.1.

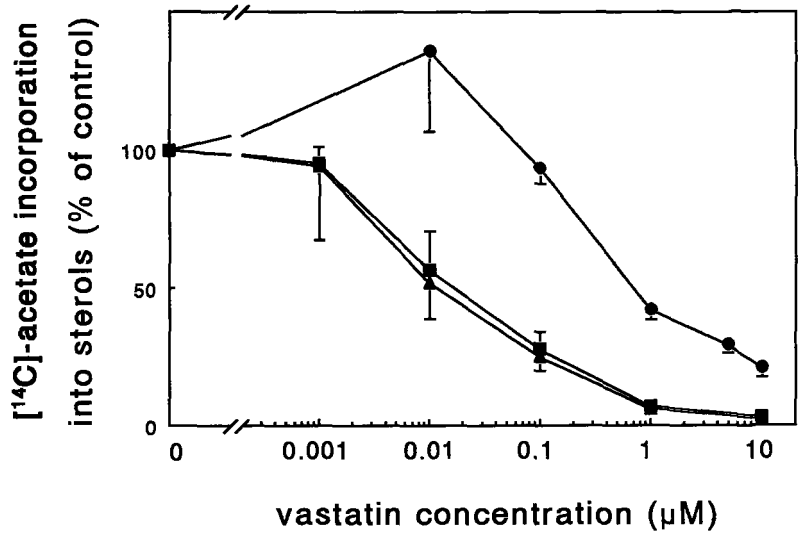

Figure 1: Inhibition of sterol synthesis in human granulosa cells by lovastatin, simvastatin and pravastatin
Synthesis was measured by incorporation of [^{14}C]-acetate into sterols and expressed as percentage of control (see Materials and Methods). The experimental conditions used were: a preincubation of 30-min with the indicated drug concentrations followed by an incubation for 3 hours in the presence of 5 μCi [^{14}C]-acetate per well. The acid forms of lovastatin (■), simvastatin (▲) and pravastatin (●) were used. The bars represent S.E.M. of three separately performed experiments (if not shown, the bars coincide with the symbols). The control mean value for [^{14}C]-acetate incorporation into sterols was 76980 ± 236791 dpm per mg of cellular protein.

The inhibition by pravastatin is much less than that of lovastatin and simvastatin. The latter two have about the same inhibiting capacity in human granulosa cells as demonstrated by the IC_{50}-values, the drug concentration at which 50% inhibition of the sterol synthesis occurs. These values were calculated from three separately obtained

curves with the aid of curve fitting through all datapoints using a dose-response equation and are presented in Table I. In the media, collected after 3.5 hours of incubation, progesterone levels were measured to see whether the vastatins had an effect on the progesterone secretion. The mean control values (± range) from two separately performed experiments were 2900 ± 750 ng of progesterone per mg of cellular protein. In the presence of 10 μM of lovastatin, simvastatin or pravastatin the mean progesterone levels (± range) were 3400 ± 300, 2680 ± 600 and 3400 ± 450 ng per mg cellular protein respectively. So no effect on the progesterone secretion was observed under these conditions at any concentration of the vastatins used (further data not shown).

TABLE I: IC_{50}-values of lovastatin, simvastatin and pravastatin for inhibition of sterol synthesis in human granulosa cells in culture

Cells were incubated for 3.5 hours with 0.001-10 μM of vastatin and [^{14}C]-acetate incorporation into sterols was measured as mentioned in Materials and Methods. The drug concentration at which 50% inhibition of the sterol synthesis occurs was calculated from three separately performed experiments with the aid of a curve-fitting programme applying a dose-response equation. The composite curves are depicted in Fig. 1.

	IC_{50}-values (nM) (mean ± S.E.M.)
lovastatin	27.0 ± 15.5
simvastatin	18.2 ± 12.2
pravastatin	977.8 ± 280.7

Effect of lovastatin, simvastatin or pravastatin on the sterol synthesis and progesterone secretion after prior depletion of sterol pools

The fact that the inhibition of sterol synthesis by vastatins was not reflected in an effect on progesterone secretion was probably due to high endogenous cholesterol pools present within these cells. This can be deduced from the high level of progesterone secreted. This situation is probably the result of the response of the patient's ovaries to hormonal pretreatment used to induce follicular growth. Therefore, under these conditions a decrease of the *de novo* cholesterol synthesis by the vastatins will not affect the progesterone secretion.

So, it is feasible that progesterone synthesis will be affected when endogenous cholesterol pools are small. This condition can be realized by treating the cells with LH/FSH to stimulate progesterone synthesis and secretion (19), thus lowering endogenous cholesterol, in the presence of LPDS instead of serum to deprive the cells of exogenous cholesterol. After culturing the cells for 7 days in medium containing human serum and new born calf serum, this medium was replaced by medium, supplemented with LPDS, and containing 30 mIU of LH/FSH per ml. The cells were cultured for 48 hours and then for further 48 hours without LH/FSH. Then, cells were preincubated with or without 1 μM of the three vastatins in the presence of LH/FSH for 24 hours. In order to determine sterol synthesis this preincubation was followed by an incubation lasting 24 hours with renewed medium containing 5 μCi of [^{14}C]-acetate per

well. Thereafter, the media were collected to measure progesterone secretion during the last 24 hours. The effect of the vastatins on the sterol synthesis after this treatment with LPDS and LH/FSH is shown in Table II. Both, lovastatin and simvastatin inhibited the sterol synthesis markedly. Pravastatin did not inhibit the sterol synthesis significantly. The values for the progesterone levels in the medium are also presented in Table II. One μM of lovastatin and simvastatin a significantly reduced progesterone secretion. A minor decrease by 1 μM of pravastatin was not significantly different from the control value.

TABLE II: Effect of vastatins on sterol synthesis and progesterone secretion in human granulosa cells after decrease of sterol pools

The cholesterol content of cells was decreased by incubation with LH/FSH and LPDS as described in the Materials & Methods section. After 48 hours of incubation with 1 μM of lovastatin, simvastatin or pravastatin, sterol synthesis, during the last 24 hours, was determined by measuring the incorporation of [^{14}C]-acetate into sterols and expressed as percentage of control. Control values (mean ± S.E.M.) of separately performed experiments were 95239 ± 12318, 672742 ± 96403, 173594 ± 11164, 3722307 ± 75785 and 6185727 ± 571492 dpm/mg of cellular protein. Values presented are mean ± S.E.M. (n = 4 to 5). Progesterone secreted in the media during the last 24 hours was measured. Mean values ± S.E.M. from 4 separately performed experiments are shown. The mean control value (mean ± S.E.M.; n = 4) was 468 ± 177 ng progesterone/mg of cellular protein.

	[^{14}C]-acetate incorporation sterols (% of control)	progesterone secreted (% of control)
control	100	100
lovastatin	34.4 ± 3.7*	66.4 ± 11.3 **
simvastatin	14.2 ± 2.0*	68.4 ± 5.3 **
pravastatin	80.2 ± 19.5	80.2 ± 15.5

*P < 0.01 compared with control.
**P < 0.05 compared with control.

Effect of exogenous cholesterol on the progesterone secretion in granulosa cells in the presence of HMG-CoA reductase inhibitors

In order to confirm that the observed decrease in progesterone secretion by lovastatin and simvastatin was dependent on the decrease of endogenous cholesterol, the same experiment was performed, but now in the presence of exogenous cholesterol in the form of human LDL. Granulosa cells were cultured as previously described (starting the incubation with LPDS and LH/FSH on day 7) with and without the addition of 100 μg of LDL/ml to the medium from day 7 until day 13. Cells were incubated for the last 48 hours with 1 μM of vastatin and the media from the last 24 hours of incubation were collected. Cholesteryl esters (CE), free cholesterol (FC) and triglyceride (TG) levels were measured in the cell lysate to see whether LDL had been taken up by these cells. In the media the progesterone levels were determined. As shown in Table III, the addition of LDL increased the CE content of the cells, whereas the TG levels remained the same. In combination with lovastatin and simvastatin the FC content increased

significantly. LDL stimulated progesterone secretion by a factor of two. In the presence of LDL the vastatins did not affect progesterone secretion, supporting the view that under conditions in which cholesterol pools had been filled, inhibition of sterol synthesis had no effect on progesterone secretion.

TABLE III: The effect of vastatins on progesterone secretion into the media in the presence of LDL; cellular contents of triglycerides (TG), free cholesterol (FC) and cholesteryl esters (CE)

Granulosa cells were incubated in the presence or absence of LDL (100 μg of protein/ml) for 144 hours and with and without 1 μM of the vastatins for the last 48 hours in medium supplemented with LPDS and LH/FSH as decribed under Materials and Methods. Progesterone secretion into the media for the last 24 hours and FG, FC and CE contents of the cells were measured. The control values (without the addition of LDL) for TG, FC and CE were 10.3 $\pm$ 0.3, 18.6 $\pm$ 0.7 and 37.5 $\pm$ 7.2 μg/mg of cellular protein, respectively. The control value for progesterone secreted into the media was 609.8 $\pm$ 122.3 ng progesterone/mg cellular protein. The mean of the percentages of control values of the incubations without LDL $\pm$ S.E.M. (n = 3 to 4) are shown.

	progesterone secreted	TG	FC	CE
		contents		
control - LDL	100	100	100	100
control + LDL	204.9 $\pm$ 31.2*	88.0 $\pm$ 10.8	106.5 $\pm$ 6.4	172.6 $\pm$ 13.8*
lovastatin + LDL	210.7 $\pm$ 23.6*	96.8 $\pm$ 8.2	132.8 $\pm$ 7.4*$^{+}$	191.9 $\pm$ 10.2*$^{+}$
simvastatin + LDL	248.5 $\pm$ 19.5*	115.2 $\pm$ 10.5	160.8 $\pm$ 10.1*$^{+}$	192.4 $\pm$ 21.3*$^{+}$
pravastatin + LDL	199.9 $\pm$ 21.3*	111.9 $\pm$ 16.9	114.3 $\pm$ 5.7	152.9 $\pm$ 21.3*

* $P < 0.05$ compared with control - LDL.
$^{+}$ $P < 0.05$ compared with control + LDL.

DISCUSSION

Within 3-6 days after isolation granulosa cells secreted high amounts of progesterone (2900 ng per mg of cellular protein/3.5 h) into the media, even in the presence of lovastatin, simvastatin and pravastatin up to a concentration of 10 μM. At this concentration sterol synthesis was strongly inhibited (Fig.1.). Similar observations were made by Tureck et al. (20). They observed a 90% inhibition of [*U*-^{14}C]-acetate incorporation into sterols by 20 μM of compactine, but no significant alteration in progesterone secretion. The high secretion of progesterone into the media suggests that these cells possess a high endogenous cholesterol pool. The same observation was done by Soto et al. (21) and by Voutilainen et al. (22). Granulosa cells were harvested from the pre-ovulatory follicles of hormone-treated women. This treatment was probably the cause of large cholesterol pools and high progesterone secretion. So it is possible that the vastatins had no effect on the progesterone secretion under these conditions due to the pretreatment of the granulosa cells before harvest.
In order to decrease endogenous cholesterol levels, granulosa cells were cultured in LPDS to exclude an exogenous cholesterol pool and were treated additionally with

LH/FSH to enhance progesterone synthesis and secretion. In conditions where endogenous pools are small, progesterone production should depend more on *de novo* cholesterol synthesis. Indeed, under these conditions lovastatin and simvastatin at a concentration of 1 μM attenuated sterol synthesis and progesterone secretion significantly. Pravastatin did not effect sterol synthesis nor progesterone secretion. In comparison, Rosenblum et al. observed in rat granulosa cells cultured in LPDS in the presence of 25 μM of compactin, a strong reduction in sterol synthesis, and also a decrease in steroid secretion (23). The conclusion that the endogenous cholesterol pools were lowered by the above-mentioned treatment was supported by the low progesterone levels secreted in the control incubations (Table II: 20 ng per mg of protein/h and in Table III: 25 ng per mg of protein/h) compared to those values observed in the initial experiments (830 ng per mg of protein/h).

The extent of sterol synthesis inhibition by the three drugs at a concentration of 1 μM after 48 hours of incubation (Table II) was smaller than the values obtained after an incubation for 3.5 hours (Fig.1.). This observation is consistent with results previously reported, which showed that longer incubation with vastatins resulted in higher IC_{50}-values (12).

To support further the thesis that inhibition of progesterone secretion by simvastatin and lovastatin was dependent on the size of the endogenous cholesterol pools, experiments were performed in the presence of exogenous cholesterol. Addition of LDL resulted in a 1.5 to 2 fold increase in CE content of the cells and concomitantly, in progesterone levels in the media which were two fold higher than those obtained without LDL. Under these conditions progesterone secretion was not influenced by 1 μM of the three drugs. In the presence of LDL the *de novo* cholesterol synthesis was markedly repressed. One μM of lovastatin or simvastatin, repressed cholesterol synthesis even more, Pravastatin was less potent in this respect (data not shown). This difference in inhibition was also reflected in the FC content of the cells. In the presence of LDL the FC content was significantly increased by lovastatin or simvastatin, but not by 1 μM of pravastatin. This can be explained by assuming that the stronger inhibition of sterol synthesis will lead to a higher upregulation of LDL-receptor activity (24). This will result in a higher uptake of exogenous LDL, and therefore, endogenous cholesterol levels will be increased.

We conclude that, when exogenous cholesterol is available, the *de novo* cholesterol synthesis seems to play a minor role in the secretion of progesterone. This situation resembles the *in vivo* situation after vascularization of the granulosa cells in the corpus luteum. In circumstances in which endogenous cholesterol pools are small, inhibition of cholesterol synthesis by lovastatin and simvastatin impaires progesterone secretion. Under the conditions used, pravastatin did not affect sterol synthesis nor progesterone secretion. Under conditions in which no or limited quantities of exogenous cholesterol are available, such as in the avascular situation prior to ovulation, inhibition of *de novo* cholesterol synthesis will affect the synthesis and secretion of progesterone.

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CHAPTER 5

COMPARISON OF THREE VASTATINS IN THEIR EFFECT ON CELL PROLIFERATION, VIABILITY AND DNA-SYNTHESIS IN HUMAN SMOOTH MUSCLE CELLS, ENDOTHELIAL CELLS AND FIBROBLASTS *IN VITRO*.

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SUMMARY

Simvastatin and lovastatin inhibited equally strongly cholesterol synthesis in cultured human smooth muscle cells from mammary artery whereas pravastatin was a much weaker inhibitor. Since 3-hydroxy-3-methyl glutaryl coenzyme A reductase inhibitors induce cellular mevalonate depletion, we investigated the anti-proliferative effects of these drugs in cornea fibroblasts, endothelial cells from umbilical vein and smooth muscle cells growing in culture. Pravastatin had no effect on proliferation (cell number), viability (MTT assay) and DNA synthesis ([^{3}H]-thymidine incorporation assay) in fibroblasts and endothelial cells up to the highest concentration used (1μM), after a 3-day incubation period. In smooth muscle cells, pravastatin inhibited proliferation after a 6-day incubation period (25% at 1 μM), but not after a 3-day incubation period. Simvastatin and lovastatin were more potent inhibitors of cell proliferation than pravastatin in the three cell types. At 1 μM concentration, the reduction in cell number was 20, 20 and 50% for lovastatin and 40, 30 and 55% for simvastatin in smooth muscle cells, fibroblasts and endothelial cells respectively, after a 3-day incubation period. Cell viability was maintained at this drug concentration. DNA synthesis was strongly inhibited by 1 μM of lovastatin and simvastatin in endothelial cells (95% inhibition). In contrast, an increase in DNA synthesis was observed in fibroblasts (70% stimulation), whereas mixed inhibition and stimulation of DNA synthesis occurred in smooth muscle cells at this concentration of the drugs. Additionally, it was observed that a change in culture conditions influenced the degree of inhibition of endothelial cell proliferation. The effect of the vastatins on cell growth and DNA synthesis was reversed by mevalonate and not by cholesterol, supporting the view that products of mevalonate are involved in this process.

INTRODUCTION

Pravastatin (1), simvastatin (2) and lovastatin (3) specifically and strongly inhibit 3-hydroxy-3-methyl glutaryl coenzyme A (HMG-CoA) reductase, a rate-limiting enzyme for the cholesterol biosynthesis. These drugs are currently used for the prevention and treatment of atherosclerosis. Several studies have shown that vastatin treatment in humans results in a reduction in the severity of arterial lesions and cardiovascular diseases (4-7). Mechanisms for prevention of restenosis by these drugs include a decrease in the serum cholesterol level but possibly also an inhibition of smooth muscle cell proliferation. Indeed, vastatins display inhibitory effects on cell growth *in vitro* which have been linked to their potency in lowering the cellular content of mevalonate, an intermediate of the cholesterol biosynthesis pathway. Certain classes of proteins are post-translationally modified by the covalent attachment of the mevalonate-derived isoprene group, farnesyl or geranylgeranyl (8-10). They include growth regulatory proteins as ras (11, 12) as well as heterotrimeric and other low molecular weight guanine-nucleotide binding proteins and nuclear lamins (13). In that respect, the control of vascular smooth muscle cell proliferation is important in preventing the formation of intimal hyperplasia in atherosclerosis (14, 15). On the other hand, inhibition of cell proliferation by vastatins is not desirable in other cell types, i.e. endothelial cells, since

cell replication is necessary for tissue repair at places of endothelial denudation in the arterial wall.

Although pravastatin, lovastatin and simvastatin are equally strong inhibitors of sterol synthesis in human hepatocytes, pravastatin exhibits a much less pronounced inhibitory activity in cultured human cells from non-hepatic tissues (16). Since the growth inhibitory activity of the HMG-CoA reductase inhibitors is related to their interference in the mevalonate synthetic pathway (17, 18), we investigated the effect of these three HMG-CoA reductase inhibitors on proliferation in cultured human fibroblasts, smooth muscle cells and endothelial cells. Our data provide evidence that, pravastatin on one hand and simvastatin and lovastatin on the other hand, exhibit differences in their growth inhibitory capacity as well as in toxicity levels within one cell type. We also observed that each drug inhibits cell proliferation to various degrees in the cell types analyzed and that this effect is dependent on culture conditions. In addition, our results confirm that cell proliferation inhibition by these drugs is dependent on cellular mevalonate depletion.

MATERIALS AND METHODS

Lovastatin, simvastatin and pravastatin (all sodium salts) were kindly donated by Sankyo Co. (Tokyo, Japan). 10 mM stock solutions were made in 100% ethanol and kept at -20°C.

Cell culture

Human smooth muscle cells (HSMC) from left internal mammary artery (LIMA)
Samples of LIMA were obtained from patients undergoing coronary artery bypass surgery (Academic Hospital Leiden, The Netherlands). Vascular specimens, discarded after the operation, were rinsed with 0.9% saline and placed in DMEM supplemented with 10% foetal calf serum (FCS), 20 mM HEPES, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamin (DMEM/10% FCS). Adhering fat and connective tissue were discarded. The adventitia was carefully dissected and the inner surface was scraped to remove endothelial cells. After mincing the medial preparations into 1-2 mm pieces, they were spread out on the surface of a 24-wells culture plate. After incubating the specimens at 37°C for 30 min in a humidified atmosphere with 5% CO_2 to allow their attachment to the plastic of the wells, the vascular pieces were covered with fresh medium and cultured further in DMEM/10% FCS or the same medium additionally supplemented with 10% human serum (HS) (DMEM/10% FCS, 10% HS). After 1-3 weeks, cells began to migrate from the explants. Cells were passaged into tissue culture flasks with a split ratio of 1:3. HSMC cultures were used within 5 passages in this study. Cells cultured with DMEM/10% FCS had a doubling time of 3 - 4 days. Cells cultured with DMEM/10% FCS/ 10% HS had a doubling time of 1.5 - 2 days.

Human cornea fibroblasts (HCF)
Pieces of cornea grafts and HCF were kindly provided by Dr. E. Pels of the Cornea Bank (Amsterdam). HCF were isolated as described by Hoppenreijs et al. (19). Cells were grown in MEM supplemented with 10% FCS, 20 mM HEPES, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine. Under these culture conditions, the cell doubling time was 2-3 days.

Human umbilical vein endothelial cells (HUVEC)
These cells were isolated from human umbilical cords as described by Jaffe et al. (20) and cultured as described by Van Hinsbergh et.al.(21). Cells were grown in either

M199 supplemented with 10% HS, 10% newborn calf serum (NBCS), 20 mM HEPES, 150 μg/ml endothelial cell growth factor, 5 IU/ml heparin, 100 IU/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine or the same medium in which NBCS and HS were replaced by 20% FCS. Cells cultured with the medium containing 10% NBCS and 10% HS had a doubling time of 3.5-4 days. Cells cultured with medium supplemented with 20% FCS had a doubling time of 1-1.8 days.

Drug incubation and determination of cell proliferation
The experimental conditions were adapted to the type of cell studied (media, length of incubation, initial seeding density of the cells). Under these conditions, the cells grew in a logarithmic growth phase and reached near-confluency at the end of the incubation period, in control conditions. After trypsinization and seeding in the plates, cells were allowed to settle 1-3 days in culture and subsequently incubated in the presence or the absence of the different concentrations of vastatins for 3 days (HCF, HUVEC and HSMC) or 6 days (HSMC) in their respective culture medium (see legends of the figures). At the end of the incubation period, cells were trypsinized and the total number of cells per well was counted with a Neubauer haemocytometer in duplicate chambers.

Determination of cellular mitochondrial dehydrogenase (MD) activity (MTT test)
At the end of the incubation period, a modified fluorometric assay (22) was performed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide salt (MTT) (Sigma, St. Louis, USA) to determine quantitatively the MD activity of the cells (viability test). The optical density (O.D.) of the emission signal was measured in duplicate in an ELISA plate reader (Titertek Multi 100-MCC 340) at 540 nm and 690 nm, the latter being substracted as the background signal and the reaction solvent being used as a blank.

Determination of cellular DNA synthesis activity
During the last 7-8 h of the incubation period, cells were incubated in the presence of [^{3}H]-thymidine (specific radioactivity 70-86 Ci/mmol, Amersham, 1 μCi/ml). After

rinsing successively with phosphate buffered saline (PBS), 10% trichloroacetic acid (TCA), and PBS, the radiolabelled DNA precipitated by TCA was solubilized in 0.3 N NaOH, counted for ^{3}H-activity in a TRICARB liquid scintillation analyzer (Packard), and the amount of [^{3}H]-thymidine incorporated into newly synthesized DNA was calculated.

Determination of sterol synthesis

This assay was performed as described previously (23). Essentially, cells were preincubated for 30 min in culture medium containing 10% human lipoprotein deficient serum in the absence or presence of the vastatins at the indicated concentrations (see legend of Fig. 4) and further incubated with [2-^{14}C]-acetate (spec. radioactivity of 52 mCi/mmol, Amersham, 5 μCi/ml) for 3 hours. Non-saponifiable lipids were separated by thin layer chromatography according to Boogaard et al. (24). Protein quantities were determined according to Lowry et al. (25). Values of sterol synthesis were calculated as ^{14}C-dpm incorporated into sterols/mg cellular protein. Curve fitting through all data points using a dose-response equation was performed to calculate the IC_{50}-value for each vastatin in each separately performed experiment.

RESULTS

Human cornea fibroblasts (HCF)

Drug toxicity

Pilot experiments showed that, under the experimental conditions used, concentrations lower than 0.1 μM had no effects on HCF proliferation for the three drugs tested (results not shown). On the other hand, at concentrations of simvastatin or lovastatin equal or higher than 5 μM, a significant number of floating cells was observed in the culture dish at the end of the 3-day incubation period. This effect was not observed with pravastatin. At 5 and 10 μM of simvastatin, the number of attached cells was decreased to 26 and 12% of control, respectively, whereas the total number of floating cells represented 9 and 30% of the total cell number in control incubations, respectively. Among these floating cells, the number of non-viable cells (as determined by trypan blue staining), at 5 and 10 μM of simvastatin, represented 5 and 23% of the total cell number in control incubations, respectively. The effect of 10 μM of simvastatin could not be reversed by supplementing the cells with exogenous human LDL at concentrations up to 50 μg/ml and, therefore, is probably not a consequence of cholesterol depletion in the culture medium.

In accordance with these results, only two drug concentrations; 0.1 and 1 μM, were tested for their effect on proliferation of HCF, HUVEC and HSMC.

Drug effect on proliferation, MD activity and DNA synthesis

Cell proliferation of HCF was inhibited after three days of incubation with 1 μM of

lovastatin or simvastatin (25% inhibition) but not pravastatin (Fig. 1A). Measurement of the MD activity showed a parallel variation with cell proliferation (Fig. 1B), indicating that the average MD activity per cell was not affected by the drug incubation. However, [^{3}H]-thymidine incorporation markedly increased at the end of 3-day incubation with 1 μM of lovastatin and simvastatin (Fig. 1C). No change in DNA synthesis was observed with pravastatin, at any concentration (Fig. 1C).

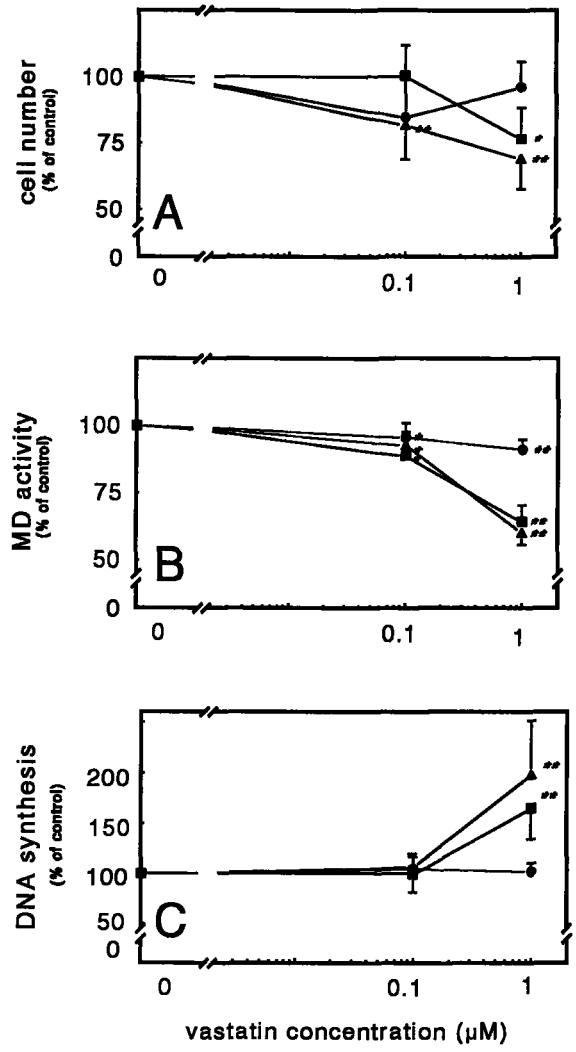

Figure 1: Effect of pravastatin (●), lovastatin (■) and simvastatin (▲) on proliferation (A), MD activity (B) and DNA synthesis (C) of HCF during a 3-day incubation period.

After seeding, cells were incubated with MEM containing 10% FCS; 24 h later the medium was replaced by fresh medium containing the indicated concentrations of drugs and the incubation continued for 3 days. Each value (mean ± S.D. of 3-4 experiments) is expressed as mean percentage of the control value (no drug): A, 53231 ± 6668 cells/well; B, 0.394 ± 0.07 O.D. units/well; C, 7135 ± 6984 dpm [^{3}H]-thymidine incorporated into DNA/well. Each individual experiment was performed in triplicate.

*, $p \leq 0.05$; **, $p \leq 0.005$.

Human umbilical vein endothelial cells (HUVEC)

Drug effect on proliferation, MD activity and DNA synthesis in the presence of FCS
After three days of incubation, a 50% decrease in the number of cells compared to the control was observed for lovastatin and simvastatin at 1 μM concentration (Fig. 2A). This growth inhibitory effect was confirmed by the same decrease in MD activity (Fig. 2B). DNA synthesis measured during the last 8 hours of incubation was also inhibited by these two drugs at 1 μM concentration (Fig. 2C), but the magnitude of the effect (98% inhibition) was much larger than the one measured in the two other determinations. No or minor effects on proliferation, MD activity or DNA synthesis were observed with pravastatin.

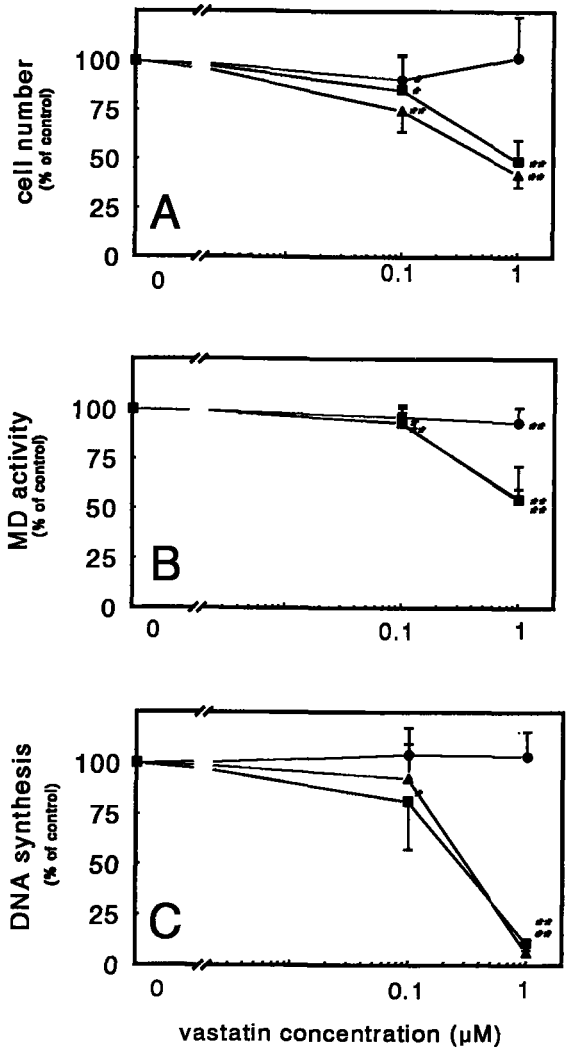

Figure 2: Effect of pravastatin (●), lovastatin (■) and simvastatin (▲) on proliferation (A), MD activity (B) and DNA synthesis (C) of HUVEC cultured in the presence of FCS, during a 3-day incubation period.
After seeding, cells were incubated with M199 containing 20% FCS and 150 μg/ml ECGF; 1-3 days later the medium was replaced by fresh medium containing the indicated concentrations of drugs and the cells were further incubated for 3 days. Each value (mean ± S.D. of 3-4 experiments) is expressed as mean percentage of the control value (no drug): A, 221958 ± 89953 cells/well; B, 0.307 ± 0.09 O.D. units/well; C, 155030 ± 31527 dpm [^{3}H]-thymidine incorporated into DNA/well). Each individual experiment was performed in triplicate. *, $p \leq 0.05$; **, $p \leq 0.005$.

Drug effect on proliferation, MD activity and DNA synthesis in the presence of HS and NBCS

In order to determine the influence of culture conditions on the vastatin effects, the same experiment was performed on HUVEC grown in medium supplemented with 10% HS and 10% NBCS instead of 20% FCS. Using this culture medium, cell growth was slower than under culture conditions where only FCS was present (3.5-4 days versus 1-1.8 days doubling time). Under these experimental conditions, the number of cells measured after three days of incubation was not significantly different from control values for lovastatin and pravastatin, at concentrations up to 1 μM (Fig. 3A) whereas 1 μM of simvastatin reduced the cell number by 20% to a significant extent ($P < 0.05$). MD activity was inhibited by 20 and 33% at 1 μM of simvastatin and lovastatin, respectively. Pravastatin had no effect (Fig. 3B).

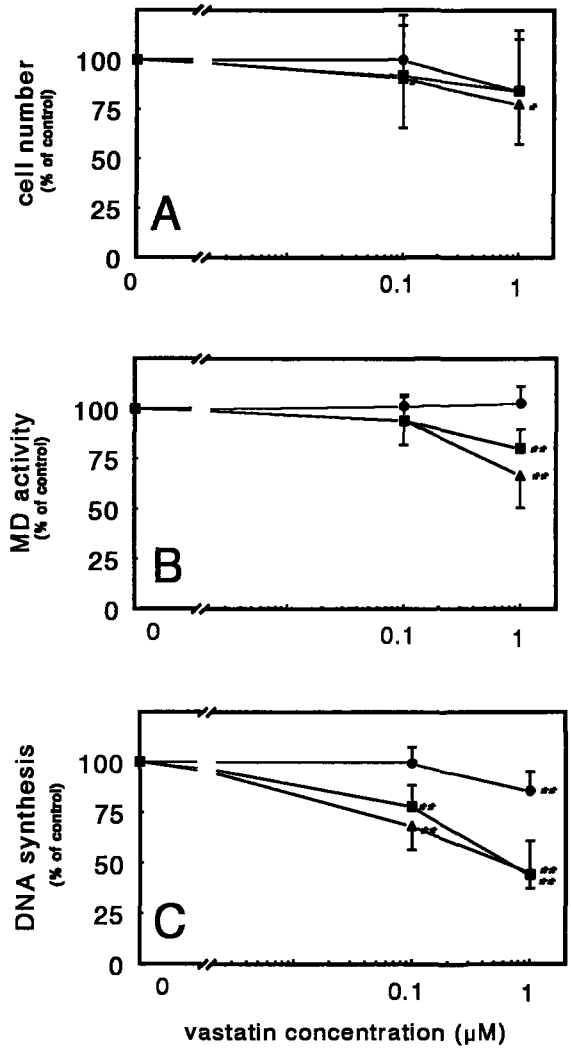

Figure 3: Effect of pravastatin (•), lovastatin (■) and simvastatin (▲) on proliferation (A), MD activity (B) and DNA synthesis (C) of HUVEC cultured in the presence of HS and NBSC, during a 3-day incubation period.

Cells were processed as described in the legend of Fig.2 except that the medium contained 10% HS and 10% NBCS instead of 20% FCS. Each value (mean ± S.D. of 3-4 experiments) is expressed as mean percentage of the control value (no drug): A, 118754 ± 54981 cells/well; B, 0.192 ± 0.195 O.D. units/well; C, 276671 ± 106185 dpm [^{3}H]-thymidine incorporated into DNA/well. Each individual experiment was performed in triplicate.*, $p \leq 0.05$; **, $p \leq 0.005$.

In contrast to these results, simvastatin and lovastatin induced a decrease in DNA synthesis at concentrations as low as 0.1 μM and up to 1 μM (50% inhibition). This effect was much larger than the one observed on cell number and MD activity. Pravastatin had a small but significant effect ($p < 0.05$) on the latter parameter at 1 μM (Fig. 3C).

These results show the same trend in the inhibitory potencies of lovastatin and simvastatin as those obtained in HUVEC grown in medium supplemented only with FCS, except that the extent of inhibition was smaller in the cells cultured in presence of HS and NBCS.

Human smooth muscle cells (HSMC) from LIMA

Inhibition of sterol synthesis

We determined the IC_{50}-values of pravastatin, lovastatin and simvastatin for inhibition of sterol synthesis after 3.5 h incubation, in HSMC. In these cells, values for pravastatin are approximately 500 times higher than the ones observed for simvastatin and lovastatin (Fig. 4). From the separately performed experiments, the IC_{50}-values were calculated. The mean values ± SD were: 16 ± 9.3, 7.7 ± 8.3 and 5869 ± 3905 nM for lovastatin, simvastatin and pravastatin, respectively.

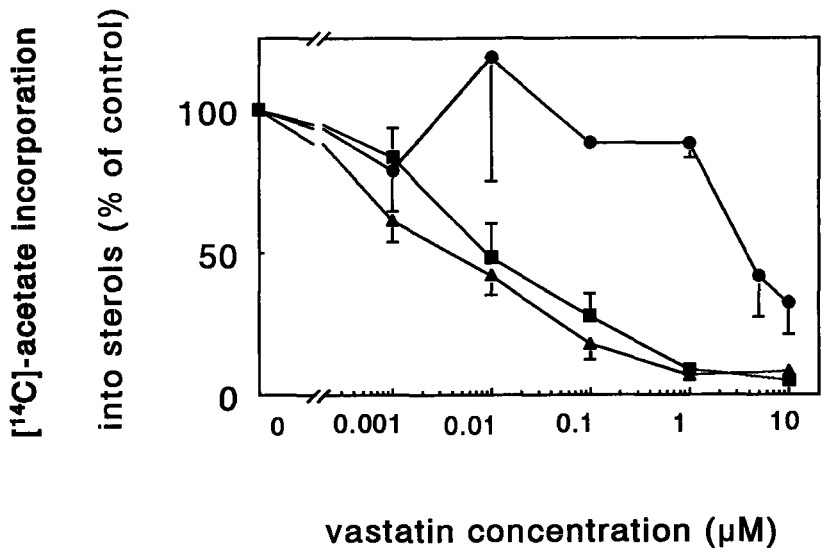

Figure 4: Inhibition of sterol synthesis by pravastatin (●), lovastatin (■) and simvastatin (▲) in HSMC. Cells were pre-incubated for 30 min with the indicated drug concentrations, then [^{14}C]-acetate was added to the medium (5 μCi/ml) and cells were further incubated for 3 hours. Each value (mean ± SEM of 3-4 experiments) is expressed as mean percentage of the control value. The control value (no drugs) was: 47570 ± 21645 ^{14}C-dpm incorporated into sterols/mg protein. Each individual experiment was performed in duplicate.

Drug effect on proliferation, MD activity and DNA synthesis in the presence of FCS and HS

Smooth muscle cell proliferation was influenced to a different extent by lovastatin, simvastatin and pravastatin after a 3-day incubation period. The cell number was reduced significantly at concentrations as low as 0.1 μM for simvastatin ($p<0.05$) and 1 μM for lovastatin ($p<0.005$) (Fig. 5A). Simvastatin displayed a stronger inhibitory potency than lovastatin (40 versus 20% inhibition at 1 μM). In the case of pravastatin, a decrease in cell number was observed at 1 μM but this decrease was not statistically significant (Fig. 5A).

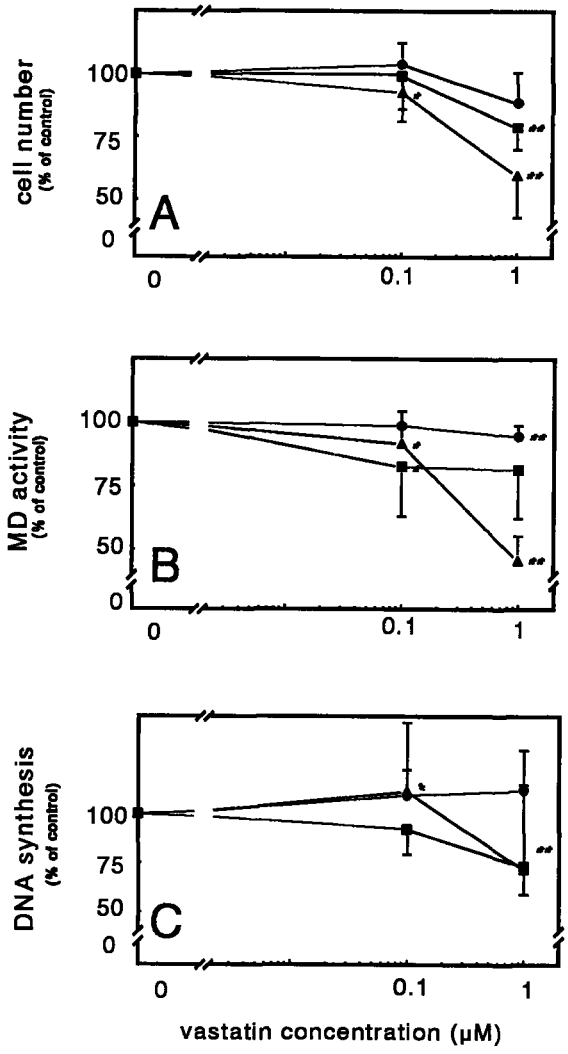

Figure 5: Effect of pravastatin (•), lovastatin (■) and simvastatin (▲) on proliferation (A), MD activity (B) and DNA synthesis (C) of HSMC cultured in presence of 10% FCS and 10% HS, during a 3-day incubation period. Twenty four hours after seeding, cells were incubated with DMEM/ 10%FCS/ 10%HS containing the indicated concentrations of drugs and further incubated for the designed period. Each value (mean ± S.D. of 3-4 experiments) is expressed as mean percentage of the control value (no drug): A, 58310 ± 17175 cells/well; B, 0.485 ± 0.32 O.D. units/well; C, 31356 ± 30729 dpm [^{3}H]-thymidine incorporated into DNA/well. Each individual experiment was performed in triplicate. *, $p \leq 0.05$; **, $p \leq 0.005$.

However, a trend in the inhibition of proliferation can be detected since higher concentrations of pravastatin induced a small but significant decrease in cell number after a 3-day drug incubation (results not shown). This is supported by a small but significant decrease in total MD activity at 1 μM of pravastatin (Fig. 5B). After 3 days of incubation with lovastatin and simvastatin, the total MD activity in the culture decreased proportionally to the number of cells, indicating that the average MD activity per cell was not affected by both drugs (Fig. 5B). [^{3}H]-thymidine incorporation into newly synthesized DNA showed mixed inhibition and stimulation in cells from separate experiments. The mean values calculated from individual experiments resulted in a general pattern of inhibition of DNA synthesis by lovastatin and simvastatin at 1 μM (Fig. 5C).

Drug effect on proliferation in the presence of FCS only

In order to investigate the effect of vastatins on smooth muscle cell proliferation under different growth conditions, we measured the cell number after a 6-day drug incubation period in the presence of FCS only. In these culture conditions, cell growth was slower than under culture conditions where HS was added to the medium (3-4 days versus 1.5-2 days doubling time). In this case, smooth muscle cell proliferation was inhibited by the three vastatins in a dose-dependent fashion (Fig. 6). Pravastatin, lovastatin and simvastatin inhibited cell proliferation to a significant extent ($p < 0.05$) at concentrations as low as 0.01 μM. At 0.1 μM, the 3 drugs displayed similar inhibitory potencies (20% inhibition) whereas at 1 μM concentration, a greater inhibition of proliferation was observed with lovastatin and simvastatin (40% inhibition) than with pravastatin (27% inhibition).

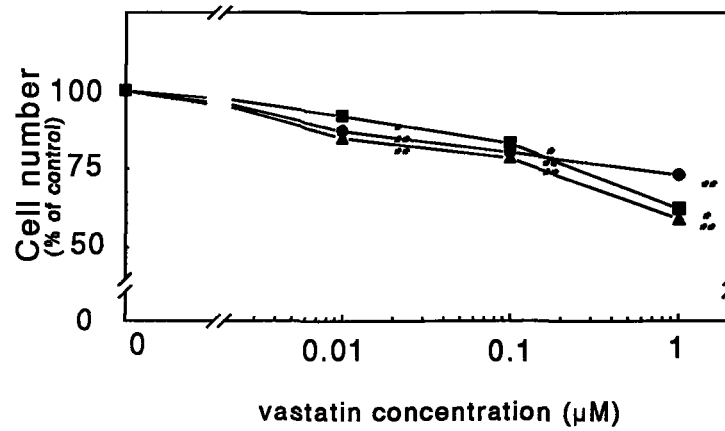

Figure 6: Effect of pravastatin (●), lovastatin (■) and simvastatin (▲) on proliferation of HSMC cultured in the presence of 10% FCS, during a 6-day incubation period.

Cells were incubated as described in the legend of Fig.5 except that the medium did not contain HS. Each value (mean ± S.D. of 2-3 experiments) is expressed as mean percentage of the control value (no drug; 31054 ± 1288 cells/well). Each individual experiment was performed in triplicate. *, $p \leq 0.05$; **, $p \leq 0.005$.

Reversal of the anti-proliferative effect of the vastatins by mevalonate

In order to test whether the effects on cell proliferation are the results of the inhibition of mevalonate production by the vastatins, cells were supplemented with mevalonate during the drug incubation period and proliferation assessed under these conditions.

In HUVEC (Table I), the decrease in cell number induced by 1 μM of simvastatin after a 3-day incubation was partially reversed by the addition of mevalonate (10 mM) while MD activity and DNA synthesis levels were brought back to controls levels. In the case of HCF (Table 1), cell number and MD activity levels were similar to control values in the presence of mevalonate. The 2-fold increase in DNA synthesis induced by simvastatin in these cells was completely prevented by the addition of 10 mM mevalonate, even resulting in a small decrease of this parameter compared to the control value.

TABLE I: Effect of mevalonate on the inhibition of HCF and HUVEC proliferation by simvastatin after 3 days of incubation.

Cells were incubated as described in the legend of Fig.1 (for HCF) and of Fig. 2 (for HUVEC) with simvastatin (1 μM) in the presence (+) or the absence (-) of mevalonate (10 mM). Each value (mean $\pm$ S.D. of 3-4 experiments) is expressed as mean percentage of the control value (no drug). The respective control values for HCF and HUVEC were expressed as the number of cells/well (125355 $\pm$ 32564; 443917 $\pm$ 179907, respectively), the O.D. units/well (0.38 $\pm$ 0.12; 0.31 $\pm$ 0.08, respectively) or the amount of dpm of [^{3}H]-thymidine incorporated into DNA/well (7149 $\pm$ 5703; 133196 $\pm$ 55938, respectively). Each individual experiment was performed in triplicate. *, $p \leq 0.05$. **, $p \leq 0.005$.

	Cell number (% control)		MD activity (% control)		DNA synthesis (% control)	
10 mM mev.	-	+	-	+	-	+
HCF	61 $\pm$ 19.6**	89.7$\pm$ 12.7	60.5$\pm$ 3.9**	95.1$\pm$ 17.7*	174.5$\pm$ 62.2**	73.3$\pm$ 13.2**
HUVEC	41.6$\pm$ 6.7**	65 $\pm$ 19.8**	55.4$\pm$ 16**	100.1$\pm$ 16.9	6.5$\pm$ 3.9**	85.3$\pm$ 20.6

DISCUSSION

It was observed in a previous study (16) that IC_{50}-values of pravastatin were approximately 200 times higher than the ones for simvastatin and lovastatin, for the inhibition of sterol synthesis in HCF and HUVEC. In the case of HSMC, our data show even larger differences between pravastatin and the two other drugs. In relation to these differences we compared the effects of pravastatin on cell proliferation of three different human extrahepatic cells *in vitro* with those of simvastatin and lovastatin. After a 3-day incubation period, pravastatin had hardly any effect on the three parameters measured (cell number, MD activity, DNA synthesis) in HUVEC, HCF and HSMC, whereas lovastatin and simvastatin reduced significantly, and to a different extent, the growth of these cells. Similarly, at a concentration of 1 μM, Corsini et al. (26) have demonstrated

using SMC from rat aorta and human femoral artery that a 3-day incubation with simvastatin, but not pravastatin, inhibited cell proliferation. Additionnally, we found that supplementation of the culture medium with mevalonate, but not cholesterol, could partially or totally reverse these anti-proliferative effects in human cells, supporting the idea that products of the mevalonate synthesis pathway are involved in this process.

We observed that the extent of the inhibition was dependent on the drug concentration but also varied between the various cell types and the culture conditions. Indeed, in HUVEC cultured in the presence of FCS, 1 μM of lovastatin or simvastatin induced a very strong inhibition of cell proliferation (50%), whereas this effect was barely detectable in cells cultured in the presence of HS/NBCS. This difference in drug inhibitory potency could be related to the fact that HUVEC cultured in the presence of FCS underwent two population doublings during the 3-day incubation period as opposed to less than one for the HUVEC cultured in the presence of HS/NCBS. In addition, the variation in sensitivity of HUVEC to simvastatin or lovastatin in terms of proliferation may be attributed to different compounds in NBCS, HS and FCS interacting with the mechanism of action of these drugs. In the case of slowly growing HSMC, cultured in the presence of FCS only, a 6-day incubation with pravastatin inhibited cell proliferation to the same extent as simvastatin, at concentrations as low as 0.01 μM (13 % inhibition). At higher drug concentrations (1 μM), inhibitory effects of simvastatin and lovastatin were larger than that of pravastatin (40 vs 27 % inhibition). It should be mentioned that, at concentrations higher than 1 μM, intracellular ATP levels in HSMC were reduced to a large extent by simvastatin and lovastatin but not by pravastatin (results not shown). This observation suggests a possible inhibition of the ubiquinone synthesis pathway (27) or cytotoxic effects of simvastatin and lovastatin at these concentrations. Also Corsini et al. (26) showed a strong reduced SMC number after a 3-day incubation with simvastatin at concentrations above 1 μM.

It is important to measure cell viability in order to discriminate between drug toxicity and anti-proliferative effects. For example, cell damage, related to elevation of intracellular calcium concentrations, has previously been detected in rat myoblasts, for high concentrations of simvastatin but not pravastatin (28). In pilot experiments, we noticed that between the second and third day of the incubation period with 5-10 μM of simvastatin and lovastatin (but not pravastatin), a significant proportion of cells rounded up and became detached from the culture dish. These observations were previously made in human SMC derived from saphenous vein cultured for 7-10 days with 10 μM lovastatin (29). As observed by other groups (29, 30), these toxic effects were not detectable within the concentration and time range used in our experiments. Additionally, as tested with an MTT viability assay, simvastatin, lovastatin and pravastatin did not affect the mitochondrial succinate-tetrazolium reductase system in our experimental conditions.

DNA synthesis in HUVEC was strongly reduced by simvastatin and lovastatin; this effect was reversible by the addition of mevalonate to the medium. The extent of this inhibition was directly influenced by culture conditions with a larger effect in HUVEC grown in FCS. In contrast to these results, Falke et al. (30) described a weak inhibition of DNA synthesis by lovastatin in HUVEC grown in FCS. However, this effect was observed after 24 h of drug incubation whereas our experiments were performed with a 3 day incubation period. Interestingly, in the two culture conditions used, the effect of simvastatin and lovastatin on DNA synthesis was always larger than that on cell

number. Since DNA synthesis was measured at the end of the incubation period, it is possible that, in non-synchronized cells, the inhibition of DNA replication (S phase) will be reflected by a similar decrease in cell number only at a later stage. It was previously demonstrated in synchronized cells that, compactin (an other HMG-CoA reductase inhibitor; 17, 18) and lovastatin (31) prevent the normal S phase burst of DNA synthesis. Paradoxically, we found that in HCF, total DNA synthesis was almost doubled in the presence of simvastatin or lovastatin (1 μM), whereas cell number decreased. The same effect was observed, to a minor extent, in HSMC where these drugs induced either a stimulation or an inhibition of DNA synthesis in separately performed experiments. According to Jakóbisiak et al. (31), lovastatin is also able to prolongate or arrest a minor fraction of cells in the G_2 phase of the cell cycle. Therefore, it is possible that some of the drug-treated cells were blocked in the G_2 phase of the cell cycle, after doubling their amount of DNA during the S phase, leading to higher levels of DNA synthesis in the culture, compared to the total cell number. On the other hand, it has been shown recently in HL-60 cells that lovastatin concentrations as low as 2 μM can induce DNA fragmentation during the first 24 h of treatment (32). We cannot exclude the possibility that in HCF, simvastatin and lovastatin induced cellular DNA degradation and that this event was counteracted by DNA repair. In human skin fibroblasts, a decrease in DNA synthesis with lovastatin was observed after 24 h treatment, at concentrations comparable with the ones used in our experiments (30). Therefore, the process of DNA fragmentation and repair could take place later on in the incubation period. Hohl et al. (33) demonstrated in leukemia cells that compactin effects on DNA synthesis could be inhibitory or stimulatory within the whole population of cells tested and that these effects were dependent on the drug concentration. Additionally, we observed that the increase in DNA synthesis induced by 1 μM of simvastatin in HCF was antagonized by the addition of mevalonate, suggesting that mevalonate interfered with the mechanism of this drug-induced effect.

In conclusion, our findings provide support for the hypothesis that mevalonate derived products, most probably isoprenylated proteins, are involved in cellular growth regulation and that the anti-proliferative effects of lovastatin and simvastatin in the cells studied are caused by mevalonate depletion. Pravastatin exhibited weaker inhibitory effects on cholesterol synthesis and cell proliferation in these extrahepatic cells. It can, however, inhibit SMC proliferation after a 6-day incubation period. Studies are in process to analyze the signal transduction pathway involved in SMC proliferation and the mechanism of action of the vastatins on this process.

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CHAPTER 6

COMPARISON OF LOVASTATIN, SIMVASTATIN AND PRAVASTATIN IN THEIR POTENCY TO INHIBIT STEROL SYNTHESIS AND THEIR ANTI-PROLIFERATIVE EFFECT IN CULTURED HUMAN MYOBLASTS

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SUMMARY

Lovastatin, simvastatin and pravastatin are fairly strong inhibitors of sterol synthesis in human myoblasts in culture. Lovastatin and simvastatin have IC_{50}-values of 19 $\pm$ 6 nM and 4.0 $\pm$ 2.3 nM, respectively. Pravastatin is a weaker inhibitor of sterol synthesis (IC_{50}-value of 110 $\pm$ 38 nM); however, compared with other extrahepatic cells this inhibition was rather strong. Therefore, the underlying mechanism was investigated in uptake studies with [^{14}C]-pravastatin and [^{14}C]-simvastatin. Both compounds showed temperature- and concentration dependent association with these cells, which is indicative of carrier-mediated transport. However, [^{14}C]-vastatin association was not saturable and a competition experiment with unlabelled pravastatin did not result in decreased association of [^{14}C]-pravastatin. The mechanism might be passive diffusion and/or transport via a low affinity carrier. [^{14}C]-simvastatin association was 9 times higher than [^{14}C]-pravastatin association measured at 37°C, using a drug concentration of 75 μM. Additionally, the more potent inhibitors of sterol synthesis, lovastatin and simvastatin, were able to inhibit the proliferation of these cells during 3 days of incubation with drug concentrations of 1 μM for lovastatin and 0.1 μM or 1μM for simvastatin. DNA synthesis was decreased by more than 80 per cent in the presence of 1 μM of lovastatin or simvastatin. In contrast, pravastatin had no influence on cell proliferation or DNA synthesis, upon extended incubation, which is probably related to the lack of inhibition of sterol synthesis by pravastatin. The three 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors did not disturb cell viability since mitochondrial dehydrogenase activity and ATP content remained proportional to the number of cells in the culture at any concentration used.

INTRODUCTION

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, lovastatin [1], simvastatin [2] and pravastatin [3], have been shown to cause a marked reduction of serum cholesterol levels and offer a new and effective approach to treatment of hypercholesterolemia. These drugs are competitive inhibitors of the enzyme HMG-CoA reductase, which converts HMG-CoA to mevalonic acid in the process of cholesterol biosynthesis. Lovastatin and simvastatin are more lipophilic in character compared with pravastatin [4,5]. Differences in the extent of inhibition of sterol synthesis in extrahepatic tissue and cells in culture have been observed for lovastatin and simvastatin on the one hand and pravastatin on the other hand [3, 6-10]. Lovastatin and simvastatin are stronger inhibitors of the sterol synthesis than pravastatin in the extrahepatic cells studied.

In contrast, in rat- [3,7] and human hepatocytes [10], the three vastatins are equally potent inhibitors of sterol synthesis. In accordance with these observations, it has been shown that pravastatin, the more hydrophilic compound, is transported into the hepatocyte via a specific transporter, which is not present in extrahepatic cells [11-13].

Side-pathways of cholesterol biosynthesis leading to e.g. ubiquinone, dolichol and the isoprene group of isoprenylated proteins, could be affected by these drugs [14].

Ubiquinone plays a role in cellular ATP generation and dolichol in protein-glycosylation; some isoprenylated proteins are involved in signal transduction pathways related to cell proliferation [15-17]. Inhibition of cell proliferation by lovastatin or simvastatin has been observed in human smooth muscle cells in culture [18-20], human and bovine endothelial cells and fibroblasts [20]. On the other hand, it was found that pravastatin did not reduce cell proliferation in rat or human arterial smooth muscle cells [19].

In clinical studies, adverse effects on muscle tissue have been reported in a small minority of patients for all three compounds [21-23]. Moreover, the incidence of associated myopathy increased when the vastatins were co-administered along with certain other drugs, such as gemfibrozil and niacin [24]. In this respect, the underlying mechanism of HMG-CoA reductase inhibitors causing myopathy has not yet been resolved. Belo et al. observed inhibition of fusion of L6 myoblasts by lovastatin at a concentration of 0.25 μM [25]. They postulated that inhibition of dolichol synthesis by lovastatin prevented the synthesis of fusogenic cell surface N-linked glycoproteins. Masters et al. observed that pravastatin was less myotoxic than lovastatin or simvastatin in neonatal rat skeletal myocytes [26]. Proliferation of myoblasts is important in the repair of damaged skeletal muscle. If a muscle is damaged, myoblasts are roused into activity; they begin to proliferate and their progeny fuse to form new muscle fibres which are not able to divide [27]. In order to get more insight into this matter, the effects of vastatins on sterol synthesis and on proliferation were investigated in cultured human myoblasts.

MATERIALS AND METHODS

Materials

Lovastatin, simvastatin, pravastatin (all sodium salts), [^{14}C]-simvastatin (spec. radioactivity of 17.1 μCi/mg) and [^{14}C]-pravastatin (spec. radioactivity of 27.8 μCi/mg), all in the lacton form, were kindly donated by Sankyo Co. (Tokyo, Japan). Stock solutions were made in 100% ethanol. Before use of the radioactive vastatins, their lacton ring was hydrolysed by a 30 minute incubation in 89% EtOH/0.11 N NaOH solution at 4°C, followed by neutralization with HCl.

Cell culture

Muscle biopsies of about 0.1 g wet weight, obtained from patients with a disc protrusion who underwent laminectomy, were used for myoblast isolation. Myoblasts were isolated as described by Yasin et al. [28]. Cells were cultured in Dulbecco's Modified Eagle's Medium containing 0.086% (w/v) $NaHCO_3$, 4 mM glutamine, 100 IU/ml penicillin, 0.1 mg/ml streptomycine and 20% fetal calf serum (Gibco, heat inactivated). In order to avoid fusion of myoblasts, cells were seeded at such a density that confluence was not reached at the end of each experiment.

Determination of sterol synthesis
Human myoblasts (in 10 cm^2 wells) were preincubated for 30 minutes in the absence or presence of 0.001, 0.01, 0.1, 1.0 and 10 μM of lovastatin or simvastatin in medium supplemented with 20% lipoprotein deficient serum. For pravastatin, 0.01, 0.1, 1.0. 5.0 and 10 μM were used. Cells were incubated further for 3 h with 5 μCi [^{14}C]-acetate (spec. radioactivity of 56.2 mCi/mmol, Amersham)/well. [^{14}C]-acetate incorporation into sterols was measured as described previously [10]. Samples were taken for protein determination [29]. Non-saponifiable lipids were separated as described by Boogaard *et al.*, using thin layer chromatography system I [30]. To calculate the IC_{50}-values of the three vastatins, curve fitting through all datapoints using a dose-response equation was performed. Sterol synthesis was also measured in cells preincubated for 2 days with 0, 0.1 and 1 μM of simvastatin or pravastatin and further incubated for 24 h in the presence of 5 μCi of [^{14}C]-acetate per well.

Determination of association of [^{14}C]-simvastatin and [^{14}C]-pravastatin in human myoblasts
Human myoblasts, preincubated for 30 minutes in Krebs-Ringer solution under 5% CO_2/95% air, were incubated with the indicated concentrations of ^{14}C-labelled vastatins (see Figure 2) for a period of 1 minute. Cells were treated further as previously described for human hepatocytes [10].

Determination of effects of vastatins on cell proliferation
To establish a growth curve of human myoblasts in culture, cells were seeded in 10 cm^2 wells on day 0. On days 4, 7 and 11, cells were trypsinized (0.5 mg trypsin/ml and 0.25 mg EDTA/ml) and the number of cells per well were counted with a Neubauer haemocytometer. For the determination of the effect of the vastatins on proliferation, cells were seeded at a density of 40,000 cells per 10 cm^2 wells on day 0 and allowed to settle for 4 days in culture. Cells were then incubated for 3 days with 0, 0.1 or 1 μM of lovastatin, simvastatin or pravastatin. At the end of the incubation period, cell number, DNA synthesis activity, mitochondrial dehydrogenase activity and ATP levels were measured in simultaneous experiments, in triplicate, for each concentration used.

Determination of DNA synthesis
DNA synthesis was measured as the incorporation of [^{3}H]-thymidine into DNA. Cells were incubated in the presence of [^{3}H]-thymidine (spec. radioactivity of 70-86 Ci/mmol, Amersham, 1μCi/well) 7 h prior to the end of the three days of incubation with the vastatins. Hereafter, cells were washed subsequently with phosphate buffered saline, 10% trichloroacetic acid and phosphate buffered saline. The precipitated DNA was dissolved in 0.3 N NaOH. After the addition of scintillation fluid (Ultima Gold, Packard), radiolabelled DNA was quantified in a liquid scintillation counter (Packard 1900 CA) and the amount of [^{3}H]-thymidine incorporated into newly synthesized DNA calculated.

Determination of cellular mitochondrial dehydrogenase (MD) activity (MTT-test)
A modified fluorometric assay [31] with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) salt (Sigma) as substrate was used to determine quantitatively the mitochondrial dehydrogenase (MD) activity of the cells. Briefly, during the last 3 hours of the incubation period, MTT was added (final concentration of 1 mg/ml) and the incubation continued at 37°C in the dark. The reaction was stopped by aspiration of the medium and solubilization of the cells by addition of lysis buffer. The optical density of the emission signal in the cell lysates was measured in a 96-well plate reader (Titertek Multi 100-MCC340) at 540 nm and 690 nm. The latter one was used as the background signal and the lysis buffer was used as the blank.

Determination of intracellular ATP levels
At the end of incubation, perchloric acid (final concentration of 3.2%) was added to the cells to precipitate cellular proteins and release the intracellular ATP. Extracts were then neutralized by 3 N KOH/ 0.3 M MOPS. A fluorometric enzymatic analysis with hexokinase and glucose-6-phosphate dehydrogenase was used to determine the cellular ATP content [32]. The fluorescent assay was also performed in the absence of hexokinase to determine the nonspecific fluorescence caused by substances in the cell extract.

Statistical analysis
The Mann-Whitney test was used to determine the statistical significance of the values obtained.

RESULTS

Effects of vastatins on the sterol synthesis in human myoblasts
The composite dose-response curves from three separately performed experiments are shown in Fig. 1. From the separate dose-response curves, using curve-fitting through all datapoints, IC_{50}-values (mean $\pm$ S.E.M.) were calculated. Lovastatin and simvastatin are strong inhibitors of the sterol synthesis in human myoblasts with IC_{50}-values of 19 $\pm$ 6 nM and of 4.0 $\pm$ 2.3 nM, respectively. Pravastatin is a somewhat weaker inhibitor with an IC_{50}-value of 110 $\pm$ 38 nM. This result indicates that, even though pravastatin is a hydrophilic compound, it is able to cross the lipid bilayer of the plasma membrane of the myoblast. To investigate the underlying mechanism, uptake studies with [^{14}C]-pravastatin and [^{14}C]-simvastatin were performed.

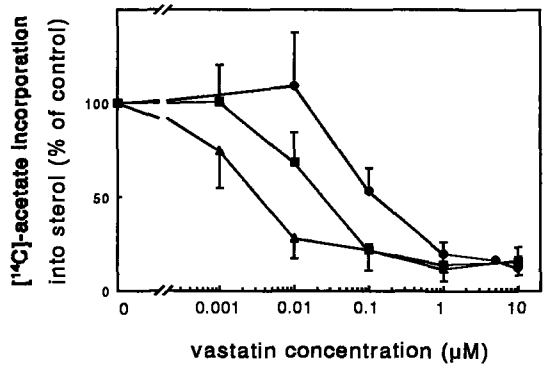

Figure 1: Inhibition of sterol synthesis by lovastatin (■), simvastatin (▲), and pravastatin (●) in human myoblasts in culture
Synthesis was measured by [^{14}C]-acetate incorporation into non-saponifiable lipids and expressed as percentage of control (see Experimental Procedures). The experimental conditions were a 30 min. preincubation, followed by a 3 h incubation in the presence of 5 μCi [^{14}C]-acetate. Data points are the mean and the bars represent S.E.M. of 3 separately performed experiments (if not shown, the bars coincide with the symbols). The mean control value was 6960 ± 2533 dpm per mg of cellular protein.

Association of ^{14}C-labelled vastatins with human myoblasts

A pilot experiment showed that the initial uptake velocity of [^{14}C]-pravastatin reached a plateau within several seconds (data not shown). For technical reasons it was decided to perform the experiments with an incubation period of 1 minute, using drug concentrations in the range of 5 - 75 μM. Temperature and concentration dependent association of [^{14}C]-pravastatin (Fig. 2A) or [^{14}C]-simvastatin (Fig. 2B) at 37°C and 4°C were observed with human myoblasts.

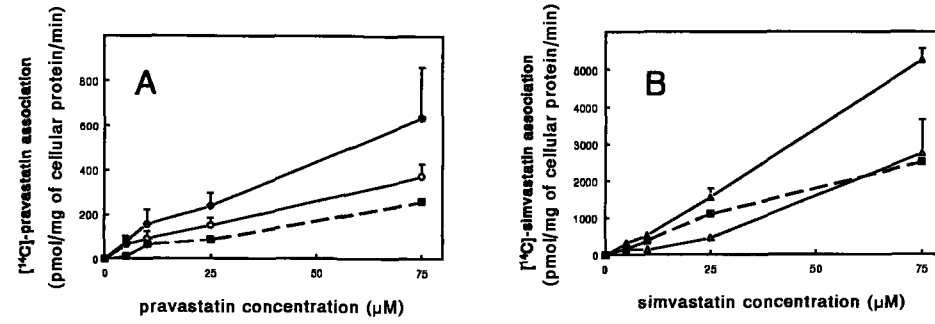

Figure 2: Concentration dependency of [^{14}C]-pravastatin (A) and [^{14}C]-simvastatin (B) association with human myoblasts in culture
Human myoblasts were preincubated for 30 min. in Krebs-Ringer solution at 37°C and incubated with the indicated concentrations of the ^{14}C-labelled vastatins at both 37 °C (●,▲) and 4°C (○,△) for 1 min. The specific vastatin associations (37°C-values minus the 4°C-values) are depicted as well (■). Mean values ± S.E.M. are presented for the [^{14}C]-pravastatin association (n=3). For [^{14}C]-simvastatin association the mean values ± range are depicted (n=2).

The association of simvastatin, measured at 37°C, was 9 times higher than that of pravastatin. The specific association (37°C-values minus the 4°C-values) of [^{14}C]-pravastatin or [^{14}C]-simvastatin was linear with the vastatin concentration and no saturation was observed.

Effects of vastatins on cell proliferation and DNA synthesis

As shown in Fig. 3, a logarithmic growth pattern was observed at cell densities between 10,000 and 200,000 cells per well.

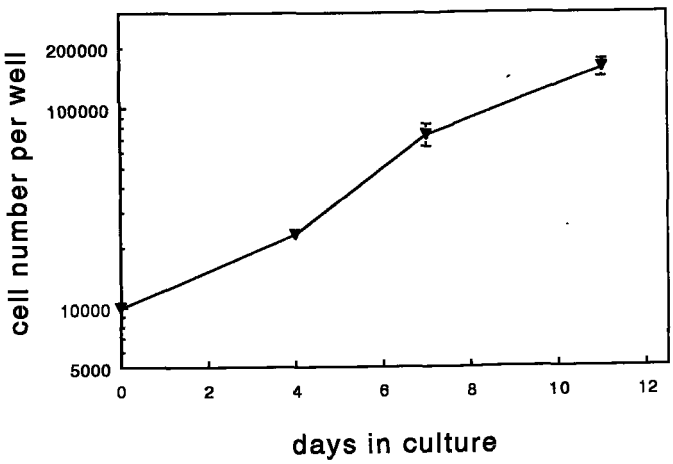

Figure 3: Representative growth curve of a human myoblast culture

Mean values of triplicate measurements within one experiment are expressed as cell number per well. The error bars represent the S.E.M. within one experiment. See the Experimental Procedures for culture conditions.

Cells divided with a doubling time of approximately 3 days. In pilot experiments, incubation of myoblasts with 5 μM of simvastatin for 3 days resulted in the detachment of 40% of the cells from the culture dish, compared with the total number of cells in the control. At a concentration of 10 μM this effect was even more pronounced. The same results were obtained with lovastatin at these concentrations, but not with pravastatin. Therefore, the effects of the drugs on cell proliferation were measured only at 0.1 or 1 μM of vastatin, for which all the cells remained attached. Pravastatin did not influence cell proliferation after 3 days of incubation (Fig.4A). 0.1 μM of lovastatin or simvastatin resulted in a small significant ($P<0.05$) decrease of the number of cells. Both compounds strongly inhibited cell proliferation to the same extent (70%) at a concentration of 1 μM. Even larger differences were observed between the vastatins in their effect on DNA synthesis (Fig.4B). At a concentration of 1 μM, [^{3}H]-thymidine incorporation was inhibited more than 80% by both lovastatin and simvastatin. Pravastatin had no effect on [^{3}H]-thymidine incorporation at any concentration tested.

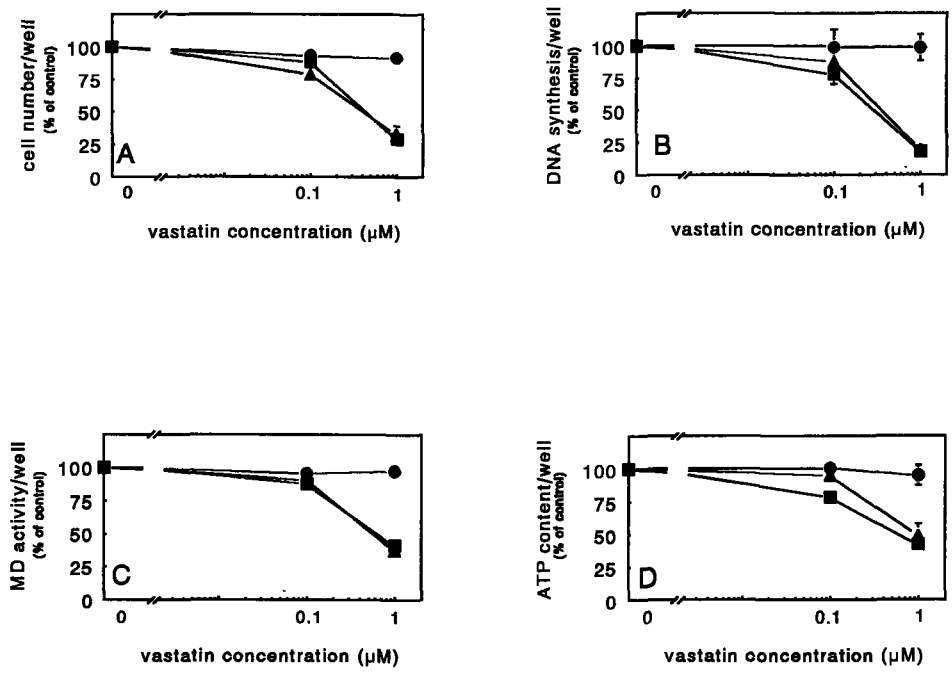

Figure 4: Effect of lovastatin (■), simvastatin (▲), and pravastatin (●) on cell number (A), DNA synthesis (B), mitochondrial dehydrogenase activity (C), and ATP content (D), after three days of incubation with 0, 0.1, or 1 μM of the three vastatins

Experiments were performed 3-4 times of which mean values ± S.E.M. are depicted. If not indicated, error bars coincide with symbol. The mean control values (± S.E.M.) of cell number, DNA synthesis, MD activity and ATP content are 154623 ± 12954 cells/well, 113024 ± 38760 dpm/well, 0.824 ± 0.109 optical density/well and 1.92 ± 0.58 nmol/well, respectively.

Effect of vastatins on two cell viability markers, mitochondrial dehydrogenase (MD) activity (MTT-assay) and intracellular ATP-levels

The MD activity was measured to determine whether cell viability was influenced by the HMG-CoA reductase inhibitors under the incubation conditions used. The MD activity was reduced to the same extent as cell proliferation in the presence of 0.1 μM and 1 μM of lovastatin or simvastatin (Fig.4C). Pravastatin at both concentrations had no effect. So, when the MD activity per cell was calculated, the activity was hardly affected at any drug concentration, for the three vastatins.

Similarly, a decrease in the amount of ATP content per well with increasing drug concentrations was observed for lovastatin and simvastatin, but not for pravastatin (Fig.4D). As for MD activity, when the ATP content per cell was calculated, it was not decreased by any vastatin, at any concentration.

Effect of simvastatin and pravastatin on [^{14}C]-acetate incorporation into sterols after 3 days of incubation

One μM of pravastatin did not have any effect on cell proliferation or DNA synthesis after 3 days of incubation, whereas after an incubation period of 3.5 hours sterol synthesis was inhibited at this concentration (Fig.1). Therefore, the inhibitory effect of pravastatin and simvastatin on sterol synthesis after 3 days of incubation was investigated. Cells were cultured and seeded as described for the proliferation experiments, incubated with 0.1 and 1 μM simvastatin or pravastatin for 3 days, after which sterol synthesis was measured. As shown in figure 5, the synthesis was inhibited 66% and 71% by simvastatin at the respective concentrations of 0.1 μM and 1 μM, whereas pravastatin did not inhibit sterol synthesis under these conditions.

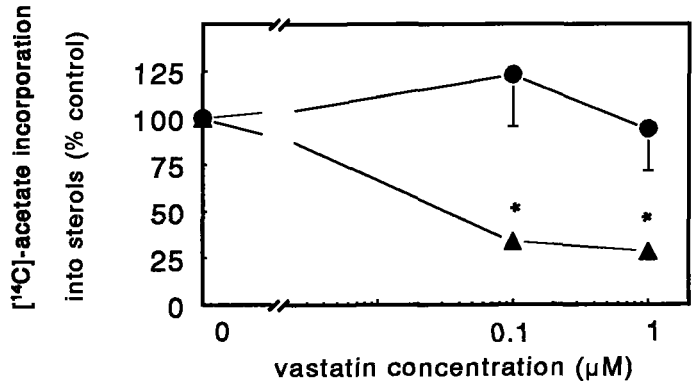

Figure 5: Inhibition of [^{14}C]-acetate into sterols in human myoblasts after three days of incubation with the indicated concentrations of simvastatin (▲) or pravastatin (●)

Depicted are the mean values ± S.E.M. of three separately performed experiments. Control mean values (± S.E.M; n=4) of each experiments are 90476 ± 21829, 213152 ± 38473 and 24834 ± 2877 dpm per mg of cellular protein. If not shown, error bars coincide with symbol (* $P<0.05$).

DISCUSSION

Lovastatin and simvastatin are strong inhibitors of sterol synthesis in human myoblasts, which is consistent with previous observations in other human cells in culture [10]. On the other hand, the inhibition of the sterol synthesis by pravastatin was stronger in myoblasts than in other extrahepatic cells in culture, as observed under similar experimental conditions [10]. The relatively low IC_{50}-value of pravastatin for the inhibition of sterol synthesis in human myoblasts might be attributed to some transport activity in these cells. However, increase of temperature dependent [^{14}C]-pravastatin association was linear with increasing concentration, not saturable (Fig.2A) and could not compete with unlabelled pravastatin (data not shown). This suggests that pravastatin is taken up in these cells by passive diffusion and/or by a low affinity carrier. The same was observed for [^{14}C]-simvastatin association. It is possible that differences in the cell

membrane structure allow pravastatin to enter myoblasts more easily than the other extrahepatic cells investigated. However, the involvement of a carrier cannot be ruled out, since association of both pravastatin and simvastatin was temperature dependent, which is indicative of carrier-mediated transport. In order to detect a possible transporter the use of radiolabelled vastatins with a higher specific radioactivity is required.

In human endothelial cells, it was observed that [^{14}C]-simvastatin association (925 pmol/ mg of cellular protein/min) was 49 times higher compared with [^{14}C]-pravastatin association (19 pmol/mg of cellular protein/min) at a concentration of 75 μM at 37°C [10]. However, in human myoblasts, [^{14}C]-simvastatin association (5256 pmol/mg of cellular protein/min) was only 9 times higher than [^{14}C]-pravastatin association (593 pmol/mg of cellular protein/min) at the same concentration and temperature. Whereas the IC_{50}-values for simvastatin are similar in both cell types, the differences in IC_{50}-values for pravastatin between human myoblasts (110 nM) and human endothelial cells (1172 nM) are relatively in agreement with the differences in association of [^{14}C]-pravastatin observed between the two cell types [10].

When sterol synthesis is strongly inhibited, not only is cholesterol synthesis decreased but non-sterol side-products can also be affected. The substrate affinity of the enzymes involved in the side pathways of cholesterol synthesis are much higher than that for the enzymes leading to cholesterol synthesis [33,34]. Therefore, only when cholesterol synthesis is strongly inhibited, can effects occur on these side pathways, which could lead for example to the inhibition of cell proliferation. After 3 days of incubation, lovastatin and simvastatin, at a concentration of 1 μM, are strong inhibitors of cell proliferation and DNA synthesis in myoblasts, whereas pravastatin did not influence cell proliferation or DNA synthesis at this concentration. Since cell viability (MD activity and the intracellular ATP levels) was not affected under the conditions used, the effect of lovastatin and simvastatin on cell proliferation and DNA synthesis cannot be attributed to toxicity of the compounds in cell culture. So the effect of the drugs on cell proliferation is probably caused by decrease of a mevalonate derived product. The strong inhibitory effect of pravastatin on sterol synthesis observed after 3.5 hours of incubation, was not reflected in an effect on cell proliferation after 3 days of incubation with pravastatin. But, after 3 days of incubation, pravastatin did not affect sterol synthesis (Fig.5), which is in agreement with the lack of effect on cell proliferation. On the other hand, simvastatin was still able to inhibit strongly sterol synthesis, after 3 days of incubation. As described previously, an increase in IC_{50}-values was observed for lovastatin, simvastatin and pravastatin in human endothelial cells, human hepatocytes [10] and Hep G2 cells [35], after extended incubation with the vastatins. This is explained by feedback regulation: inhibition of cholesterol synthesis leads to a decrease in the synthesis of regulatory sterols, which results in an increase in HMG-CoA reductase mRNA and subsequently in higher HMG-CoA reductase enzyme levels [14]. So, to suppress HMG-CoA reductase, more inhibitor is needed. In conclusion, these data show that under the conditions used, proliferation is affected by the more potent inhibitors of sterol synthesis in these cells. Even though pravastatin displayed lower IC_{50}-values in human myoblasts versus other human extrahepatic cells, no effect was observed on cell proliferation.

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CHAPTER 7

INDICATIONS FOR CARRIER MEDIATED TRANSPORT OF PRAVASTATIN AND SIMVASTATIN IN HUMAN HEPATOCYTES IN CULTURE

LEVER

Men heeft inmiddels al geleerd
Hoe men de lever transplanteert.
Geen donor echter is - bij leven -
Bereid zijn lever af te geven.
En dus bestaat geen zekerheid
Omtrent de leverlevertijd.

Theo van de Leur
OPRISPINGEN
- Dichterlijke vrijheden -
spelen met taal
Uitgeverij Michon B.V.
Helmond

SUMMARY

The mechanism of pravastatin and simvastatin association with human hepatocytes was studied. Concentration and temperature- dependent association with human hepatocytes was observed and seems to consist of at least two components for both compounds. Both compounds are possibly associated by passive diffusion as well. The specific association of pravastatin, obtained from one experiment, had the following characteristics: K_m-values of 1.5 and 35 μM, with V_{max}-values of 14 and 89 pmol per mg of cellular protein per minute, respectively. In the presence of metabolic inhibitors such as potassium cyanide, rotenone, N-ethyl maleimide and oligomycine A, ATP levels and [^{14}C]-pravastatin association were decreased. A pilot experiment studying the competition with unlabelled simvastatin and pravastatin resulted in a decreased association of 25 μM of [^{14}C]-pravastatin.
So these data support the statement that pravastatin is in part associated with human hepatocytes by at least two carrier-mediated mechanisms, of which one or more may be ATP-dependent. The specific association of [^{14}C]-simvastatin also seems to consist of more than one component. The K_m-value of the low affinity component for the specific association of simvastatin was 155 μM, with a V_{max}-value of 1808 pmol of [^{14}C]-simvastatin associated per mg of cellular protein per minute obtained from one typical experiment. Simvastatin transport across the hepatocyte cell membrane seems to be ATP-independent using either 5 or 25 μM of [^{14}C]-simvastatin. Unlabelled pravastatin was not able to compete with radiolabelled simvastatin but, unlabelled simvastatin did. So in human hepatocytes, simvastatin association seems to be also in part carrier-mediated, temperature-dependent, but ATP-independent.

INTRODUCTION

A high level of low density lipoprotein (LDL) cholesterol in the circulation is a potential risk factor for the development of atherosclerosis. Drug intervention can reduce this risk. One group of drugs used to lower plasma cholesterol are the vastatins. These drugs are competitive inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. This enzyme converts HMG-CoA to mevalonic acid in the cholesterol biosynthetic pathway. Through inhibition of this conversion, less cholesterol is synthesized, resulting in an induction of LDL receptors on the liver, which increases the uptake of LDL cholesterol from the circulation, thus lowering the risk. The target tissue for these drugs is the liver, for the liver is able to metabolize and convert cholesterol.

Three vastatins used for drug intervention are lovastatin (1), simvastatin (2) and pravastatin (3). These drugs are very similar in structure, however they differ in hydrophobicity (4,5). Lovastatin and simvastatin are lipophilic compared with pravastatin which is more hydrophilic. Due to these differences, vastatins inhibit the *de novo* cholesterol synthesis to a different extent in human extra hepatic cells in culture (3,6-10), i.e. pravastatin inhibits sterol synthesis much less than lovastatin or simvastatin. This was also observed in the human hepatoma cell line Hep G2, often used as a model for the human hepatocyte (11). However, in human hepatocytes the

three vastatins are equally potent inhibitors of cholesterol synthesis (10). Hepatocytes seem to be able to transport pravastatin, the more hydrophilic vastatin, across the plasma membrane, whereas Hep G2 cells seem to have lost this ability (11,12).
In rat hepatocytes in culture it was shown that pravastatin is transported across the plasma membrane via a sodium independent organic anion transporter (13-16). It was suggested that at least the more hydrophilic compound pravastatin is taken up in human hepatocytes by a liver specific transporter, for in a previous pilot experiment it was observed that both pravastatin and simvastatin were associated to a similar extent. Pravastatin hardly associated with human endothelial cells, while simvastatin the more lipophilic compound was associated to a higher extent (10).

In order to investigate further the presence of a particular transporter for these compounds in human hepatocytes, additional uptake experiments were performed with [^{14}C]-pravastatin and [^{14}C]-simvastatin.

MATERIALS AND METHODS

Pravastatin and simvastatin (all sodium salts), [^{14}C]-pravastatin (spec. radioactivity of 27.8 μCi/mg) and [^{14}C]-simvastatin (spec. radioactivity of 17.1 μCi/mg) (radioactive compounds all in lactone form) were kindly provided by the Sankyo Co. (Tokyo, Japan). Stock solutions were made in ethanol. Before use the lactone ring of the [^{14}C]-labelled drugs was hydrolysed either by a 30-minute incubation in 0.1 M NaOH at 37°C or at 4°C for 30-minutes in 89% EtOH/0.11 N NaOH solution, followed by neutralization with 0.1 N HCl. Rotenon and potassium cyanide (KCN) were purchased from Janssen Chimica, N-ethylmaleimide (NEM), oligomycine A (oligo A) and glucose-6-phosphate from Sigma, hexokinase and ATP from Boeringer Mannheim GmbH and fructose from Fluka.

Purity test of [^{14}C]-pravastatin and [^{14}C]-simvastatin by TLC-analysis

[^{14}C-labelled drugs, both lactone- and sodium salt forms as compared with the unlabelled drugs were tested for their purity by TLC (silica gel 150A, Whatman) before each experiment. The running solvent used for pravastatin was a mixture of chloroform, acetic acid and methanol with ratio 9:1:1 (v/v). The plate with simvastatin was developed in toluene, acetone and acetic acid (50:50:3, v/v). Radioactive spots were made visible by autoradiography. Unlabelled spots were visualized by charring after spraying with 10% H_2SO_4. Compounds amounted to more than 95% in the sodium salt form after the opening of the lacton ring.

Isolation and culture of human hepatocytes

Human hepatocytes were isolated from part of the livers from donors, which could not be used for transplantation due to technical problems. The isolation and culturing of human hepatocytes was as described by Rijntjes *et al.* (17), except that during culture fungizone, gentamycin, tetracyclin and vancomycin were replaced by 100 IU of penicillin per ml, and 100 μg of kanamycin and streptomycin per ml. Cultures were

used within 4 days after isolation.

Determination of uptake of ^{14}C-labelled vastatins in human hepatocytes

Human hepatocytes (1.3 x 10^6 viable cells) seeded in 10 cm^2 wells were incubated for 1 minute with ^{14}C-labelled vastatins (sodium salts) in Williams E medium (Gibco) at both 37°C and 4°C. We showed previously (10), that the association of 15 μM of both radiolabelled pravastatin and simvastatin in human hepatocytes, was linear up to two minutes. The vastatin concentrations used were 1, 2, 5, 10, 25, 50 and 100 μM. Incubation was stopped by the addition of 1 ml of ice-cold 0.5% bovine serum albumin (Sigma) in Krebs-Ringer solution (Sigma). This medium was promptly removed and the cells were subsequently washed with 2 ml of the same solution, and three times with 1 ml ice cold Krebs-Ringer solution. Cells were lysed with 300 μl of 0.2 N NaOH per well. The lysate was collected and neutralized by adding 30 μl of 2 N HCl. Samples were taken for the determination of protein (18). To 250 μl of cell lysate 10 ml scintillation fluid (Ultima gold, Packerd) was added and radioactivity was counted in a Packard 1900 CA liquid scintillation counter.

To study the influence of the cellular ATP-level on the radiolabelled vastatin association, KCN, rotenon and fructose at concentrations as indicated in the legends of the figures, were added in combination with the labelled drugs. N-ethylmaleimide and oligomycine A were added 5 minutes prior to the incubation with the radiolabelled drugs. The specific association (37°C-values minus the 4°C-values) and the substrate concentrations were plotted according to Lineweaver and Burk for determination of the K_m- and V_{max}-values.

Determination of cellular ATP levels

In order to determine the cellular ATP content, uptake experiments with metabolic inhibitors and fructose were performed as described above, but now in the presence of unlabelled pravastatin or simvastatin. Briefly, at the end of incubation, perchloric acid (2.3% final concentration) was added to the cells to precipitate cellular proteins and to release the intracellular ATP. Extracts were then neutralized by 3 N KOH/ 0.3 M MOPS. The cellular ATP contents was measured using a fluorimetric enzymatic analysis with hexokinase and glucose-6-phosphate dehydrogenase (19). Optimum conditions for measurements in our culture system were 340 nm for excitation and 470 nm for emission. The fluorescent assay was also performed in the absence of hexokinase to compensate for nonspecific fluorescence caused by substances in the cell extracts.

RESULTS

[^{14}C]-vastatin association with human hepatocytes

To support the existence of a hepatic transporter, saturation of the transporter involved should occur. Therefore, the association of [^{14}C]-pravastatin and [^{14}C]-simvastatin in a concentration range of 1 - 100 μM was investigated in human hepatocytes. Incubations

were performed at 37°C and at 4°C for 1 minute. The association at 4°C is thought to be a combination of diffusion and aspecific adherence of the compound. The 37°C-association values minus the 4°C-association values is supposed to represent specific carrier mediated association. Figure 1A depicts a typical example of the dose-dependent association of [^{14}C]-pravastatin with human hepatocytes. The association is concentration- and temperature dependent. The specific association is not linear with increasing drug concentration, indicating saturation of a transporter. These data plotted according to Lineweaver-Burk (Fig.1B) showed a two-phase curve: one component had a K_m-value of 1.5 μM with a V_{max}-value of 14 pmol/mg of cellular protein/min. The other had a K_m-value of 35 μM with a V_{max}-value of 89 pmol/mg of cellular protein/min. Similar K_m and V_{max}-values were obtained in a second experiment using another batch of human hepatocytes for both components.

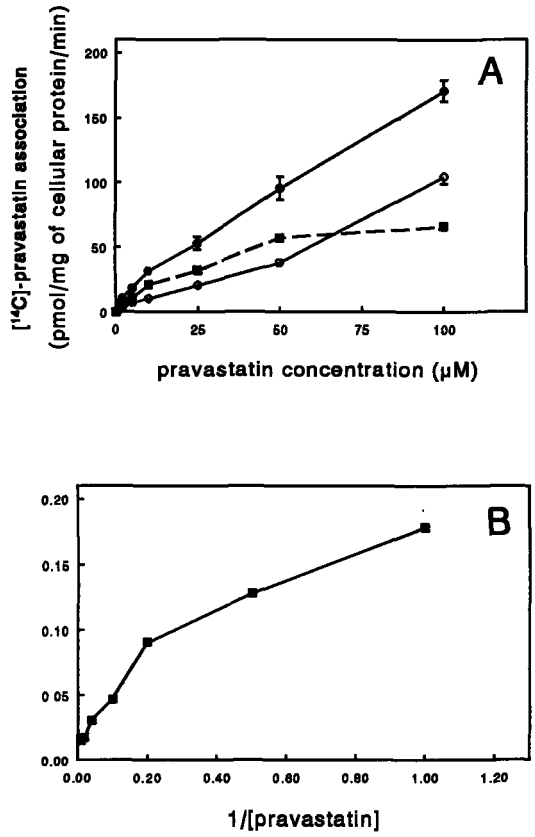

Figure 1: Concentration dependency of pravastatin association with cultured human hepatocytes

(A) Human hepatocytes were incubated with the indicated [^{14}C]-pravastatin concentrations at 37°C (●) and 4°C (○) for 1 minute in Williams E solution. The specific association (37°C-values minus the 4°C-values) is also depicted (■). The mean values of [^{14}C]-pravastatin association of duplicate measurements are expressed. The error bars depict the range between the duplicate measurements within the experiment. (B) The specific association presented in a Lineweaver-Burk plot.

The specific association of [^{14}C]-simvastatin in human hepatocytes was determined in a similar way (Fig.2) and was also temperature and concentration dependent. The specific association does not seem to be linear with increasing [^{14}C]-simvastatin concentration (Fig.2A). In this experiment the low affinity component had a K_m value of 155 μM and a V_{max}-value of 1808 pmol/mg of cellular protein/min (Fig.2B). In three other experiments using different batches of hepatocytes, the comparable K_m-value and V_{max}-value of the low affinity component were obtained. The mean K_m and V_{max}-values ($\pm$ S.E.M) obtained from these four separately performed experiments were 95 $\pm$ 23 μM and 1495 $\pm$ 345 pmol/mg of cellular protein/min, respectively.

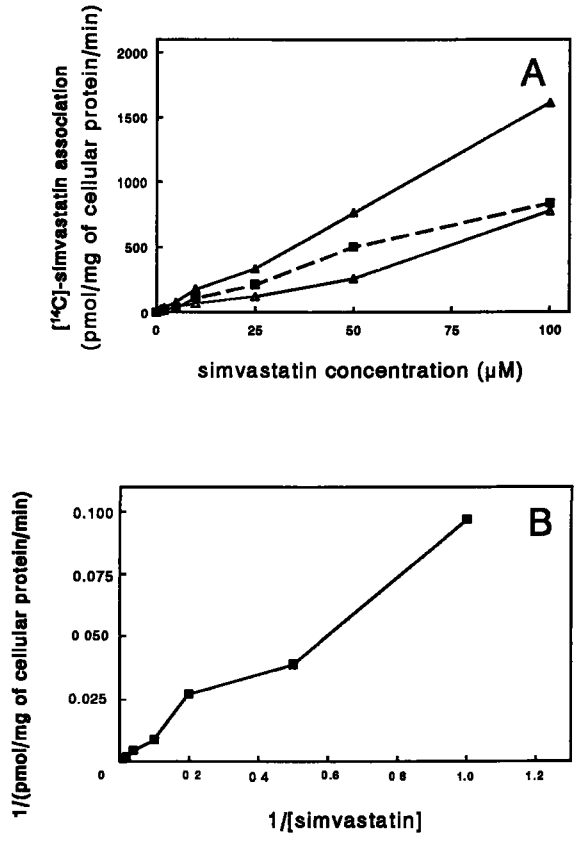

Figure 2: Concentration dependency of simvastatin association with human hepatocytes in culture
(A) Concentration dependent association of [^{14}C]-simvastatin in human hepatocytes in culture at 37°C (▲), at 4° (△) and the specific association (37°C-values minus the 4°C-values) are presented (■). Each point represents the mean $\pm$ range of one separately performed experiment. If not shown error bars coincide with the symbol. (B) The specific association depicted in a Lineweaver-Burk plot.

Effect of cellular ATP reduction on pravastatin and simvastatin association with human hepatocytes

If a transporter is involved, this can either be ATP-dependent or ATP-independent. By lowering the ATP-content in the cells, less compound will be transported across the plasma membrane when the system is ATP-dependent. To lower ATP-levels, human hepatocytes were incubated with different metabolic inhibitors having different targets in the respiratory chain or with fructose. Fructose is converted to fructose-6-phosphate, hence lowering cytosolic ATP levels. Hereafter, the medium was discarded and cells were incubated for one minute with either 5 or 25 μM of ^{14}C-labelled vastatins (to determine vastatin association) or with unlabelled vastatins (to determine ATP levels) in the presence of the metabolic inhibitors or fructose. In one experiment using 5 μM of pravastatin ATP-levels were decreased, using 30 μM of rotenon, 1 mM of KCN and 10 mM of fructose by 43%, 34% and 23% respectively; in the cells incubated at 4°C compared with the 37°C control value, no difference in ATP-content was observed (Fig.3A). In this experiment [^{14}C]-pravastatin again showed temperature-dependent association, but additionally ATP-dependent association (Fig. 3B). This association in the presence of metabolic inhibitors was reduced to approximately 50% compared with control.

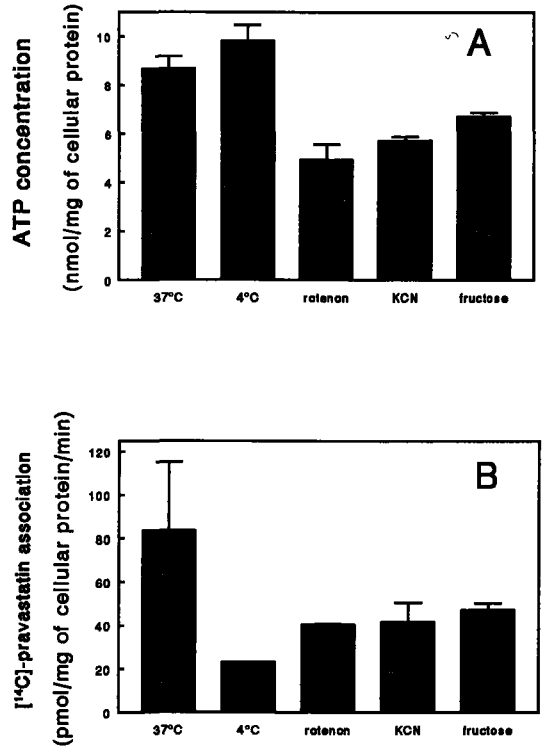

Figure 3: Pravastatin association in the presence of metabolic inhibitors and fructose

Human hepatocytes were incubated respectively with 5 μM of unlabelled pravastatin (A) or 5 μM of [^{14}C]-pravastatin (B) at 37°C for 1 minute with and without 30 μM of rotenone, 1 mM of KCN or 10 mM of fructose. The vastatin association of the control was also measured at 4°C. (A) In the experiment performed without radioactivity cellular ATP contents were measured. (B) The [^{14}C]-pravastatin asssociation is expressed as pmol/mg cellular protein/min. The bars indicate the range between the duplicate measurements of one experiment.

In order to reduce the ATP levels to a more pronounced extent, cells were preincubated for 5 minutes in this experiment with the metabolic inhibitors N-ethylmaleimide and oligomycine A in the presence of 5 μM of unlabelled simvastatin or [^{14}C]-simvastatin. The reduction in cellular ATP levels by the metabolic inhibitors (Fig.4A), did not result in a decreased [^{14}C]-simvastatin association (Fig.4B).

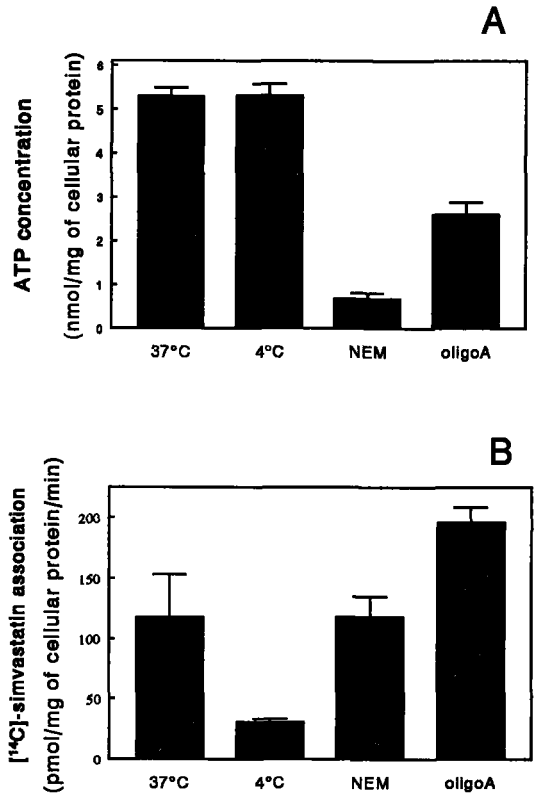

Figure 4: Effect of oligomycine A and N-ethylmaleimide on ATP levels and [^{14}C]-simvastatin association with human hepatocytes

Incubations with the metabolic inhibitors were preceded by a preincubation for 5 minutes. Human hepatocytes were incubated with 5 μM of unlabelled simvastatin (A) or with 5 μM of [^{14}C]-simvastatin at 37°C for 1 minute without and with 1 mM of N-ethylmaleimide or with 13 μM of oligomycine A. The ATP level and simvastatin association of the control was also measured at 4°C. (A) In the experiment performed without radioactivity cellular ATP levels were measured. (B) The [^{14}C]-vastatin association is expressed as pmol/mg of cellular protein/min). The bars indicate the difference between the duplicate measurements of one experiment.

In a separately performed experiment, using 25 μM of pravastatin, in combination with 13 μM of oligomycine A or 1 mM of N-ethylmaleimide for ATP-measurements (Fig.5A), and for [^{14}C]-pravastatin association (Fig.5B), again a reduced pravastatin association was observed with a decrease in cellular ATP levels. Association of 25 μM of unlabelled simvastatin or [^{14}C]-simvastatin in the presence of metabolic inhibitors did result in a decrease in cellular ATP levels (Fig.5C), but not in a reduced association of radiolabelled simvastatin (Fig.5D).

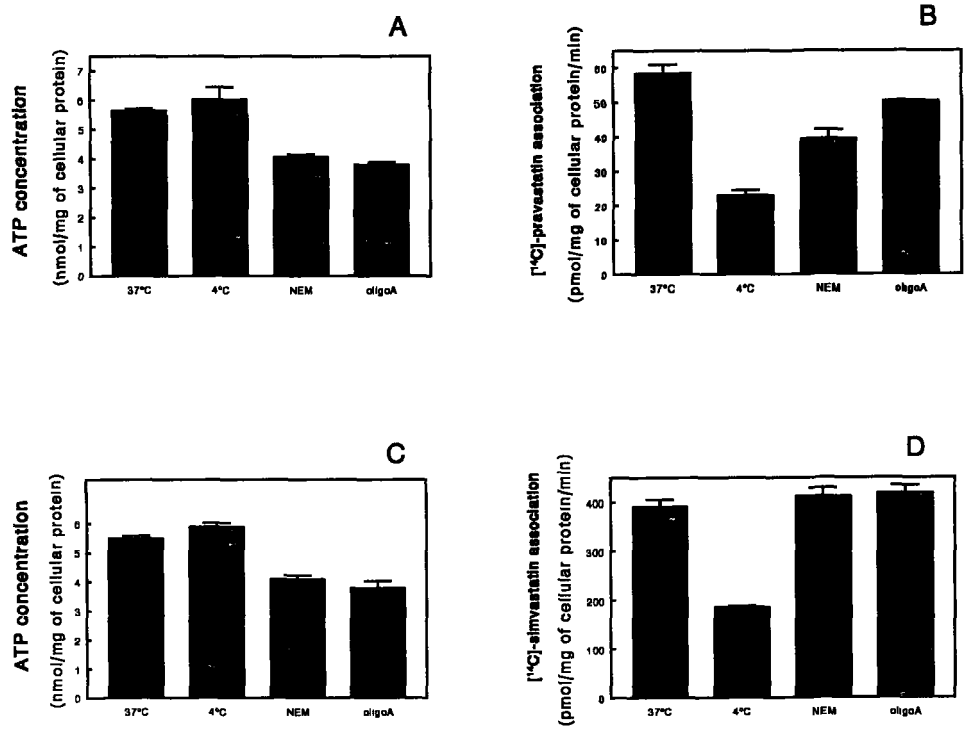

Figure 5: Effect of metabolic inhibitors on cellular ATP content and association of 25 μM of radiolabelled pravastatin or simvastatin

Human hepatocytes were incubated at 37°C for 1 minute in the absence or presence of 1 mM of N-ethylmaleimide or 13 μM of oligimycine A, respectively with 25 μM of unlabelled pravastatin (A), 25 μM of [^{14}C]-pravastatin (B), unlabelled simvastatin in a concentration of 25 μM (C) and 25 μM of ^{14}C-labelled simvastatin (D). Incubations were preceded by a preincubation for 5 minutes with the metabolic inhibitors. In the experiments performed with the unlabelled vastatins (A,C) cellular ATP contents were measured. The [^{14}C]-vastatin association is expressed in pmol/mg cellular protein/min (B,D). Bars indicate the range between the duplicate measurements of one experiment.

Effect of unlabelled pravastatin and simvastatin on the ^{14}C-labelled vastatin association in human hepatocytes.

When a carrier is saturated, substrates transported by the same transporter will compete with one another. A competition experiment was performed with unlabelled pravastatin or simvastatin in combination with 5 or 25 μM of ^{14}C-labelled vastatins. Addition of 10 or 100 μM of unlabelled pravastatin to 5 μM of [^{14}C]-pravastatin did not show competition between unlabelled and labelled pravastatin (Fig.6A). Addition of unlabelled simvastatin (10 and 100 μM) resulted in a decrease of [^{14}C]-pravastatin association of approximately 30% for both simvastatin concentrations. However, when using 25 μM of labelled pravastatin, the addition of unlabelled pravastatin (10 μM) resulted in an decrease of 16%, an excess of 4-times resulted in a 33% inhibition compared with control (Fig.6B).

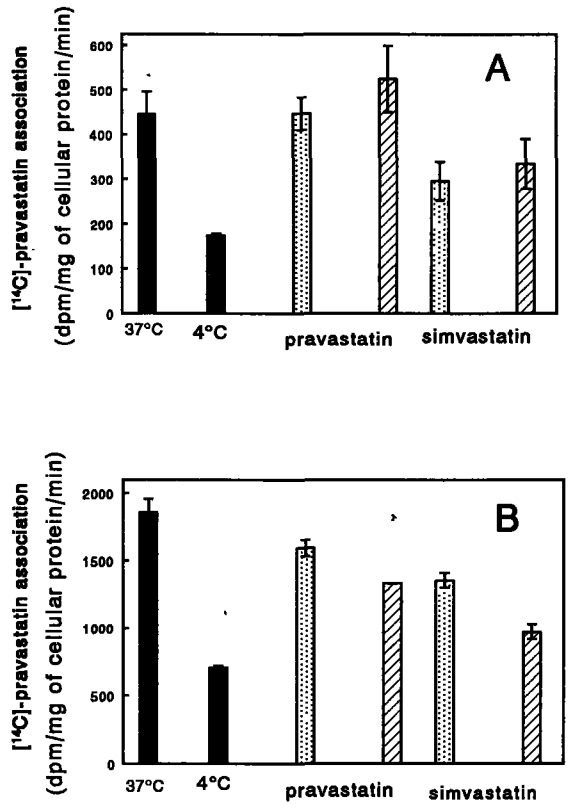

Figure 6: Effects of HMG-CoA reductase inhibitors on [^{14}C]-pravastatin association

In two separately performed experiments, human hepatocytes were incubated with 5 μM - (A) or 25 μM of [^{14}C]-pravastatin (B) at 37°C for 1 minute in the presence or absence of 10 μM (dotted bars) or 100 μM (hatched bars) of unlabelled pravastatin or simvastatin. The control association was also measured at 4°C. Bars indicate the range between the duplicate measurements of one experiment.

Unlabelled pravastatin did not compete with [^{14}C]-simvastatin association (Fig.7). At a low concentration of [^{14}C]-simvastatin (5μM), the addition of unlabelled simvastatin (10 or 100 μM) resulted in a decrease of 14% and 27% respectively (Fig.7A). In contrast, at 25 μM of [^{14}C]-simvastatin addition of 10 or 100 μM of unlabelled simvastatin resulted in an increase of [^{14}C]-simvastatin association of 20% and 75% respectively, compared with control (Fig.7B).

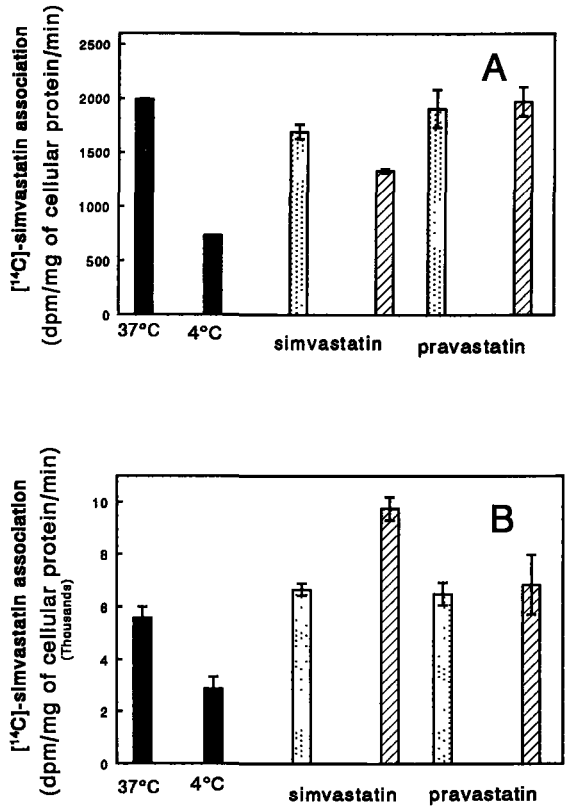

Figure 7: Effects of unlabelled simvastatin and pravastatin on [^{14}C]-simvastatin association
Human hepatocytes were incubated for 1 minute with 5 μM of [^{14}C]-simvastatin (A) or with 25 μM of [^{14}C]-labelled simvastatin (B) in the presence or absence of unlabelled simvastatin or pravastatin at concentrations of 10 μM (dotted bars) or 100 μM (hatched bars). Controls were incubated at 37°C and at 4°C, the other incubations were performed at 37°C. The mean values ± range of duplicate measurements of one experiment are depicted.

DISCUSSION

Pravastatin and simvastatin are associated with human hepatocytes in a concentration - and temperature dependent manner. Their association in human hepatocytes seems to be at least partly carrier mediated and possibly by passive diffusion as well. Pravastatin transport in this batch of human hepatocytes consisted of at least two components with K_m-values of 1.5 μM and 35 μM, and with V_{max}-values of 14 and 89 pmol/mg cellular protein/min, respectively. Similar data were obtained with a different batch of human hepatocytes. The transporter with the low K_m-value has a high affinity for pravastatin. At a concentration of 5 μM of [^{14}C]-pravastatin the high affinity component will contribute to a greater extent to the association than the low affinity component, however at an increasing concentration the contribution of the latter one will be higher. The high affinity component however, can only be extensively studied when the specific radioactivity of the compound is higher than that of the one used.

Saturation kinetics for pravastatin were observed with rat hepatocytes. Ziegler *et al.* reported that pravastatin is taken up by one transporter with a Km-value of 27 μM and a V_{max}-values of 537 pmol/mg cellular protein/min (16). For pravastatin association in human hepatocytes, using the same concentration range as Ziegler *et al.* (16), a K_m-value of 35 μMolair with a V_{max}-values of 89 pmol/mg cellular protein/min was

observed. This indicates that the affinity of the pravastatin carriers in human hepatocytes and rat hepatocytes, is in the same order of magnitude, however, the transport capacity of human hepatocytes is much lower. Reduced uptake of taurocholic acid, ouabain and organic cations, in human hepatocytes compared with that in rat hepatocytes has been reported (20). In general, it seems that human hepatocytes have a lower transport capacity for various compounds, than rat hepatocytes.

In the one experiment performed, 25 μM of unlabelled pravastatin was able to compete with [^{14}C]-pravastatin. However, at 5 μM of unlabelled pravastatin no competition was observed. More, experiments need to be performed in order to draw conclusions about the type of transporter involved for the different drug concentrations used.

Simvastatin seems to be able to lower the association of [^{14}C]-pravastatin. This has also been reported for rat hepatocytes (15). It is possible that simvastatin has a higher affinity for this transporter. For [^{14}C]-simvastatin association, we observed two phases in the concentration curve. This might indicate that [^{14}C]-simvastatin is transported via at least two components into the hepatocyte. As observed for the high affinity component of pravastatin, the possible high affinity carrier(s) of simvastatin can only be investigated when the specific radioactivity of the radiolabelled compound is increased.

A difference in the association of pravastatin and simvastatin is, that pravastatin is at least partially ATP-dependently transported. [^{14}C]-simvastatin transport did not seem to require ATP. [^{14}C]-simvastatin association was not inhibited by unlabelled pravastatin suggesting a lower affinity of pravastatin for the carrier involved. Unlabelled simvastatin competed with [^{14}C]-simvastatin at a concentation of 5 μM. Competition of unlabelled simvastatin with 25 μM of labelled simvastatin resulted in an increase of [^{14}C]-simvastatin association. The reason for this increased simvastatin association is not clear yet. Additional experiments will have to be performed to confirm the results obtained in this study so far. Only then can conclusions be drawn about the nature of these transporters in human hepatocytes.

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CHAPTER 8

SUMMARY AND GENERAL DISCUSSION

Most biochemical studies with vastatins have been performed in animals, and in cell cultures of animal origin. Our interest has focussed on the effects of three HMG-CoA reductase inhibitors: lovastatin, simvastatin and pravastatin. The effect of these compounds on cholesterol synthesis has been assessed in cultured human hepatocytes, versus several human extrahepatic cells in culture, in combination with their effects on other processes related to the cholesterol synthesis.

As the liver is the main target for lipid lowering drugs, the action of vastatins was therefore investigated in a human hepatoma cell line (Hep G2)(Chapter 2), which is often used as a representative model for the human hepatocyte, and in cultured human hepatocytes (Chapter 2 and 7). The extrahepatic human cells studied in this respect were umbilical endothelial vein cells (Chapter 2 and 5), retinal pigment epithelial cells (Chapter 2), corneal fibroblasts (Chapter 2 and 5), granulosa cells (Chapter 2 and 4), smooth muscle cells (Chapter 5) and myoblasts (Chapter 6). The extrahepatic cells examined were chosen for the following reasons: the inner lining of blood vessels consists of endothelial cells, which are therefore in direct contact with the drugs in the blood circulation; retinal pigment epithelial cells play an active role in the normal function of photoreceptor cells; corneal fibroblast and granulosa cells are deprived of exogenous cholesterol, in the sense that they exist within an avascular surrounding depending on their *de novo* cholesterol synthesis for a sufficient supply of cholesterol; cell proliferation is a very important mechanism for repairing damaged skeletal muscle tissue and smooth muscle cell migration, and proliferation plays a very important role in the formation of atherosclerotic plaques.

As shown in Chapter 2, the three drugs inhibited HMG-CoA reductase in Hep G2 cell homogenate and human hepatocytes to a similar degree. However, in intact Hep G2 cells, pravastatin was a less potent inhibitor of the sterol synthesis than either lovastatin or simvastatin. This was also observed in all the extrahepatic cells studied. The IC_{50}-values, the drug concentration at which 50% inhibition of sterol synthesis occurs, of lovastatin, simvastatin and pravastatin in the various cultured human cells studied, are presented in Table I. The differences observed between human hepatocytes and other human cell types, may be explained by supposing that pravastatin is transported into the hepatocyte via specific hepatic transporter(s). The hepatoma cell line Hep G2 may behave similarly to extrahepatic cells, in that pravastatin does not inhibit sterol synthesis as it does in cultured human hepatocytes. The lack of such a hepatic transporter in Hep G2 cells indicates that this particular cell type is not suitable for studying liver related uptake of certain compounds. Pilot uptake experiments, using [^{14}C]-simvastatin and [^{14}C]-pravastatin, in human hepatocytes versus human umbilical vein endothelial cells, as an example of an extrahepatic cell type, support this view (Chapter 3). [^{14}C]-simvastatin was associated with both human hepatocytes and endothelial cells. [^{14}C]-pravastatin hardly associated with endothelial cells, but did so with human hepatocytes, to a similar extent as did [^{14}C]-simvastatin. The association of both vastatins was further investigated in human hepatocytes (Chapter 7). It seems that both compounds may be transported into the human hepatocyte via two or more transport components. The association of pravastatin appeared to be at least partially ATP-dependent, while the association of simvastatin did not require energy. However, the high affinity carrier(s), for both compounds, can only be investigated when the specific radioactivity of the compounds is increased. The nature of these transporters needs

to be further investigated with competition studies using unlabelled vastatins, or compounds which are known to be transported by the liver, such as disulfobromophathalein, taurocholate or cholate.
Human hepatocytes have proved to be useful for uptake studies, although, transport activity decreases with prolonged culturing times. Uptake studies in human hepatocytes, using cells in culture suspension, instead of adherent cells in culture, may be a better way to study hepatic uptake mechanisms.

The availability of these cells and the reproducibility of experiments with human hepatocytes are problematic. Therefore, one might consider performing uptake studies with these radiolabelled vastatins using vesicles consisting of isolated hepatocyte membranes. Basolateral and canalicular membranes can be isolated, from the human hepatocyte (1). In this way, more information about the actual carriers involved in basolateral or canalicular uptake of human hepatocytes may be obtained.

Cryopreservation and long-term storage of isolated hepatocytes is also possible (2). This opens the way to create human liver stores, from which hepatocytes can be retrieved upon request, without being dependent on the immediate availability of human liver donors. It is not yet clear whether cryopreserved hepatocytes lose their transport capacity for bile acids, as was observed in the prolonged culturing of isolated rat hepatocytes.

The liver is able to transport many structurally different compounds across the cell membrane, e.g. bile acids, toxins, sugars. Bile acid transporters have been utilized to target bile acid derived HMG-CoA reductase inhibitors to the liver (3). In this way, targeting of lipid lowering drugs to the liver will presumably result in less adverse side-effects. Studies have demonstrated that liver specificity of a given HMG-CoA reductase inhibitor can be increased by combining it with bile acid structural elements, and making use of the specific bile acid uptake mechanisms present in this organ.

In human myoblasts, the IC_{50}-value of pravastatin was only six-fold higher, as compared with the IC_{50}-values of either lovastatin or simvastatin. In all the other extrahepatic cells investigated, a difference of at least 100-fold was observed between pravastatin on one hand and lovastatin or simvastatin on the other. This suggests that pravastatin can cross the cell membrane of myoblasts more readily, possibly due to some form of transport activity present in these cells.
Therefore, uptake studies with [^{14}C]-pravastatin and [^{14}C]-simvastatin were performed. From these experiments, it cannot be concluded that either compound is taken up by a carrier-mediated process. The mechanism or transporter involved is possibly passive diffusion and/or a low affinity carrier, suggesting that the membrane of myoblasts is different from the other extrahepatic cells investigated. This could well be, as myoblasts have the ability to fuse and form muscle cells. However, the possible involvement of a carrier cannot be ruled out, since both pravastatin and simvastatin are temperature-dependently associated, which is indicative of carrier-mediated transport. As observed in uptake studies with human hepatocytes, a higher specific radioactivity is necessary for the investigation of a possible transporter for these compounds in human myoblasts. Transport capacity for simvastatin acid has been observed in bovine endothelial cells. In this particular case, the transporter involved in association is probably a monocarboxylic acid transporter, which is most likely to be present in several other, if not all, extrahepatic cell types.

Hep G2 cells are subject to feedback regulation of regulatory enzymes of the cholesterol biosynthetic pathway such as HMG-CoA reductase and squalene synthase, when incubated with lovastatin or simvastatin, but to a lesser extent by pravastatin. Furthermore, it was found that longer incubation with the vastatins resulted in higher IC_{50}-values. In order to show that the feedback mechanism for HMG-CoA reductase was involved in this phenomenon, mRNA levels were measured in human endothelial cells after incubation with the vastatins for 3.5 hours and 20 hours. Indeed, prolonged incubation with either lovastatin or simvastatin led to higher levels of HMG-CoA reductase mRNA, indicating the existence of a feedback mechanism.

The influence of the vastatins on the secretion of progesterone, for which cholesterol is the precursor, was investigated in cultured human granulosa cells (chapter 4). These cells are located in the mammalian ovary, within a mature Graafian follicle. Before ovulation, these cells are in an avascular surrounding, deprived of exogenous cholesterol as a possible source of progesterone synthesis. Large amounts of progesterone have been found in the follicular fluid prior to ovulation. It might be that under avascular conditions, the *de novo* cholesterol synthesis plays an essential role in the production of progesterone. After ovulation, vascularization of the corpus luteum provides the means by which exogenous cholesterol is delivered to luteinized granulosa cells, thereby allowing progesterone synthesis from this precursor source. Lovastatin and simvastatin inhibited progesterone secretion into the media of cultured granulosa cells, under conditions in which endogenous cholesterol pools were decreased. Pravastatin did not affect progesterone secretion. When pools were filled, by the addition of exogenous cholesterol (LDL), progesterone secretion was not affected by either of the drugs. It seems possible that under conditions where exogenous cholesterol is not available (or only in limited amounts) such as prior to ovulation, inhibition of *de novo* cholesterol synthesis may affect the synthesis and secretion of progesterone. Vastatins are still contraindicated during pregnancy, and in adolescents, and their safety and efficiency on this particular issue is yet to be established.

Clinical trials with the three vastatins have revealed that inhibition of sterol synthesis also leads to regression of atherosclerotic plaques. The mechanism involved may be that inhibition of sterol synthesis affects proliferation of smooth muscle cells, and thus regression of plaque formation. The isoprene group of, for example the ras protein or lamin B, both of which are involved in the process of cell proliferation, is derived in both cases from the cholesterol biosynthesis pathway. If inhibition of cell proliferation by vastatins represents a general phenomenon, this could also lead to adverse side-effects. The effect of lovastatin, simvastatin and pravastatin on cell proliferation and DNA synthesis was therefore investigated in cultured human smooth muscle cells, human umbilical endothelial cells, human corneal fibroblasts (chapter 5), and human myoblasts (chapter 6). In addition, the effect of these compounds on the cell viability of these cells was investigated, by measuring the mitochondrial dehydrogenase activity. ATP levels were additionally determined as a cell viability marker in human myoblasts. Endothelial cells, fibroblasts, smooth muscle cells and myoblasts were incubated for 3 days with the different vastatins, at concentrations of either 0.1 or 1 μM. Additionally, smooth muscle cells were incubated for 6 days with the drugs used, in a concentration range of between 0.001-10 μM.

Lovastatin and simvastatin, at a concentration of 1 μM, inhibited cell proliferation in all four cell types, after 3 days of incubation. In smooth muscle cells, endothelial cells and myoblasts, inhibition of DNA synthesis was observed. However, in fibroblasts the opposite was observed in terms of [^{3}H]-thymidine incorporation, which was stimulated with lovastatin or simvastatin at 1 μM. This phenomenon may in part be due to some form of repair mechanism of damaged DNA, initiated by the action of the strong cholesterol synthesis inhibitors, lovastatin and simvastatin and/or by inhibition of the G2 phase of the cell cycle (4). Pravastatin did not affect cell proliferation or [^{3}H]-thymidine incorporation into DNA of dividing smooth muscle cells, endothelial cells, corneal fibroblasts or myoblasts up to a concentration of 1 μM. However, in smooth muscle cells, incubated for 6 days, pravastatin inhibited cell proliferation at a concentration of 0.1 μM, but to a lesser extent than simvastatin or lovastatin. Mitochondrial dehydrogenase activity was not affected by the three vastatins in any cell type used. Cellular ATP levels of myoblasts were also not affected by the HMG-CoA reductase inhibitors, under the conditions used. It appeared that the synthesis of ubiquinone was not affected by the strong inhibitors of the isoprenoid synthesis, lovastatin and simvastatin. It seems likely, that sufficient ubiquinone is synthesized for ATP generation, but it is also possible that mitochondrial oxidative phosphorylation does not play an essential role in the ATP generation of cultured cells.

The inhibitory effect of the vastatins on cell proliferation was not due to the toxicity of the drugs. The anti-proliferative effect of the vastatins could partially be reversed by the addition of mevalonate to the culture medium, suggesting that a decrease in metabolites of mevalonate are the cause of this inhibitory effect. Further experiments are in progress to investigate the signal transduction pathway(s) involved in smooth muscle cell proliferation and the mechanism of action of the vastatins on this process.

It should be considered that inhibition of smooth muscle cell proliferation and endothelial cells in the formation of an atherosclerotic plaque may lead to a less stable plaque, which ruptures easily, leading to a thrombus or even to occlusion of a blood vessel.

The frequent association of mutated, oncogenic forms of cellular ras proteins with a broad spectrum of human malignancies has prompted intensive investigations into their role in normal cellular physiology, and establishing the contribution of aberrent ras function to human tumorigenesis. Several *in vitro* and *in vivo* studies have shown that inhibition of cholesterol synthesis, leading to the inhibition of farnesylation of ras protein in particular, inhibits tumour cell proliferation (5,6). However, the use of HMG-CoA reductase inhibitors in cancer chemotherapy has several drawbacks. Firstly, since mevalonate is the precursor for all isoprenoid synthesis, including cholesterol, these inhibitors may affect other isoprenylation pathways, such as cytoskeletal organization and the regulation of intracellular vesicular transport. Secondly, it has been shown that cholesterol synthesis is 100-fold more sensitive to the use of vastatins than is protein prenylation. Thus, a very high drug dose is necessary to inhibit isoprenylation, which could lead to the adverse effects decribed above. Finally, ras proteins represent only a small percentage of isoprenylated proteins. Thus, the relative non-specific action of these mevalonate inhibitors may limit their usefulness for blocking oncogenic ras function in tumours (7). Recently,

great efforts were made to develope inhibitors of the enzyme farnesyl protein transferase (8,9). This enzyme catalyses the farnesylation of the thiol group of cysteine, located at the fourth amino acid position from the ras C-terminus. Specific inhibitors of ras farnesyl transferase may one day provide effective and selective inhibition of tumour cells harbouring oncogenic ras proteins.

The squalestatins (Zaragozic acids) represent a new class of cholesterol lowering drugs (10,11). These compounds inhibit the enzyme squalene synthese in the cholesterol biosynthesis pathway. Inhibition of squalene synthase has an advantage over HMG-CoA reductase inhibitors, in that squalestatines do not affect synthesis of products from side pathways involved in cholesterol biosynthesis. Extended research with this class of lipid lowering drug is necessary. Antioxidants, such as vitamin E, and ubiquinone, have been put forward as antiatherosclerotic drugs (12). The working hypothesis is that they may act by inhibiting the oxidation of low density lipoproteins, particles associated with plaque formation and a known risk factor of increased incidence of atherosclerosis.

Inhibitors of cholesterol absorption, such as phytosterols and acyl coenzyme A cholesterol acyltransferase inhibitors, have been reported to reduce serum cholesterol levels (13). ACAT inhibitors are also expected to prevent foam cell formation as the result of inhibition of cholesterol esterification in macrophages as well as intimal smooth muscle cells in the arterial wall. The use of gene therapy as a succesful intervention in the field of atherosclerosis is likely to happen at the end of this decade. Introduction of a selected gene into the tissue of interest is being investigated at present, for example by the introduction of the LDL receptor gene into the liver of patients suffering from familial hypercholesterolemia, or genes that interact upon the complex expression of cytokines, growth factors, adhesion molecules, and their respective cell-surface receptors, by the arterial wall and blood cells (14).

Recently, it was observed that long term simvastatin and pravastatin treatment improved mortality in patients with coronary heart disease. Up to the present time, vastatins have proved to be useful and safe hypocholesterolemic drugs. It is not expected that any of the newly claimed strategies for lowering plasma lipid levels will have a clear and easy passage through clinical trial and onto the market. The new cholesterol lowering drugs will have to show improved safety and better lipid lowering profiles, compared with the HMG-CoA reductase inhibitors already in use. An important advantage of the existing HMG-CoA reductase inhibitors is the low price compared with the newly developed lipid lowering drugs. In periods of recession, public health care is one of the first areas to suffer economic cutback.

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SAMENVATTING

Atherosclerosis kan leiden tot coronaire hartziekten en is daardoor de meest voorkomende oorzaak van sterfte in Westerse landen. Een belangrijke risicofactor in het ontstaan van atherosclerosis is een hoog LDL-cholesterol gehalte in het bloed. Vastatines, remmers van het enzym HMG-CoA reductase, de eerste snelheids-bepalende stap in de cholesterol synthese, zijn in staat om zeer efficiënt het LDL-cholesterol in het bloed te verlagen, door middel van verhoogde expressie van de LDL-receptor op de lever. Naast het gunstige cholesterolverlagend effect zijn er echter ook negatieve bijwerkingen gemeld bij gebruik van deze HMG-CoA reductase remmers, onder andere spierklachten en lever-aandoeningen (Hoofdstuk 1). In het begin van dit onderzoek was er weinig bekend over de invloed van deze stoffen op de cholesterol synthese in human cellen.

De effecten van drie vastatines: lovastatine, simvastatine en pravastatine op de cholesterol synthese en de daaraan gelieerde processen worden daarom onderzocht in verschillende humane cellen in kweek.

De IC_{50}-waarde van de drie vastatines, de vastatine concentratie waarbij 50% remming van de cholesterol synthese plaatsvindt, wordt vastgesteld in cel-homogenaten van Hep G2 cellen en in de volgende humane cellen in kweek: Hep G2 cellen, humane hepatocyten (menselijke levercellen), navelstreng endotheel cellen, cornea fibroblasten, granulosa cellen, retina pigment epitheel cellen, gladde spiercellen en myoblasten (Tabel I). Deze cellen zijn om verschillende redenen gekozen. Aangezien de lever het doelorgaan is van de vastatines, zijn de effecten van deze remmers op de sterol synthese in Hep G2 cellen, een veelvuldig gebruikt modelsysteem voor de humane hepatocyt, vergeleken met die in humane hepatocyten. Endotheel cellen bekleden de binnenkant van de bloedvaten en zijn daardoor in direct contact met de drugs in de circulatie. Cornea fibroblasten en granulosa cellen bevinden zich in een a-vascular milieu, waardoor deze cellen afhankelijk zijn van de *de novo* cholesterol synthese om in hun cholesterolbehoefte te voorzien. Granulosa cellen daarentegen zijn tevens in staat om cholesterol om te zetten tot het steroid hormoon, progesteron. Proliferatie en migratie van gladde spiercellen speelt een rol in de vorming van atherosclerotische plaques. In myoblasten, de voorloper cel van dwars gestreept spierweefsel, speelt proliferatie ook een belangrijke rol, namelijk in het herstel van beschadigd spierweefsel.

Lovastatine en simvastatine zijn zeer sterke remmers van de cholesterol synthese in alle bovengenoemde cel typen. Pravastatine, is een zwakkere remmer van de cholesterol synthese in de onderzochte extrahepatische cellen en in Hep G2 cellen. In human hepatocyten blijken de drie vastatines echter in staat om de sterol synthese in gelijke mate te remmen. Dit leidt tot de veronderstelling dat pravastatine wordt opgenomen in hepatocyten door een transporter die uitsluitend voorkomt in levercellen. Uit een vooronderzoek met radioactief gemerkte pravastatine en simvastatine in humane hepatocyten versus humane endotheel cellen blijkt dat pravastatine geassocieerd is met hepatocyten, maar niet of nauwelijks met endotheel cellen, dit in tegenstelling tot simvastatine, dat geassocieerd is met beide cel typen (Hoofdstuk 3).

Hep G2 cellen blijken deze transporter niet meer te bezitten. Er zijn indicaties dat zowel pravastatine als simvastatine via een transporter worden opgenomen in de humane hepatocyt, door minimaal twee transport-systemen (Hoofdstuk 7). De associatie van pravastatine is mogelijk gedeeltelijk ATP-afhankelijk, terwijl die van simvastatine ATP-onafhankelijk lijkt te zijn.

In tabel I valt op te merken dat de IC_{50}-waarde van pravastatine voor myoblasten lager is dan in de andere extrahepatische cel typen. Dit kan te verklaren zijn door de aanwezigheid van een transporter in myoblasten, die instaat is om pravastatine te transporteren over het cel membraan. Opnamestudies met radioactief pravastatine en simvastatine kunnen echter geen transporter aan tonen in deze cellen (Hoofdstuk 6).

Remming van de cholesterol synthese in Hep G2 cellen leidt tot verhoging van het mRNA nivo van HMG-CoA reductase en tot verhoogde activiteit van het enzym squaleen synthase, waarbij lovastatine en simvastatine, de sterkere HMG-CoA reductase remmers, een grotere inductie veroorzaken dan pravastatine (Hoofdstuk 2). In endotheel cellen en humane hepatocyten is waargenomen dat verlenging van de incubatie tijd (20 uur in plaats van 3,5 uur) met de vastatines leidt tot een verhoging in de IC_{50}-waarden (Hoofdstuk 3). In endotheel cellen wordt onderzocht of ook hier het mRNA nivo van HMG-CoA reductase verhoogd is. Inderdaad, wordt een verhoogd mRNA nivo waargenomen voor HMG-CoA reductase na 20 uur incubatie met lovastatine en simvastatine ten opzichte van de 3,5 uur incubatie, de sterke remmers van de cholesterol synthese. De gevonden verhoging kan verklaard worden doordat er minder regulerende sterolen gevormd worden onder invloed van de vastatines. Deze sterolen bevorderen de degradatie van het enzym en remmen de translatie, waardoor er minder mRNA gevormd wordt. In het geval van remming van de cholesterol synthese door de vastatines vindt dus het omgekeerde plaats, namelijk een verhoging van het enzym activiteit, door een verhoogde translatie activiteit en verminderde afbraak van het enzym.

Naast het feit dat de cholesterol synthese geremd wordt door deze vastatins, kunnen processen waarbij cholesterol gemetaboliseerd wordt, ook beïnvloedt worden. In het ovarium zou de synthese van progesteron, een steroid hormoon waarvan cholesterol de precursor is, eventueel beïnvloed kunnen worden door remming van de cholesterol synthese. In hoofdstuk 4 wordt aangetoond dat onder omstandigheden waar de endogene cholesterol pools laag zijn, lovastatine en simvastatine naast een cholesterol verlagend effect ook een reducerend effect hebben op de progesteron secretie door human granulosa cellen. Pravastatine beïnvloedt de cholesterol synthese en de progesteron secretie niet onder gelijke condities. In aanwezigheid van voldoende exogeen cholesterol wordt er geen verlagend effect meer waargenomen op de progesteron secretie in aanwezigheid van de vastatines. De omstandigheden zijn hierbij gelijk aan die in het vorige experiment.

In hoofdstuk 5 en 6 word aangetoond dat de HMG-CoA reductase inhibitoren, lovastatine en simvastatine, gedurende een 72 uurs-incubatie, een negatieve invloed hebben op de cel proliferatie en op de incorperatie van [^{3}H]-thymidine in DNA van humane endotheel cellen, gladde spiercellen, cornea fibroblasten en myoblasten. Echter in cornea fibroblasten wordt een verhoogde [^{3}H]-thymidine incorporatie waargenomen met lovastatine en simvastatine bij een concentratie van 1 μM. Pravastatine beïnvloedt deze processen niet of nauwelijks. De vitaliteit van de cellen onder de gekozen experimentele omstandigheden wordt niet beïnvloed door de

vastatines. Na verlenging van de incubatie tijd tot 144 uur wordt er nu ook een verlagend effect op de cel proliferatie waargenomen in gladde spiercellen (25%) met 1 μM pravastatine. Mevalonzuur additie is in staat om het effect veroorzaakt door de vastatines op proliferatie en DNA synthese grotendeels te voorkomen.

Bovenstaand samenvattend, blijkt dat in het gebruikte *in vitro* humane cel-systeem er geen verschillen zijn tussen lovastatine, simvastatine en pravastatine op de sterol synthese in humane hepatocyten. Echter, de effecten van deze vastatines op de sterol synthese en op processen gekoppeld aan de cholesterol synthese in extrahepatische cellen, laten duidelijke verschillen zien hoofdzakelijk tussen lovastatine en simvastatine enerzijds en pravastatine anderzijds. Pravastatine beïnvloedt de gemeten parameters niet of nauwelijks in extrahepatische cellen. Lovastatine en simvastatine beïnvloeden echter wel de sterolsynthese en daaraan gelieerde processen in deze cellen.

TABEL I: Inhibitie van de sterol synthese door lovastatine, simvastatine en pravastatine, in verschillende humane cellen in kweek.

	Incubatie tijd (uur)	IC_{50}-waarden (nM)		
		lovastatine	simvastatine	pravastatine
Hep G2 homogenaten		61	18	95
Hep G2 cellen	3.5	24	34	1900
Hepatocyten	3.5	4.1 ± 1.3	8.0 ± 4.2	2.0 ± 0.1
	18	n.d	23.0 ± 10.0	105 ± 30
Endotheel cellen	3.5	2.4 ± 0.5	5.5 ± 2.5	1172 ± 247
	20	79.3 ± 12.8	56.1 ± 35.4	8520 ± 3673
Cornea fibroblasten	3.5	15.0 ± 4.8	4.6 ± 2.0	1340 ± 595
Retina pigment epitheel cellen	3.5	17.5 ± 9.1	8.0 ± 2.4	4119 ± 2173
Granulosa cellen	3.5	27.0 ± 15.5	16.3 ± 10.3	1539 ± 779
Gladde spiercellen	3.5	15.6 ± 4.6	7.7 ± 4.2	5869 ± 2255
Myoblasten	3.5	19 ± 6	4.0 ± 2.3	110 ± 38

ABBREVIATIONS

ACAT	acyl CoA-cholesterol acyltransferase
CE	cholesteryl esters
CEH	cholesteryl ester hydrolase
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
DMEM	Dulbecco's modified Eagle's medium
ER	endoplasmatic reticulum
FC	free cholesterol
FCS	foetal calf serum
FSH	follicle stimulating hormone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCF	human cornea fibroblasts
HDL	high density lipoproteins
HGC	human granulosa cells
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HPL	hepatic lipase
HRPEC	human retinal pigment epithelial cells
HUVEC	human umbilical vein endothelial cells
HS	human serum
HSMC	human smooth muscle cells
IDL	intermediate density lipoproteins
KCN	potassium cyanide
KR	Krebs-Ringer solution
LCAT	lecithin:cholesterol acyltransferase
LDL	low density lipoproteins
LDL-C	low density lipoprotein cholesterol
LH	luteinizing hormone
LIMA	left internal mammary artery
LPDS	lipoprotein depleted serum
LPL	lipoprotein lipase
MD	mitochondrial dehydrogenase
MEM	minimal essential medium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NEM	N-ethylmaleimide
oligoA	oligomycine A
PBS	phosphate buffered saline
PL	phosholipids
rem	chylomicron remnant
SRE	sterol regulatory element
SREBP	sterol regulatory element binding proteins
TCA	trichloroacetic acid
TG	triglycerides
VLDL	very low density lipoproteins
WE	Williams E medium

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 2 februari 1963 om 9:53 uur des morgens te Leiden. Na het doorlopen van de MAVO en respectievelijk de HAVO te Grave, volgde zij van 1981 tot 1984 een HBO-B opleiding tot Biochemisch analiste. Haar stageplaats bevond zich destijds aan de Rijksuniversiteit van Limburg. Hier werd hartbeschadiging veroorzaakt door een hartstilstand aan de hand van het vrijkomen van enzymen na reperfusie van het hart volgens Langendorff onderzocht.

Na een jaar werkzaam te zijn geweest aan onderzoek op het Nederlands Kanker Instituut te Amsterdam - targeting van drugs via liposomen ter bestrijding van longkanker - werd in 1985 gestart met een studie Biologie aan de Rijksuniversiteit Groningen te Groningen. Deze studie werd in 1990 afgesloten met het behalen van de bul. Het laatste deel van deze periode bestond uit werkzaam zijn op drie verschillende stageplaatsen. De eerste stageplaats was een 8 maanden durende studie bij de vakgroep Microbiologie, op zoek naar de sleutelpositie en zuivering van D-aminozuur oxidase in de metabole afbraak van D-alanine als enige koolstof-, stikstof- en energie bron, in peroxisomen van de gist Candida boidinii. De tweede stageplaats was een 7 maanden durend onderzoek aangaande de activatie van rat Kupffer cellen met vrij of liposomaal ingesloten immuunmodulatoren tot tumor-toxiciteit en tumor necrosis factor secretie *in vitro*, uitgevoerd bij de vakgroep Fysiologische Chemie van de universiteit. De derde stageplaats was een onderzoek naar de physiologische consequentie van het overproduceren van een heterogeen eiwit in Escherichia coli, verricht gedurende 5 maanden bij Shell Research Limited te Sittingbourne (Engeland).

Van 1991 tot 1994 was Arlène van Vliet werkzaam als Assistent In Opleiding aan de Rijksuniversiteit Leiden en gedetacheerd bij het Gaubius Laboratorium TNO-PG te Leiden. Het verrichte onderzoek aldaar staat beschreven in dit proefschrift.