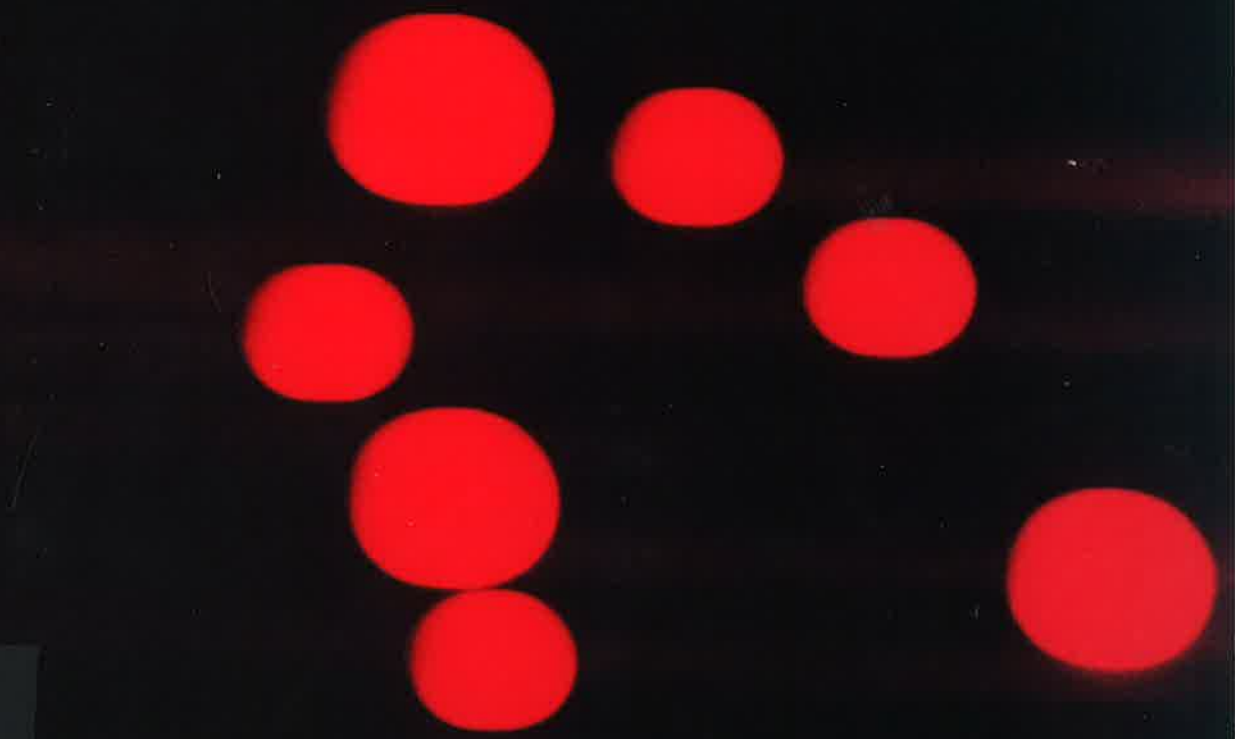
Regulation of the Synthesis and Secretion of Apolipoprotein A–I, Apolipoprotein B100 and Lipoprotein(a) in Cultured Hepatocytes



Allard Kaptein

Regulation of the synthesis and secretion of apolipoprotein A-I, apolipoprotein B100 and lipoprotein(a) in cultured hepatocytes

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STELLINGEN

behorende bij het proefschrift

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In het openbaar te verdedigen op dinsdag 22 juni 1993
des namiddags te 16.15 uur door

Allard Kaptein

1. De suggestie van Cianflone et al. dat alleen de beschikbaarheid van cholesterol esters bepalend is voor de secretie van apolipoproteïne B100 door hepatocyten, is gebaseerd op een niet adekwate experimentele opzet.
Cianflone et al. J. Lipid Res. 1990; 31: 2045-2055

2. De experimenten op basis waarvan Jevnikar et al. de hypothese postuleren dat cyclosporine A de secretie stimuleert van apolipoproteïne B100-bevattende lipoproteïnen door de lever, sluiten geenszins uit dat cyclosporin A juist de klaring remt van deze lipoproteïnen.
Jevnikar et al. Transplantation 1988; 46: 722-725

3. Een correcte vouwing van apolipoproteïne B100 tijdens de synthese in hepatocyten is van belang voor de uiteindelijke hoeveelheid gesecreteerd eiwit.

4. Het verdient aanbeveling om bij de presentatie van de Lp(a) concentratie in serum of plasma, duidelijk te vermelden of hiermee de totale Lp(a) massa of de apo(a) massa wordt bedoeld.

5. De sterke genetische controle van de Lp(a) concentratie in het bloed betekent niet dat het niveau van dit lipoproteïne niet reguleerbaar is.

6. De bevinding dat apolipoproteïne A-I in het medium van Hep G2 cellen en primaire apehepatocyten voornamelijk als een lipide-arm deeltje wordt gevonden en niet als bestanddeel van HDL_2 of HDL_3, zoals in het bloed, zegt meer over hoe apolipoproteïne A-I *in vivo* door de lever wordt uitgescheiden dan over eventuele beperkingen van deze modelsystemen voor studies naar de produktie van apolipoproteïne A-I.

7. Bij het onderzoek naar de rol van transcriptiefactoren bij de expressie van verschillende genen, worden vaak supra-fysiologische concentraties van de transcriptiefactoren gebruikt, waardoor de vraag blijft of deze factoren *in vivo* ook van belang zijn.

8. Het oordeel dat wordt gegeven over een bevinding of mening hangt af van de sympathie voor de bron.
gebaseerd op "De theorie van de opinïerende identificatie-reflex" van Dirk Ayelt Kooiman

9. Bij het verdwijnen van de landsgrenzen binnen Europa zijn minstens zo veel nieuwe grenzen ontstaan.

10. De uitdrukking van Martin Luther King "Oog om oog maakt iedereen blind" zou onder ogen moeten worden gebracht van iedereen die ziende blind is.

11. Een negatief beeld is voor een fotograaf niet altijd negatief.

12. Plantaardig is niet altijd mensaardig.

Regulation of the synthesis and secretion of apolipoprotein A-I, apolipoprotein B100 and lipoprotein(a) in cultured hepatocytes

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op dinsdag 22 juni 1993
te klokke 16.15 uur

door

Allard Kaptein

geboren te Den Ham (Ov.) in 1962

Promotiecommissie:

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	Prof.dr. D.D. Breimer
	Dr. A.F.H. Stalenhoef (Katholieke Universiteit Nijmegen)

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Kaptein, Allard

Regulation of the synthesis and secretion of
apolipoprotein A-I, apolipoprotein B100 and
lipoprotein(a) in cultured hepatocytes / Allard Kaptein.
- [S.l. : s.n.]
Proefschrift Leiden. - Met lit. opg.
ISBN 90-5412-012-6 geb.
Trefw.: levercellen ; stofwisseling

The study presented in this thesis was performed at the IVVO-TNO, Gaubius Laboratory, and at the former Gaubius Institute TNO, and was supported by the Netherlands Heart Foundation (grant 87.035).
Financial support by the Netherlands Heart Foundation and the Gaubius Laboratory for the publication of this thesis is gratefully acknowledged.

Cover design: Anja van der Poel

Anderen knippen met hun vingers, zie:
Er valt een wonder uit hun hand.
Ik zwoeg gestaag, verbrand mijn energie,
.....

.....
Toch kan ik mijn geluk bijna niet op.

(Fragment uit het gedicht "Zondagskind" van Gerrit Komrij
uit de bundel "De os op de klokketoren".)

CONTENTS

CHAPTER 1
General Introduction

THE LIPID METABOLISM

Introduction

During the last decades scientific effort has been focussed on finding therapies to lower the risk for atherosclerosis, as this disease is the major cause of death in the Western Society. Important risk factors for the development of atherosclerosis are elevated levels of cholesterol and/or triglycerides. In humans cholesterol is a physiologically important compound, used for the synthesis of plasma membranes, steroid hormones and bile acids, while triglycerides are sources of energy for muscle and various other tissues. Excess triglycerides may be stored in adipose tissues. Although these two lipids are essential components of the human body, high levels are threatening because of their relation with atherosclerosis [1-4].

Cholesterol and triglycerides are hydrophobic molecules, and insoluble in a hydrophilic environment like the blood compartment. Because of their hydrophobicity cholesterol and triglycerides are transported in the blood together with proteins in a complex, called lipoproteins. Lipoproteins are spherical particles composed of a polar outer shell of phospholipids, free cholesterol and proteins (named apolipoproteins), whereas the core is composed of the non-polar triglycerides and cholesteryl esters. The lipoproteins are divided into four major classes according to their buoyant density, determined by ultracentrifugation: chylomicrons, very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL). These lipoproteins also differ in size, composition, electrophoretic mobility (Table 1) and the presence of different apolipoproteins of which several types have been characterized (Table 2). In addition to these major lipoproteinfractions, another lipoprotein particle can be found in humans, i.e. lipoprotein(a) (Lp(a)), which is composed of LDL and a high molecular weight glycoprotein called apolipoprotein(a).

The lipoprotein metabolism (Fig. 1) can be divided into three different pathways: 1) the metabolism of exogenous lipids (dietary fat), 2) the metabolism of endogenously synthesized lipids and 3) the cholesterol transport from peripheral tissues to the liver (reverse cholesterol transport, for reviews see references 5-9). The metabolism of the lipids and apolipoproteins on Lp(a) does not seem to interact with the three pathways mentioned above and will be discussed separately.

Exogenous lipid transport

Dietary triglycerides and cholesterol are absorbed and packed in the intestine into chylomicrons. Chylomicrons, the largest and least dense lipoproteins, are very rich in triglycerides and contain apolipoprotein (apo) A-I, apo A-IV and apo B48. The chylomicrons are secreted into the lymph. Upon entering the circulation, the chylomicrons

Table 1. Composition of human plasma lipoprotein classes. (ref. 10-16).

	Chylomicron	VLDL	LDL	HDL	Lp(a)
Density (g/ml)	< 0.96	0.96-1.006	1.04 (1.019-1.063)	1.063-1.21	1.06 (1.03-1.10)
Size (nm)	75-1200	30-80	18-30	5-12	25
Electrophoretic mobility	Origin	pre-β	β	α	pre-β
Triglycerides (% weight)	80-95	45-65	4-8	2-7	3-11
Phospholipids (% weight)	3-6	15-20	18-24	26-32	15-22
Free cholesterol (% weight)	1-3	4-8	6-8	3-5	6-8
Cholesteryl esters (% weight)	2-4	6-10	45-50	15-20	32-50
Proteins (% weight)	1-2	6-10	18-22	45-55	20-26
Major apolipoproteins	A-I, A-IV, B48, C-II, C-III, E	B100, E, C-I C-II, C-III	B100	A-I, A-II, E	B100, apo(a)

Table 2. Properties and functions of the major apolipoproteins (ref. 10,12,15,17-19).

Apolipoprotein	Molecular weight (kD)	Plasma concn. (g/l)	Metabolic function
A-I	28.3	1.00-1.20	LCAT activation, HDL formation
A-II	17.4	0.30-0.50	HL inhibitor
A-IV	44.5	0.12-0.20	LCAT activator
B48	264.0	0.03-0.05	Chylomicron formation
B100	549.0	0.70-1.00	LDL-receptor ligand, VLDL and LDL formation
C-I	6.6	0.04-0.06	LCAT activator (moderate)
C-II	8.9	0.03-0.05	LPL activator
C-III	8.8	0.12-0.14	LPL and HL inhibitor
E	34.2	0.03-0.05	ligand for remnant and LDL-receptor
apo(a)	300-700	0.0-0.3*	?

*Isoform of molecular mass 500 kD

HDL = high density lipoprotein, LDL = low density lipoprotein, VLDL = very low density lipoprotein, LPL = lipoprotein lipase, HL = hepatic lipase, LCAT = lecithin:cholesterol acyl transferase.

loose part of their apo A-I and apo A-IV and acquire apo C-I, apo C-II, apo C-III and apo E. Simultaneously, the triglycerides are rapidly hydrolyzed by the enzyme lipoprotein lipase (LPL), present on the endothelial surface, with apo C-II serving as a co-factor. Apo A-I, apo A-IV and excess surface phospholipids are split off from the chylomicrons as surface

remnants to form HDL, whereas additional apo E is transferred from HDL to chylomicrons. The resulting chylomicron remnants are taken up by the liver probably via a still unidentified apo E receptor (also named remnant receptor). The dietary cholesterol taken up by the liver through this pathway is utilized for the synthesis of bile acids and the assembly of VLDL.

Endogenous lipid transport

Triglycerides and cholesterol are secreted from the liver into the circulation in the triglyceride-rich lipoprotein VLDL, containing the apolipoproteins B100, C-I, C-II, C-III and E. The VLDL particles are hydrolyzed by LPL, resulting in the formation of intermediate density lipoprotein (IDL, also called VLDL-remnants). These IDL particles can be cleared from the circulation via the LDL-receptor or the apo E-receptor. The IDL particles that remain in the circulation are further hydrolyzed into LDL by a second lipolytic enzyme, hepatic lipase (HL). During this hydrolysis, IDL loses the apo C's and apo E, leaving apo B100 as the sole protein on LDL. Apo B100 serves as the ligand for receptor-mediated clearance of LDL from the circulation via the LDL-receptor, present on liver cells but also on extra-hepatic tissues (e.g. muscle, adipose tissues, steroid hormone producing glands, skin). LDL may also be modified, for instance by oxidation [20]. This modified LDL is cleared from the circulation by Kupffer cells in the liver, via the scavenger receptor, but also by macrophages in the intima leading to foam cell formation in the intimal layers of the bloodvessels. The latter process is thought to be one of the major initial steps in the formation atherosclerotic lesions [21,22].

Reverse cholesterol transport

Cholesterol present in extra-hepatic cells can be transported to the liver by the lipoprotein HDL. Nascent HDL is secreted by the liver as lamellar disks, containing apo A-I, apo A-II and phospholipids. Nascent HDL increases in size by transfer of surface remnants from chylomicrons and VLDL which are released from these particles during lipolysis. Subsequently, HDL takes up free cholesterol from extra-hepatic tissues. This cholesterol is esterified by the enzyme lecithin:cholesterol acyltransferase (LCAT), which needs apo A-I as a co-factor. This finally results in a small spherical HDL particle called HDL_3. Further uptake of cholesterol, and the action of LCAT leads to the formation of the larger, more cholesteryl ester-rich HDL_2. The cholesteryl esters in HDL_2 are exchanged for triglycerides of VLDL, IDL and LDL, by the action of the cholesteryl ester transfer protein (CETP). HDL_2 can be converted back to HDL_3 by depletion of triglycerides by the action of HL. The cholesteryl esters transferred to VLDL, IDL or LDL can be taken up by the liver via the internalization of these lipoproteins. HDL_2 may also release its cholesteryl esters directly to the liver. To do so HDL_2 binds to the liver via the apo A-I specific

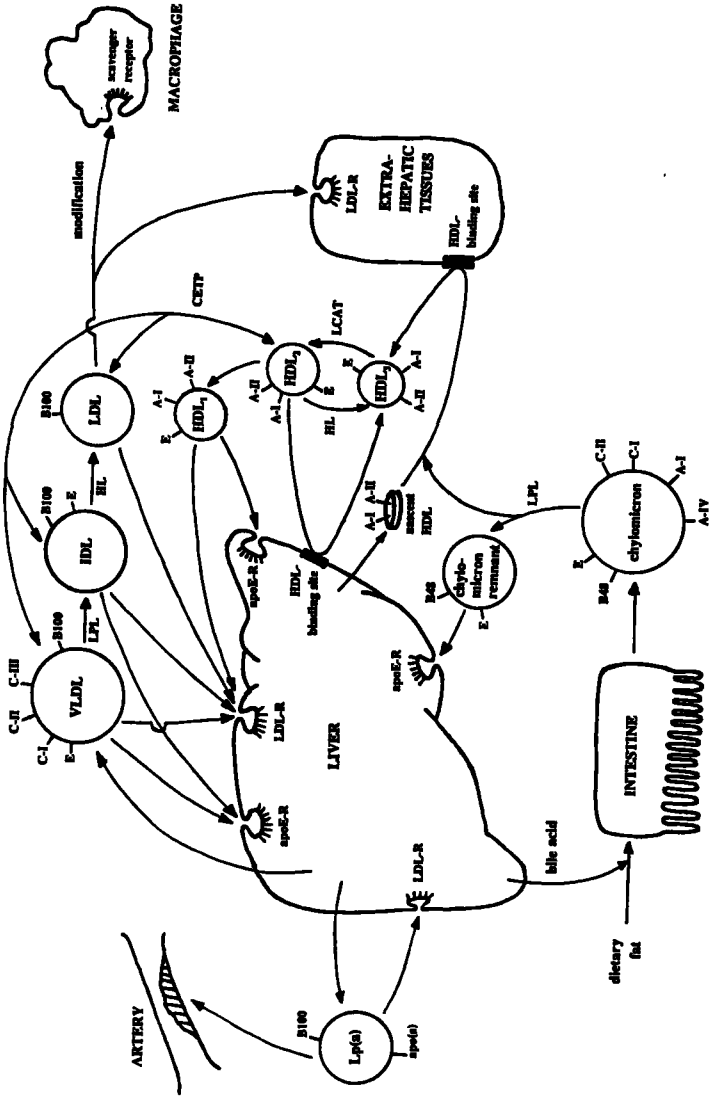

Figure 1. Schematic representation of the metabolic pathways of lipoproteins. The apolipoproteins present on the different lipoproteins are indicated on the lipoproteins. LPL = lipoprotein lipase, HL = hepatic lipase, LCAT = lecithin:cholesterol acyl transferase, CETP = cholesterol ester transfer protein, LDL-R = LDL-receptor, apoE-R = remnant receptor.

binding site (also called HDL-receptor) and leaves the liver again after the release of cholesteryl esters [23,24]. Another possible pathway for the uptake of cholesteryl esters, present in HDL, by the liver, is by conversion of HDL_2 into HDL_1 which is enriched in apo E. This apo E-rich HDL_1 can be cleared from the circulation via the LDL-receptor or the apo E receptor.

Lipoprotein(a)

The metabolic pathway of the lipids and apolipoproteins on lipoprotein(a) (Lp(a)) does not seem to interact with the lipid pathways described above. The glycoprotein apolipoprotein(a) (apo(a)) in Lp(a) is synthesized (almost) exclusively by the liver [25,26]. At present it is still unknown whether the liver secretes Lp(a) as a lipoprotein or only the protein apo(a) or both. Lp(a) can be taken up by the liver or extra-hepatic tissues via the LDL-receptor, although the affinity of Lp(a) for the LDL-receptor is lower than that of LDL [27-33]. Apo(a) and Lp(a) were also found associated with triglyceride-rich lipoproteins [34-37]. It has been suggested that Lp(a) present in triglyceride-rich lipoproteins may be cleared from the circulation via the scavenger receptor pathway [34,37]. Lp(a) also binds to glycosaminoglycans present in the human aorta [38] or proline- or hydroxyproline-rich proteins [39] that are exposed to the circulation after disruption of the endothelium (e.g. collagen and elastin). The latter findings may explain why Lp(a) is present in atherosclerotic lesions in relatively larger amounts than other lipoproteins [40,41]. Apo(a) shows strong homology to plasminogen both at DNA and protein level [42,43]. Apo(a) consits of the protease domain, kringle 5 domain and a variable number of kringle 4 domains of plasminogen. The number of kringle 4 domains determines the size polymorphism of apo(a) (see section "Regulation of Lp(a) levels" for additional information). Lp(a) and apo(a) compete with plasminogen for binding to the plasminogen receptor [44-46], and interact with fibrin or fibrinogen on the same site where plasminogen binds [47-49]. Interaction of Lp(a) with the plasminogen receptor present on the endothelium of bloodvessels, increases the risk that Lp(a) passes the endothelium. This provides an additional explanation for the preferential accumulation of Lp(a) in the intima. When Lp(a) competes with the binding of plasminogen to fibrin, Lp(a) may prevent the degradation of thrombi by plasmin. This indicates that Lp(a) not only is an atherosclerotic risk factor but also a thrombolytic risk factor (for reviews see refs. 15,50,51).

REGULATION OF LEVELS OF APO A-I, APO B100 AND Lp(a)

Introduction

Elevated levels of cholesterol are associated with an increased risk for coronary heart disease [1,2]. The risk of cholesterol on the development of atherosclerosis depends on the distribution of cholesterol over the various lipoproteins. In humans, under normal conditions, the majority (60-70%) of plasma-cholesterol is present in LDL. Elevated levels of LDL-cholesterol are associated with increased incidence of coronary heart disease [52]. In contrast, elevated levels of HDL-cholesterol are associated with a reduced risk [53]. High plasma levels of Lp(a) are positively associated with the development of coronary heart disease and cerebrovascular disease in men and women, especially when plasma levels exceed 0.20-0.30 g/l [54-57]. An even higher risk has been reported when high Lp(a) levels are associated with increased levels of total cholesterol or LDL-cholesterol [30,57].

Next to the distribution of cholesterol over the various lipoproteins, the relation between the apolipoprotein concentration and coronary heart disease has been investigated. Especially the levels of apo A-I, the major protein on HDL, and apo B100, the sole protein on LDL were studied. These apolipoproteins are the most abundant apolipoproteins in human blood. Just as the cholesterol levels of the lipoproteins of which they are a constituent the concentration of apo A-I and apo B100 are negatively and positively associated, respectively, with the risk of developing coronary heart disease. In various studies apo A-I and apo B100 showed to be better predictors for atherosclerosis than the cholesterol level of the lipoproteins of which they are a constituent [10,58-62].

Regulation of the hepatic synthesis or secretion of apo A-I and apo B100

The above indicates that regulation of the secretion of apo A-I and apo B100 containing lipoproteins may be a powerful tool in manipulating the risk for coronary heart disease. Changes in plasma levels of apo A-I and apo B100 by current therapies may be indicative for a possible effect of such a therapy on the secretion of these two apolipoproteins. However, next to synthesis and secretion the clearance of apo A-I and apo B100 from the circulation controls the plasma levels of these two apolipoproteins. The synthesis and secretion of apo A-I and apo B100 can be studied *in vivo* with the use of stable isotopes or by kinetic studies. These type of experiments are difficult to interpret and do not allow the investigation of molecular factors that may affect the synthesis and secretion of apo A-I and apo B100. Study of the regulation of the synthesis and secretion of apo A-I and apo B100 in the organs that synthesize these two apolipoproteins may lead to a better insight. Apo A-I is synthesized by the liver and intestine, whereas apo B100 is synthesized only by the liver. In order to investigate the secretion of these two apolipoproteins simultaneously, we focussed our attention on the liver. Regulation of the hepatic synthesis or secretion of apo

A-I and apo B100 has been studied by analysis of the liver after *in vivo* experiments, in perfused livers and in hepatocyte cultures (both primary cultures of human and animal origin, and with the human hepatoma cell line Hep G2). In the next two paragraphs the potential regulatory effects will be discussed of: 1) lipids (including the effects of hypolipidemic drugs applied in clinical practice) and 2) hormones.

Lipids

Apolipoproteins are secreted from the liver as a constituent of lipoproteins. Apo A-I may be secreted lipid-free or in a lipid-poor particle [63-65], but apo B100, which is not soluble in a hydrophilic environment, must be assembled, together with lipids into a lipoprotein. The synthesis and further processing of apo B100 in the endoplasmic reticulum is illustrated in Fig. 2 (for reviews see references 8,66-68). During translation apo B can be diverted into two distinct directions; 1) integration into the cytoplasmic site of the endoplasmic reticulum membrane [69,70] which leads to its degradation or 2) translocation of apo B across the endoplasmic reticulum membrane, followed by integration in the lumenal site of the endoplasmic reticulum membrane [70,71]. The apo B100, that is now at the lumenal site of the endoplasmic reticulum, is assembled together with lipids into a VLDL particle before it is ejected into the secretory pathway. In studies with primary cultures of rat hepatocytes or the human hepatoma cell line Hep G2 the major part of apo B that is synthesized is not secreted but intracellularly degraded [69,72-77]. Next to an impaired translocation [76] this may be caused by a limited availability of lipids.

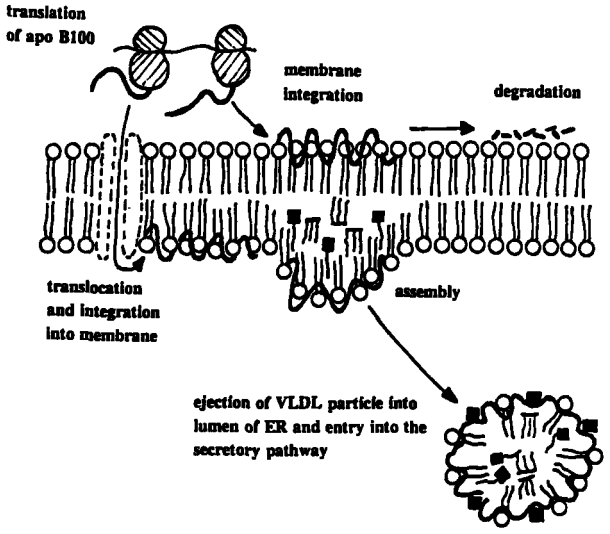

Figure 2. Schematic representation of the assembly VLDL in the endoplasmic reticulum (adapted from Davis [66]). = phospholipid, = triglyceride, ■ = cholesterol, = cholesteryl ester.

One type of the lipids that is assembled together with apo B100 into a lipoprotein particle are phospholipids. In primary cultures of rat hepatocytes, isolated from rats with choline deficiency, the apo B secretion was inhibited [78]. In rats with the choline deficiency, the phospholipid phosphatidylcholine is not synthesized, indicating that the secretion of apo B is dependent on the synthesis of phosphatidylcholine.

Another type of lipids that is assembled together with apo B100 before it is secreted from the hepatocyte are triglycerides. Intracellular levels of triglycerides in hepatocytes and secretion of triglycerides from hepatocytes can be increased by incubation of the cells with fatty acids or butyrate. Perfusion of livers from fasted rats [79] in the presence of oleic acid or incubation of Hep G2 cells with oleic acid or butyrate [64,65,69,77,80] not only resulted in an increased secretion of triglycerides but also of apo B. The apo B100 mRNA level in Hep G2 cells was not affected by oleic acid or butyrate [77,80]. Pulse-chase studies showed that triglycerides formed from oleic acid and butyrate partly prevent the intracellular degradation of apo B100 [75,77].

Also cholesterol and cholesteryl esters, which form the apo B100-containing lipoprotein, are secreted by hepatocytes. In *in vivo* studies with humans [81,82], miniature pigs [83] and rats [84], where the synthesis of cholesterol was inhibited by the HMG CoA-reductase inhibitor lovastatin, there was a decreased secretion of VLDL into the circulation. This observation is in accordance with the positive association between HMG-CoA reductase activity and VLDL secretion in rats *in vivo* and in perfused livers from rats [85] and the inhibition of the apo B100 secretion in Hep G2 cells by lovastatin [86] or simvastatin [87]. An increased secretion of apo B100 was found in perfused livers from rats fed a cholesterol-rich diet [88] and in primary human hepatocytes [89] and Hep G2 cells [90] loaded with cholesterol. Similar to the intracellular triglycerides concentration, an elevated cellular cholesterol content in Hep G2 cells does increase the apo B100 secretion by inhibiting the intracellular degradation of apo B100 [74]. Since cholesterol is stored in hepatocytes as cholesteryl esters, it may well be that not cholesterol but cholesteryl ester levels do regulate the apo B secretion. This contention is supported by a reduced secretion of apo B100 from Hep G2 cells when the cholesteryl ester synthesis is blocked by inhibiting the enzyme ACAT (acyl-CoA:cholesterol acyltransferase) [86].

However, there have also been reports showing that oleic acid did not affect the apo B secretion in Hep G2 cells [91-93] or rat hepatocytes [94-96], indicating that the availability of triglycerides may not be that important. The same comment can be made for the importance of the cellular content of cholesterol or cholesteryl esters. In Hep G2 cells where the cholesterol or cholesteryl ester synthesis was inhibited [80,97] or in Hep G2 cells loaded with cholesterol alone [92] no effect was found on the secretion of apo B100.

The discrepancies in the regulatory effect of triglycerides, cholesterol and cholesteryl esters on the secretion of apo B100 in cultured hepatocytes may result from the different

experimental conditions used by the investigators. A regulatory effect of one of the lipids will be only observed when there is no limitation in the availability of the other lipids, indicating that a mixture of various lipids are essential for regulating the apo B secretion from hepatocytes. This view is in accordance with the stimulation of the apo B secretion when cholesteryl esters and triglycerides are added together, in chylomicrons or β-VLDL, to Hep G2 cells [92] or primary rabbit hepatocytes [98]. In the same study [92] it was shown that the intracellular content of solely triglycerides or cholesterol in Hep G2 cells did not result in an increased secretion of apo B100.

Apo A-I secretion from cultured hepatocytes requires no or only small amounts of lipids. This indicates that the cellular availability of lipids for the assembly of lipoproteins will only have minor effects on the secretion of apo A-I. Both in Hep G2 cells [65,93] and rat hepatocytes [94] oleic acid did not alter the apo A-I secretion, indicating that triglycerides do not limit the apo A-I secretion. However, there are also reports showing that in animals the hepatic apo A-I mRNA level is affected by diets enriched with different oils. Olive oil fed rats had an enhanced apo A-I mRNA level as compared to corn oil fed rats [99], whereas in hepatocytes, isolated from fish oil fed rats the apo A-I mRNA was reduced [100]. In cebus monkeys an increased apo A-I mRNA level was observed in coconut oil fed group as compared to a corn oil fed group [101]. In the latter study this increase in the apo A-I mRNA was associated with an increased plasma level of apo A-I.

The cellular content of cholesterol seems not important for the secretion of apo A-I. Inhibition of the cholesterol synthesis in Hep G2 cells [97] or incubation of Hep G2 cells with LDL [74] or cholesterol rich liposomes [92] did not affect the apo A-I secretion. This is in accordance with *in vivo* experiments with rats [102] and monkeys [101,103] where a cholesterol rich diet did not affect the apo A-I synthesis. However, in one study where Hep G2 cells were incubated in the presence of LDL a small increase in the secretion of apo A-I was observed, associated with an enhanced apo A-I mRNA level [104].

As stated above HMG-CoA reductase inhibitors (vastatins) do not have a clear effect on the hepatic synthesis and secretion of apo B100 and have no effect on apo A-I secretion. Another set of hypolipidemic drugs, which have been investigated for their effect on the synthesis of apo A-I and apo B100, are the fibrates. Fibrates are used in clinical practice to lower the triglyceride level in hypertriglyceridemic patients. *In vivo* studies in humans indicate that the most important effect of fibrates is an increased fractional catabolic rate of VLDL [105-107]. This may be caused by a stimulation of lipoprotein lipase and hepatic lipase activity [108-110]. Additionally, fibrates reduce the catabolization of LDL in hypertriglyceridemic patients [106,107,111]. For bezafibrate an inhibition of the synthetic rate of VLDL was found in humans *in vivo* [107]. This observation is in accordance with an inhibition of the apo B100 secretion by Hep G2 cells treated with clofibrate, fenofibrate or gemfibrozil [112,113]. A comparable effect was found for bezafibrate in primary human

hepatocytes [114]. There is some discrepancy whether the fibrates do have a direct effect on the synthesis of apo B100. With clofibrate and fenofibrate no effect was observed on the synthesis but only on the accumulation of apo B100 in the culture medium [112], whereas with gemfibrozil also a lower apo B100 mRNA level was found [113].

In hypertriglyceridemic patients treated with fibrates, elevated HDL levels were observed. This effect may be secondary to an increased lipolysis of VLDL. However, an increased HDL level may also result from a stimulation of the synthesis of apo A-I by fibrates. *In vivo* studies with hypertriglyceridemic patients show an increased synthesis of apo A-I after treatment with gemfibrozil [108]. This observation is in accordance with the slightly stimulated synthesis of apo A-I in Hep G2 cells treated with fenofibrate and gemfibrozil [112,113], which was associated with an elevated apo A-I mRNA level [113]. Clofibrate did not affect the synthesis of apo A-I [112].

Until now it is not clear whether the effect of fibrates on the apo A-I and apo B100 synthesis is mediated through an effect of fibrates on lipid levels in particular triglycerides, or through a direct effect on the synthesis of both apolipoproteins.

Hormones

Various hormones have been investigated for their possible effect on the hepatic synthesis and secretion of apo A-I and apo B100. In Table 3 the effects of the hormones: insulin, the corticosteroid dexamethasone, steroid hormones, and the thyroid hormone, are listed. The changes in the synthesis and secretion of apo A-I and apo B100 by the different hormones are described in more detail in separate paragraphs underneath. As stated before, next to synthesis and secretion, the clearance of apo A-I and apo B100 from the circulation determines the plasma levels of these apolipoproteins. In order to get a better understanding of the effect of these hormones on the plasma levels of apo A-I and apo B100, a possible effect on the clearance of these apolipoproteins is also shown for the different hormones.

Insulin

The hormone insulin has a number of well known effects on the fatty acid metabolism, i.e. firstly, increased fatty acid synthesis; secondly, a stimulation of lipoprotein lipase in adipose tissue leading to an increased flux of fatty acids to adipose tissue and third, an increased flux of glucose into adipose tissues enhancing the reesterification of intracellular free fatty acids to triglycerides. Considering these effects of insulin on the fatty acid metabolism, this hormone may have a potential role in modulating lipoprotein synthesis and secretion. Various reports show that insulin inhibits the secretion of apo B by primary cultures of rat hepatocytes [73,115-118], human hepatocytes [119] and the human hepatoma cell line Hep G2 [64,80,93]. Sparks et al. [116] showed that insulin inhibits the VLDL

Table 3. Effects of hormones on the hepatic synthesis and secretion of apo A-I and apo B100

3A. apo A-I

Hormone	System or animal	Effect on apo A-I	Reference
Insulin	Rat hepatocytes	Increased secretion	123
	Rat hepatocytes	Increase in mRNA	124
	Rat hepatocytes	Decreased secretion	125
	Hep G2 cells	Decreased secretion	64
Dexamethasone	Rat hepatocytes	Increased secretion	123,125
	Rat hepatocytes	Increase in mRNA	124,125
	Hep G2 cells	Increased secretion	132
	Hep G2 cells	Increase in mRNA	132
	Rats	Increase in hepatic mRNA level	134
17-β estradiol	Hep G2 cells	Increased secretion	144
	Hep G2 cells	Increase in mRNA	145
ethinylestradiol	Rats	Increase in hepatic mRNA level	146,147
testosterone	Hep G2 cells	Antagonizes the effect of 17-β estradiol	144,148
thyroid hormone	Rats	Increase in hepatic synthesis	156-160
	Rats	Increase in transcription apo A-I gene	157,158

3B. apo B100

Hormone	System or animal	Effect on apo B	Reference
Insulin	Rat hepatocytes	Decreased secretion	73,115-118
	Human hepatocytes	Decreased secretion	119
	Hep G2 cells	Decreased secretion	64,80,93
Dexamethasone	Rat hepatoctyes	Increased secretion	118
	Hep G2 cells	Decreased secretion	132
	Rats	No or minor effect on hepatic mRNA level	134
17-β estradiol	Hep G2 cells	Increase in mRNA	148
estrogen	Avians	Increased production	149
	Rats	Increased production	150
	Humans	Increased synthetic rate VLDL	140,151
thyroid hormone	Hep G2 cells	Increased secretion	163
	Hep G2 cells	Increase in mRNA	163
hypothyrodism	Rats	Increase hepatic mRNA level	160
hyperthyrodism	Rats	No change in hepatic mRNA level	160

secretion, but at the same time stimulates the triglyceride synthesis. The inhibition of the VLDL secretion by insulin may be a result of an altered partitioning of triglycerides in the hepatocyte, in a storage pool (cytoplasm) and a secretory pool (VLDL). A decrease of triglycerides in the secretory pool may lead to an increased intracellular degradation of apo B [73,117]. Reduced plasma concentrations of apo B-containing lipoproteins may also be the result of an increased clearance of these lipoproteins from the circulation. An enhanced LDL-receptor activity was observed in rat hepatocytes [12,120] and Hep G2 cells treated with insulin [121]. In Hep G2 cells this increase in the LDL-receptor activity was accompanied by an enhanced LDL-receptor mRNA level [122].

The effect of insulin on the hepatic synthesis and secretion of apo A-I is not clear. Stimulation of the apo A-I secretion [123] and apo A-I mRNA level [124] have been reported in primary rat hepatocytes. This is in complete contrast with the decreased apo A-I secretion in Hep G2 cells [64] and in a second study with rat hepatocytes [125].

Corticosteroids

Another set of hormones that have been investigated frequently for a possible effect on apolipoprotein synthesis are the corticosteroids. Treatment of humans with corticosteroids does increase plasma levels of HDL-cholesterol and apo A-I but also of VLDL- and LDL-cholesterol and apo B [126-131]. For apo A-I these changes can be explained by an enhanced hepatic synthesis. The synthetic corticosteroid dexamethasone stimulated the apo A-I secretion in primary rat hepatocytes [123,125] and Hep G2 cells [132]. The increased secretion was associated with an elevated apo A-I mRNA level [124,125,132]. Next to dexamethasone, other corticosteroids, i.e. triamcinolone and hydrocortisone, and adrenocorticotropin (ACTH), which stimulates the corticosteroid production, also increased the plasma levels of apo A-I in rats *in vivo* [133,134]. For hydrocortisone, dexamethasone and triamcinolone the increased plasma levels of apo A-I were accompanied by an enhanced hepatic apo A-I mRNA level [134].

Corticosteroids only have minor effects on the hepatic apo B mRNA level in rats [134], whereas the apo B secretion in Hep G2 cells was inhibited by dexamethasone [132]. This is in contradiction with the increased secretion of VLDL triglyceride [135] and secretion of newly synthesized apo B [118] by rat hepatocytes treated with dexamethasone. The increased plasma VLDL and LDL levels in patients treated with corticosteroids are, most likely, not caused by an altered hepatic synthesis and secretion of apo B but by an inhibited LDL-receptor mediated binding and uptake of VLDL and LDL [120,136,137].

Steroid hormones

In women the risk for coronary heart disease is lower than in men of the same age. This seems to be related to the elevated plasma HDL-cholesterol concentration [138] and the

reduced LDL-cholesterol level [139]. The increased serum apo A-I levels after estrogen treatment in humans [140-143] suggests that steroid hormones may regulate the synthesis of apo A-I. Treatment of Hep G2 cells with 17-β estradiol resulted in an increased secretion of apo A-I [144], which was associated with an enhanced apo A-I mRNA level [145]. This is in accordance with the elevated hepatic apo A-I mRNA level in rats treated with ethinylestradiol [146,147]. The androgen testosterone antagonized the effect of 17-β estradiol on the secretion of apo A-I by Hep G2 cells [144,148].

Next to apo A-I, 17-β estradiol stimulated the secretion of apo B100 in Hep G2 cells by increasing the apo B100 mRNA level [148] but only at high concentration of 17-β estradiol. These findings are in accordance with the increased production of VLDL and apo B in response to high doses of estrogen in avians [149] and rats [150]. Similar results were observed for the synthetic rate of VLDL in humans [140,151]. However, estrogens also stimulated the clearance of VLDL and LDL from the circulation [151,152]. The overall effect of estrogens *in vivo* is a lowering of the concentration of apo B-containing lipoproteins in the blood. The increased clearance of VLDL and LDL from the circulation can be explained by an upregulation of the LDL-receptor [146,152-155].

Thyroid hormone

The last hormone that has been studied frequently for its effect on apolipoprotein synthesis is thyroid hormone. In rats the hepatic apo A-I synthesis is positively correlated with the thyroid hormone level [156-160]. The increased synthesis of apo A-I is regulated at the transcriptional level [157,158].

Thyroid hormone has an intriguing effect on the synthesis of either apo B100 or apo B48. In humans apo B100 is synthesized only in the liver, whereas apo B48 is synthesized only in the intestine [161]. Thyroid hormone administration to rats resulted in a simultaneous synthesis of apo B100 and apo B48 by the liver [156,162]. The hormone regulates the postranscriptional editing of the apo B mRNA leading to the synthesis of either apo B100 or apo B48. Whether thyroid hormone affects the total amount of apo B synthesized remains to be elucidated. Theriault [163] observed a stimulation of the apo B100 secretion in Hep G2 cells treated with thyroid hormone, which could be explained, in part, by an increased apo B mRNA level. This is in contradiction with the elevated hepatic apo B mRNA level in hypothyroid rats, whereas in hyperthyroid rats the apo B mRNA level was not affected [160].

The effect of thyroid hormone on the LDL-receptor activity may be more important for the concentration of apo B-containing lipoproteins in the circulation. In rats [164,165] hypothyroidism leads to a decreased catabolic rate of LDL, whereas the hepatic LDL receptor mRNA level in rats increased after thyroid hormone administration [160]. This is in accordance with the increased LDL-receptor activity in rat hepatocytes after thyroid

hormone treatment [12,166], which was accompanied by an increased LDL-receptor mRNA level [167].

Regulation of Lp(a) levels

As mentioned earlier plasma levels of Lp(a) vary strongly between individuals. The differences in Lp(a) levels can be explained partially by the different isoforms of apo(a). These isoforms differ in size and are encoded by different alleles at a single LPA gene locus on the long arm of chromosome 6 [168]. This size polymorphism is directly correlated with the number of kringle 4 domains in apo(a) and inversely correlated with the plasma level of Lp(a) [169-173]. The size polymorphism can explain 19-70% of the variability of the Lp(a) levels found [174,175], whereas the genetic control accounts for greater than 90 % of the variation in Lp(a) levels [176-178]. Other genetic factors affecting Lp(a) levels may be the degree of glycosylation of apo (a) [179] and the apolipoprotein E phenotype [180].

However, this does not mean that Lp(a) levels can not be regulated by non-genetic factors. Until now a limited number of studies have been published in which changes in plasma Lp(a) levels were analyzed after dietary and pharmacological intervention.

Treatment of persons with nicotinic acid (analogues) [181-183] and fibrates [184,185] decreased the plasma levels of Lp(a). These drugs are normaly used to lower triglyceride levels in hypertriglyceridemic patients, suggesting that the metabolism of triglycerides and Lp(a) may be associated. In population studies a significant negative correlation was observed between plasma Lp(a) levels and triglycerides [54]. The same trend was observed by others [178,186-188]. This again suggests a relation between the metabolism of Lp(a) and triglycerides. This contention was also supported by studies in which the diet is supplemented with different fatty acids. In humans a diet enriched in palm oil [189] or fish oil [190,191] lowered Lp(a) levels. However, in other studies with fish oil or n-3 polyunsaturated fatty acids no effect was seen on the Lp(a) level [192,193]. In a diet study with monkeys changes in the amount of saturated or unsaturated fatty acids had no effect on plasma Lp(a) levels [194]. The latter findings do not support the hypothesis that the metabolism of Lp(a) and triglycerides are associated.

In population studies a positive correlation was observed between cholesterol and Lp(a) levels [54,56,174]. This observation supports the finding that Lp(a) is taken up via the LDL-receptor, although less efficiently than LDL [27-33]. However, when the plasma level of total cholesterol was corrected for the amount of cholesterol present in Lp(a), the correlation between cholesterol and Lp(a) disappeared [195] or was present only in females [178]. Treatment of hypercholesterolemic patients with HMG-CoA reductase inhibitors (vastatins) either had no effect or increased the Lp(a) level [196,197]. Leren et al. [198] reported that prolonged treatment of hypercholesterolemic patients with lovastatin resulted

in a reduced Lp(a) level. In the latter study the changes in Lp(a) did not correlate with the changes in LDL-cholesterol level. These observations are in contradiction with the possible involvement of the LDL-receptor in the clearance of Lp(a) from the circulation.

In males treated with estrogen [199,200] reduced Lp(a) levels were observed. The decrease in the Lp(a) levels did not correlate with the reduction in LDL-cholesterol, again suggesting that the LDL-receptor activity is not decissive for the Lp(a) level in the circulation. As mentioned earlier estrogens do upregulate the LDL-receptor [146,153-155].

Other compounds that reduce Lp(a) levels are the anabolic steroid stanozolol [201] and ethanol [202]. N-acetylcysteine, which cleaves the disulfide bridge between apo(a) and apo B100 was also reported to lower the Lp(a) levels in the circulation [203]. However, others showed that N-acetylcysteine only had a limited capacity to lower Lp(a) levels [204].

Leren et al. [198] suggest that the inhibitory effect of prolonged treatment of hypercholesterolemic patients with lovastain on plasma Lp(a) levels is caused by a decreased production, rather than by an increased clearance. This is accordance with other reports [205,206] showing that *in vivo* plasma levels of Lp(a) were predominantly a result of the synthetic rate. Because until now no good *in vitro* model was available it was impossible to study the regulation of the hepatic synthesis and secretion of Lp(a) or the binding and uptake of Lp(a). Two *in vitro* systems do synthesize Lp(a), the primary hepatocyte cultures from baboons [207] or cynomolgus monkeys [208]. Future research with these model systems, and maybe other cell culture systems, will be important for a better understanding of the regulatory mechanisms involved in the synthesis and secretion, or the clearance of Lp(a) from the circulation.

OUTLINE OF THIS THESIS

The studies presented in this thesis were initiated by the question whether the synthesis and secretion of apo A-I and apo B100 by hepatocytes can be regulated and how regulation takes place. The first aim of the study was to find compounds which may be of interest for regulation of the synthesis of (apo)lipoproteins. The second aim of the study was to get a better understanding of the regulatory mechanisms that are involved in the synthesis and secretion of apo A-I and apo B100. During the investigations also Lp(a) was included in the studies. For Lp(a) the attention was focussed on the exploration of a suitable model system that could be used to study the secretion of Lp(a).

Two model systems were used to investigate the synthesis and secretion of apo A-I, apo B100 and Lp(a) by hepatocytes; i.e. the human hepatoma cell line Hep G2 and primary hepatocyte cultures from the cynomolgus monkey (*Macaca fascicularis*).

Using the human hepatoma cell line Hep G2 we investigated the regulatory mechanism

involved in the effect of sodium butyrate on the secretion of apo A-I and apo B100 (Chapter 2 and 3).

A second study with Hep G2 cells is described in Chapter 4, in which the effect of the immunosuppressive drug cyclosporin A was investigated. Cyclosporin A is widely used in organ transplantation. Treatment of patients with this drug results in an increase in the LDL-cholesterol level. In the study described in chapter 4 we investigated whether this increase in LDL might be caused by an increased hepatic production of apo B100-containing lipoproteins.

With primary hepatocyte cultures from the cynomolgus monkey and Hep G2 cells we investigated the effect of retinoids (vitamin A and vitamin A analogues) on the secretion of apo A-I and apo B100. The mechanism of regulation and the time course of the regulatory effect of retinoids in cynomolgus hepatocytes was further investigated (Chapter 5 and 6).

Primary hepatocyte cultures from cynomolgus monkeys were also investigated for the synthesis of Lp(a) and the possibility of regulating this synthesis (Chapter 7).

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CHAPTER 2

BUTYRATE STIMULATES THE SECRETION OF APOLIPOPROTEIN A-I AND APOLIPOPROTEIN B100 BY THE HUMAN HEPATOMA CELL LINE Hep G2. INDUCTION OF APO A-I mRNA WITH NO CHANGE OF APO B100 mRNA.

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Biochemical Journal 1991; 278: 557-564.

Also published as a capsule in:

- Molecular Biology of Atherosclerosis. Proceedings of the 57th European Atherosclerosis Society Meeting, ed. M.J. Halpern, John Libbey & Company Ltd., 1992, 87-90.
- Clinical Biochemistry 1992; 25: 317-319.

ABSTRACT

Addition of sodium butyrate to the medium of the human hepatoma cell line Hep G2 resulted in a time- and dose-dependent increase in the secretion of apolipoprotein A-I (apo A-I) and apolipoprotein B100 (apo B100). After a 24 h preincubation period, a 2.4- and 2.2-fold increase in the secretion of apo A-I and apo B100, respectively, was obtained during the next 24 h in the presence of 2 mM sodium butyrate. Secretion of albumin, fibrinogen or [^{35}S]-methionine labelled, newly-synthesized proteins was unaffected or only marginally affected, indicating that the effect of butyrate on apo A-I and apo B100 is not part of a general effect on protein synthesis and secretion. In structure-function studies butyrate was found to be the most potent inducer among various straight-chain carboxylic acids. Hydroxylated, aminated, and otherwise modified butyrate-derivatives were inactive.

The enhanced accumulation of apo A-I and apo B100 in the culture medium could not be explained by changes in the uptake and degradation of the synthesized apolipoproteins or by alterations in the secretion of possible intracellular pools. In addition, [^{35}S]-methionine incorporation studies indicated that synthesis and/or secretion of newly-synthesized apo A-I and apo B100 is enhanced in the presence of butyrate. The apo A-I mRNA level was increased 2.3-fold upon treatment with 2 mM butyrate for 48 h, suggesting regulation at (post-)transcriptional level. In contrast, no change in the level of apo B100 mRNA in butyrate-treated cells was observed, indicating regulation at translational or co- or post-translational level. We propose that the effect of butyrate on the secretion of apo A-I and apo B100 by Hep G2 results from two different regulatory mechanisms.

INTRODUCTION

Plasma levels of low density lipoprotein (LDL) cholesterol and high density lipoprotein (HDL) cholesterol are, respectively, positively and negatively related to the development of atherosclerosis [1-3]. The proteins which are part of these lipoproteins, i.e. the apolipoproteins, are also used in risk-assessment for cardiovascular disease. The plasma levels of apolipoprotein B100 (apo B100) and apolipoprotein A-I (apo A-I) have been reported to be even more discriminatory in determining the risk on developing coronary heart disease than the cholesterol concentration in the lipoprotein from which they are a constituent, i.e. LDL and HDL [4-6].

A vast number of reports describes regulation of the synthesis and secretion of apo A-I and apo B100, as a way of altering the amounts and composition of lipoproteins in the blood. Since the liver is the central organ in the metabolism of these two apolipoproteins,

many of the studies were done with perfused livers and primary cultures of hepatocytes from animal origin, due to the limited availability of human liver. More recently, the human hepatoma cell line Hep G2 has been used as a model to study the apolipoprotein and lipoprotein synthesis and catabolism in human hepatocytes. This cell line has been shown to synthesize a number of apolipoproteins, including apo A-I and apo B100 [7]. Apo A-I is secreted as a HDL-like particle, but is also found in a lipid-poor or lipid-free form [8], whereas apo B100 is secreted as a constituent of a LDL-like particle enriched in triglyceride. In this respect, secretion of apo B100 differs from the *in vivo* situation, possibly as a result of the reduced amount of smooth endoplasmatic reticulum in Hep G2 cells [8], which may lead to a defective lipoprotein assembly and secretion in these cells [9]. Furthermore, the availability of fatty acids may be of importance [10]. Nevertheless, the Hep G2 cell line was found to be a suitable model for investigating the regulation of the synthesis and/or secretion of apo A-I and apo B100 in response to lipoproteins [11,12], fatty acids [9,10,13-15], insulin [9,13,14], and steroid hormones [16].

In many of these reports supply of lipids and changes in the assembly of lipoproteins are thought to be responsible for the alterations in the synthesis and secretion of apo A-I and apo B100. In search for compounds, which may affect the synthesis and/or secretion of these two apolipoproteins by another regulatory mechanism, we investigated the effect of butyrate. Butyrate is known to induce specific alterations in different cell types, including hepatoma cell lines and hepatocytes (for a review see Kruh [17]). One of the most evident changes brought about is the hyperacetylation of histones [18] due to inhibition of the enzyme histone deacetylase [19]. This process has been shown to affect gene expression [20-23].

In this paper we describe the effects of butyrate on apo A-I and apo B100 secretion by Hep G2 cells. The secretion of these proteins into the medium was found to be enhanced more than two-fold upon incubation with butyrate. This increase was accompanied by a comparable stimulation of the apo A-I mRNA without a change in the apo B100 mRNA level.

METHODS

Materials

α-[^{32}P] dCTP (3000 Ci/mmol), [^{35}S]-methionine (> 1000 Ci/mmol), and Na-[^{125}I] (100 mCi/ml) were obtained from Amersham International plc (Buckinghamshire, England, U.K.). Sodium butyrate and butyrate analogues (as listed in Table 3 in the "Results" section) were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.) except for sodium acetate which was purchased from Merck (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) was purchased from Flow

Laboratories (Irvine, Scotland, U.K.), foetal bovine serum, penicillin, and streptomycin were from Boehringer Mannheim, (Mannheim, Germany).

Cell culture

The established Hep G2 cell line, derived from a human liver tumour, was obtained from Dr. B.B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA, U.S.A.). The cells were cultured as described [24] at 37°C, under air/CO_2 (19/1), in 25 cm^2 flasks containing 0.1-0.2 ml of culture medium/cm^2. Standard medium used was DMEM supplemented with 20 mM Hepes, 10 mM $NaHCO_3$, 10% (v/v) foetal bovine serum (heat inactivated for 30 min at 56°C), 2 mM glutamin, penicillin (100 U/ml), and streptomycin (100 μg/ml). The medium was renewed twice a week. At 4-5 days before the start of the experiment, the cells were treated with trypsin and transferred to six-well-cluster plates (10 cm^2/well, Costar Cambridge, MA, U.S.A.) with a split ratio of 1:6, and were grown to confluency before starting the experiments, in order to study the effect of butyrate on protein synthesis, which is not due to arrest of cell growth.

Except where otherwise described the effect of sodium butyrate and related analogues was studied over a 24 h period, after a 24 h preincubation with the same compound at the same concentration. At the end of the incubation period, medium was collected and centrifuged for 1-2 min in an Eppendorf centrifuge (type 5414) to remove detached cells and debris. The supernatant was frozen immediately in liquid nitrogen and stored at -20°C, until measurement of apo A-I and apo B100 concentration. Cells were washed three times with cold PBS (phosphate buffered saline; sodium/potassium phosphate buffer (11 mM, pH 7.5) containing 150 mM NaCl) and were harvested by scraping in water. The cellular protein was determined according to Lowry [25].

Enzyme-linked immunosorbent assay (ELISA) of apo A-I

Specific IgGs against human apo A-I were isolated from goat antiserum (raised in our institute) by affinity chromatography using purified apo A-I crosslinked to Sepharose 4B (Pharmacia), according to Bury and Rosseneu [26]. Part of the isolated anti-apo A-I was conjugated to horse radish peroxidase (EC 1.11.1.7) by the SPDP (N-succinimidyl 3-(2-pyridyldithiopropionone) method, essentially according to the Pharmacia procedure. Immunoblotting of human serum and foetal bovine serum, after sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis showed that anti-apo A-I was monospecific for human apo A-I.

The isolated anti-apo A-I and the peroxidase anti-apo A-I conjugate were used in a "sandwich" ELISA, according to the procedure described by Bury and Rosseneu [26], with a few modifications. In short, polystyrene micro-titre plates ("Nunc Immuno" plates, "high-binding" quality; Intermed, Roskilde, Denmark) were coated with anti-apo A-I, 10 μg IgG/ml in PBS, containing NaN_3 (1 g/l) (120 μl/well). Blocking of the residual binding sites was done with 0.1% (w/v) casein in PBS (150 μl/well). Samples for apo A-I determination were diluted in the same buffer as used for blocking (100 μl of diluted sample added per well). Peroxidase-anti-apoA-I conjugate was diluted in blocking buffer containing 0.05% (w/v) Tween 20 (100 μl of diluted conjugate added per well). The final IgG concentration of the peroxidase-anti-apo A-I conjugate used in the assay was 25 ng/ml. The peroxidase-labelled conjugate was visualized using 3,3',5,5'-tetramethylbenzidine and H_2O_2 as the substrate mixture [27].

The working range of the ELISA is 20-150 ng/ml and as little as 10 ng apo A-I/ml can be

detected. The standard curves for purified apo A-I, apo A-I in human serum, and apo A-I in the medium of Hep G2 cell cultures were parallel. Apo A-I concentration in sera using the ELISA were identical to values found with radial immunodiffusion (R.I.D.) [28].

Enzyme-linked immunosorbent assay (ELISA) of apo B

Specific IgGs against human apo B100 were isolated from rabbit antiserum (raised in our institute) by affinity chromatography, exactly as described above for the purification of anti-apo A-I, using purified, apo E-free LDL crosslinked to Sepharose 4B (Pharmacia). LDL was purified from human serum by density gradient ultracentrifugation according to Redgrave et al. [29]. The LDL was aspirated from the gradient between densities 1.030 and 1.050 g/ml, adjusted with KBr to a density of 1.21 g/ml, layered with a KBr solution in water, of the same density, and ultracentrifuged to further purify and concentrate LDL. SDS/polyacrylamide gelelectrophoresis of the LDL showed that apo B100 was the only protein present in the isolated LDL, as determined by silver staining according to Paleologue [30]. SDS/polyacrylamide gel electrophoresis was performed under reducing conditions according to the method of Laemmli [31], with resolving gels containing a gradient of 4-20% (w/v) acrylamide and stacking gels of 3.5% (w/v) acrylamide. Immunoblotting of human serum and foetal bovine serum, after SDS/polyacrylamide gelelectrophoresis, showed that the isolated antibodies were monospecific for human apo B.

Part of the isolated anti-apo B was conjugated to horse radish peroxidase and used together with anti-apo B in a "sandwich" ELISA. The ELISA procedure was similar to the ELISA procedure for apo A-I, except that the final IgG concentration in peroxidase-anti-apo B conjugate was 50 ng/ml. The working range of the assay was 100-1000 ng apo B/ml and as little as 50 ng /ml could be detected. The standard curves for LDL (isolated from human serum by density gradient ultracentrifugation), apo B in human serum, and apo B in medium of Hep G2 cells were parallel. Apo B concentration measured in sera using the ELISA showed a linear correlation with values found with R.I.D. [32].

Association and degradation of apo B100 (LDL) and apo A-I (HDL)

Association and degradation of HDL and LDL by Hep G2 cells was measured according to Dashti et al. [33] and Goldstein et al. [34], respectively, over a 24-h period. The concentration of [^{125}I]-labelled apo B100 (in LDL) and apo A-I (in HDL) added to the medium was 2.0 μg/mg cell protein and 2.5 μg/mg cell protein, respectively, as measured by ELISA procedure. These concentrations are comparable to the amounts of apo A-I and apo B100 secreted by Hep G2 cells during a 24-h incubation in control medium.

Measurement of the secretion of albumin and fibrinogen

Accumulation of albumin in the medium of Hep G2 cell cultures was measured by rocket immunoelectrophoresis according to the method described by Laurell [35] using rabbit anti-human albumin (Dakopatts, Glostrup, Denmark).

Accumulation of fibrinogen in the medium was measured using an ELISA procedure as described [36]. Degradation of fibrinogen was prevented by adding trasylol and heparin to the medium, both at a concentration of 10 U/ml. This addition did not affect basal or butyrate stimulated secretion of apo A-I and apo B100 in the culture medium.

Protein synthesis

Overall secretion of newly synthesized proteins was measured essentially as described by Kooistra et al. [37]. The metabolically radiolabelled proteins secreted in the medium were analyzed by SDS/polyacrylamide gel electrophoresis under reducing conditions as described above.

Protein Mr standards (Bio-Rad, Richmond, CA, U.S.A.) were used for calibration of the gel. For autoradiography the gel was treated with an autoradiography enhancer (En^{3}Hance, NEN DuPont, Boston, MA, U.S.A.) according to the manufacturers' instructions, dried and placed on X-ray film (Kodak X-Omat AR films, Eastman-Kodak Company, Rochester, NY, U.S.A.), and stored at -80°C for the appropriate time.

RNA hybridization

Total RNA was isolated from Hep G2 cells according to the method of Chomczynski and Sacchi [38]. After washing the RNA pellets with 70% (v/v) ethanol, RNA samples were dissolved in H_2O. The RNA concentration in each sample was determined spectrophotometrically, assuming that one A260 unit is 40 μg/ml RNA.

Equal amounts of total RNA from different incubations were fractionated by electrophoresis in a 0.8% (w/v) agarose gel containing 1 M formaldehyde, and transfered to Hybond N (Amersham) according to the manufacturers' instructions. RNA-blots were hybridized with different probes at 65°C in a sodium phosphate buffer (0.5 M, pH 7.5), containing 7% (w/v) SDS and 1 mM EDTA. DNA fragments used as probes were isolated from low melting point agarose [39]. One blot was hybridized with 25 ng of probe, labelled by the random primer method (Multi-prime, Amersham) to approximately 2 x 10^8-10^9 cpm/μg DNA.

After hybridization, the blots were washed twice with 2 x SSC/0.1% SDS (30 min at 65°C) (1 x SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), twice with 1 x SSC/0.1% SDS (30 min at 65°C), and twice with 0.1 x SSC/0.1% SDS (30 min at 65°C), successively. The blots were exposed to Hyperfilm MP (Amersham), together with an intensifying screen (Eastman-Kodak Company), for 15-75 h at -80°C. For quantification of the relative amounts of mRNA the autoradiographs were scanned with a Shimadzu CS 910 chromatograph scanner and areas under the peaks were integrated and plotted with the aid of a data processor (Shimadzu Corporation, Kyoto, Japan). Quantitation of the mRNA levels was done using three different amounts of total RNA loaded on the gel, giving a linear relation between the specific mRNA signal and the amount of RNA applied. For apo A-I and albumin mRNA linearity was present with 1.5, 3 and 6 μg of total RNA. For apo B100 and GAPDH mRNA there was linearity using 3, 6 and 12 μg of total RNA.

The following DNA fragments were used as probes in the hybridization experiments: a 2.2 kb Pst I fragment of the human apo A-I genomic DNA (kindly provided by Dr. S.E. Humphries [40]), a 2.7 kb Hind III fragment located at the 3' end of human apo B100 cDNA (kindly provided by Dr. J. Scott [41]), a 740 bp Hind III/Pst I fragment located at 3' end of human serum albumin cDNA, and a 1.2 kb Pst I fragment of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (kindly provided by Dr. R. Offringa [42]). The human serum albumin clone was obtained from Dr. H. Pannekoek, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands.

Statistical analysis

Statistical significance of differences was calculated using Student's t-test for paired data with the level of significance selected to be $p < 0.05$. Values are expressed as means ± S.D..

RESULTS

Time-course and dose-dependency of the effect of butyrate on the accumulation of apo A-I and apo B100 in the cellular medium of HepG2 cell cultures

In Fig. 1 a typical experiment is shown of the time-course of the butyrate effect on apo A-I and apo B100 secretion by Hep G2 cells. With control medium apo A-I and apo B100 increased linearly with time over a 48-h period. With 2 mM Na-butyrate present in the medium an accelerated accumulation of apo A-I and apo B100 was observed after a lag time of 16-24 hours. In two additional time-course experiments an identical lag time was seen both for apo A-I and apo B100 (data not shown). Therefore, in subsequent experiments the effect of butyrate on Hep G2 cell cultures was studied over a 24 h period, after a 24 h preincubation period in the presence of the same butyrate concentration.

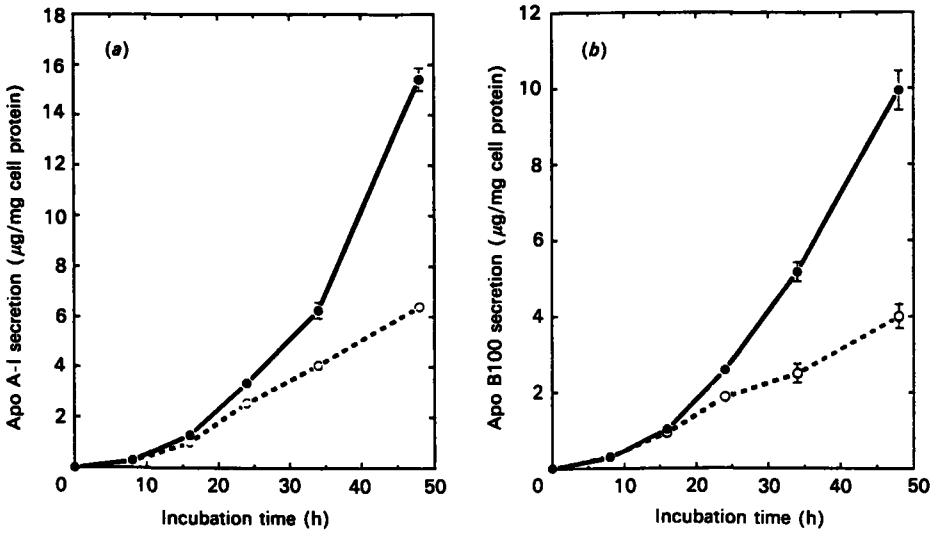

Figure 1. Time course of the effect of butyrate on apo A-I (*a*) and apo B100 (*b*) accumulation in the medium of Hep G2 cells. Hep G2 cells were cultured for 48 h on standard medium without (○) and with (●) sodium butyrate (2 mM). At various times, samples of medium were taken and analysed for apo A-I and apo B100. Apo A-I and apo B100 concentrations in the culture medium were measured as described in the Materials and Methods section. Data are from one representative of three independent experiments. The values are means ± range of triplicate assays of two separate wells.

As shown in Fig. 2, addition of increasing amounts of butyrate resulted in an increase in the secretion of apo A-I and apo B100 in the medium of Hep G2 cell cultures. The effect on apo A-I and apo B100 were comparable in magnitude for the various butyrate concentrations. A significant increase was only found with concentrations of 1 mM of Na-butyrate or higher (paired t-test, $p < 0.05$). Judging from the changes in cell morphology and loss of cells from the culture dishes, butyrate concentrations $\geq$ 3 mM were cytotoxic to Hep G2 cell cultures. Therefore, further experiments were performed at a concentration of 2 mM butyrate. At this concentration no adverse effect was observed on the cell morphology, cell protein present in the culture dishes at the end of the incubation, or on the release of the cytoplasmic enzyme lactate dehydrogenase.

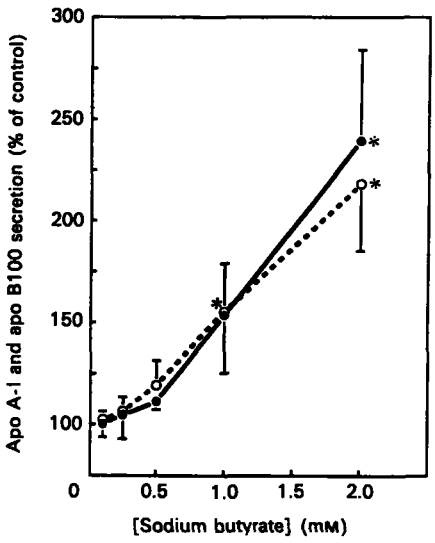

Figure 2. Effect of butyrate on the secretion of apo A-I and apo B100 in the medium of Hep G2 cell cultures. Hep G2 cells were incubated for 24 h with various concentrations of sodium butyrate, after a 24 h preincubation with the same concentration of sodium butyrate. Apo A-I and apo B100 concentrations in the culture medium were measured as described in the Materials and Methods section. Results were normalized for cell protein in culture dishes and are expressed as a percentage of control, and are means ± S.D. for three to five independent experiments. Apo A-I (●) and apo B100 (○) production by Hep G2 in control medium was 2.9 ± 1.2 and 1.9 ± 0.3 μg/24 h per mg of cell protein respectively (means ± S.D. for five experiments): *indicates a significant difference ($P < 0.05$) between control and treated cells.

Butyrate stimulates the secretion of newly-synthesized apo A-I and apo B100

Since Hep G2 cells are capable of binding and internalizing LDL [43] and HDL [33], inhibition of the uptake and degradation might have caused the observed changes in the accumulation of apo B100 and apo A-I in the culture medium. The results listed in Table 1 for the accumulation and for the binding and degradation of apo B100 and apo A-I show that uptake and degradation is far smaller than the accumulation in the medium of both apolipoproteins over a 24-h period. Addition of butyrate (2 mM) to the medium resulted in reduced binding and degradation of apo A-I and apo B100, but this does not explain the increases found in the accumulation.

Table 1. The effect of butyrate on the secretion and on the association and degradation of apo B100 (LDL) and apo A-I by Hep G2 cells.

	DMEM/FBS	DMEM/FBS + 2 mM Na-butyrate
apo B100 secretion* (μg apo B100/mg cell protein/24h)	1.9 ± 0.3	4.2 ± 0.7
[^{125}I]-apo B100 (LDL) cell associated** (μg apo B100/mg cell protein)	0.17 ± 0.01	0.10 ± 0.01
[^{125}I]-apo B100 (LDL) degradation** (μg apo B100/mg cell protein/24 h)	0.24 ± 0.01	0.16 ± 0.01
apo A-I secretion* (μg apo A-I/mg cell protein/24 h)	2.9 ± 1.2	7.0 ± 1.3
[^{125}I]-apo A-I (HDL) cell associated** (μg apo A-I/mg cell protein)	0.019 ± 0.001	0.016 ± 0.001
[^{125}I]-apo A-I (HDL) degradation** (μg apo A-I/mg cell protein/24 h)	0.007 ± 0.003	0.004 ± 0.001

The secretion, binding and degradation of apo A-I and apo B100 was measured over a 24-h period in the presence or absence of 2 mM Na-butyrate after a 24-h preincubation on the same medium. In binding and degradation experiments 2.0 and 2.5 μg/mg cell protein apo B100 (in LDL) and apo A-I (in HDL), respectively, were added to the medium of Hep G2 cells. * indicates the means ± S.D. for five experiments. ** indicates means ± S.D. for one experiment performed in quadruplicate.

In addition, changes in the secretion of possible intracellular storage pools of apo A-I and apo B100 can also not explain the differences in accumulation due to butyrate. After a 24-h incubation of Hep G2 cells in standard medium the apo A-I and apo B100 concentrations were measured in the culture medium and in the cells. The cells were solubilized in PBS (phosphate buffered saline) containing 0.5% Triton X100. Apo A-I and apo B100 were measured by the ELISA procedure. The addition of Triton X100 had no effect on the ELISA results. For apo A-I and apo B100 we found that 95% and 85-90%, respectively, of the total amount of the apolipoproteins was present in the medium. These experiments indicate that the effect of butyrate on the accumulation of apo A-I and apo B100 is caused by increases in the secretion of newly-synthesized apolipoproteins.

Specificity of the stimulation of apo A-I and apo B100 secretion by butyrate

To see whether the effect of butyrate on the secretion of apo A-I and apo B100 is part of a general effect of butyrate on Hep G2 cell cultures, secretion of albumin and fibrinogen

in the culture medium was measured. The results are shown in Table 2. Only minor differences were found for the secretion of albumin and fibrinogen upon incubation of cells in standard medium with 2 mM butyrate.

Table 2. The effect of butyrate on the secretion by Hep G2 cells of albumin, fibrinogen, and [^{35}S]-methionine labelled proteins.

	DMEM/FBS	DMEM/FBS + 2 mM Na-butyrate
albumin secretion (μg/mg cell protein/24h)	16.9 ± 1.3	13.2 ± 0.2
fibrinogen secretion (μg/mg cell protein/24h)	0.64 ± 0.03	0.77 ± 0.09
[^{35}S]-methionine incorporation in secreted proteins (% of incubation on standard medium)	100%	92 ± 7%

Hep G2 cells were incubated for 24 h in the presence or absence of 2 mM Na-butyrate after a 24-h preincubation on the same medium. Results are means ± range of two independent experiments, performed in duplicate.

In addition to the effect of butyrate on the albumin and fibrinogen secretion, the total protein secretion by Hep G2 cell cultures was studied using [^{35}S]-methionine incorporation in newly-synthesized and secreted proteins. Little effect was observed on the secretion of total newly-synthesized proteins (see Table 2). To explore the possibility that butyrate may alter the pattern of the proteins secreted into the culture medium, the medium of the experiment described above was separated on a SDS/polyacrylamide gel and autoradiographed (Fig. 3). Hep G2 cells incubated with butyrate secreted a similar pattern of proteins in the culture medium as control cells, with the exception of a few proteins (indicated by the arrows in Fig. 3) which showed an altered intensity. After specific immunoprecipitation the proteins with a molecular weight of approximately 500 and 28.5 kDa were identified as apo B100 and apo A-I, respectively.

Structural specificity studies

Table 3 shows the effects of various butyrate analogues, all tested at a concentration of 2 mM, on the secretion of apo A-I and apo B100 in the medium of Hep G2 cell cultures.

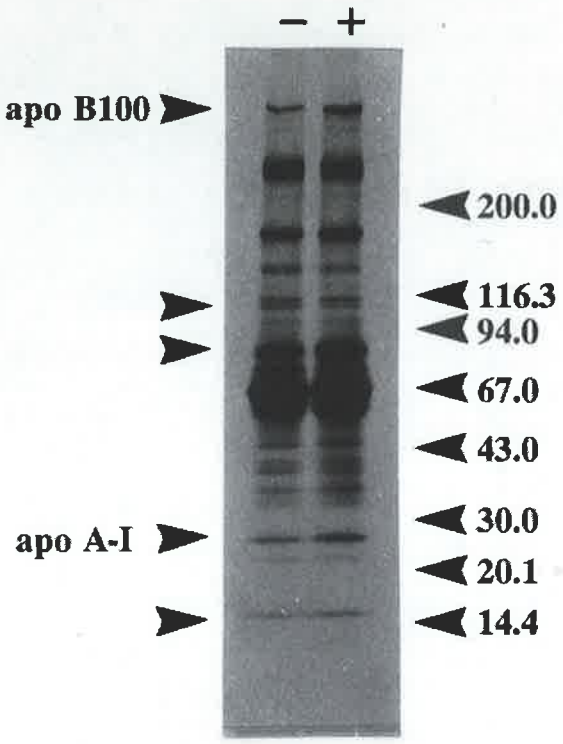

Figure 3. SDS/PAGE and autoradiography of metabolically radiolabelled proteins in the medium of Hep G2 cell cultures. Hep G2 cells were incubated for 24 h in standard medium with or without 2 mM sodium butyrate, containing 50 μCi of [^{35}S]-methionine/ml, after a 24 h preincubation in the same medium without labelled methionine. Molecular-mass standards (kDa) are indicated on the right of the autoradiograph. At the left side of the autoradiograph the positions of apo A-I and apo B100 and of other proteins with altered intensity after butyrate treatment are indicated. + or - indicates incubation with or without butyrate respectively.

The results for the various butyrate analogues used were comparable for apo A-I and apo B100. Butyrate was the most potent inducer. Other straight chain, unsubstituted carboxylic acids, propionate (C_3) and valerate (C_5) also gave a significantly increased secretion of both apolipoproteins. They were, however, less effective than butyrate. The straight chain carboxylic acids acetate (C_2) and hexanoate (C_6) were either uneffective or only marginally effective. No effects were found with hydroxylated or aminated butyrate derivatives, isobutyrate, the branched chain analogue of butyrate), crotonate (which contains a double band between the second and third carbon atom in comparison with butyrate), butanol, γ-butyrolactone (intramolecular lactone), 2-oxobutyrate, and succinate, all differently oxidated C_4-analogues.

Effect of butyrate on apo A-I and apo B100 mRNA levels

To observe whether the accelerated secretion of apo A-I and apo B100 results from changes in mRNA levels, the effect of butyrate on the mRNA level of both apolipoproteins was assessed by Northern blot hybridization (Fig. 4). Apo A-I mRNA levels increased with time, as compared with the albumin and GAPDH mRNA levels which were not affected. A significant increase of the apo A-I mRNA level was seen when Hep G2 cells were treated with 2 mM Na-butyrate for 16 h and reached maximally 2.3-fold stimulation after

48 h. In contrast to the apo A-I mRNA level the apo B100 mRNA level was not significantly affected by butyrate during the 48 h incubation period.

Table 3. Structural specificity of butyrate.

compound added to the medium	apo A-I secretion (% of control)	apo B100 secretion (% of control)
Acetate	101 ± 10	102 ± 13
Propionate	128 ± 13•	131 ± 22•
Butyrate	239 ± 45•	218 ± 33•
Valerate*	173 ± 23•	181 ± 47•
Hexanoate*	129 ± 26	124 ± 1
2-Hydroxybutyrate	101 ± 11	105 ± 11
3-Hydroxybutyrate	114 ± 12	106 ± 14
4-Hydroxybutyrate	120 ± 18	132 ± 18
2-Aminobutyrate	103 ± 10	94 ± 3
3-Aminobutyrate	98 ± 8	83 ± 9
4-Aminobutyrate (GABA)	107 ± 1	89 ± 9
Isobutyrate*	116 ± 26	125 ± 14
Crotonate*	106 ± 15	126 ± 20
Butanol	128 ± 29	123 ± 3
Butyrolactone*	110 ± 25	108 ± 6
2-Oxobutyrate	125 ± 5	128 ± 36
Succinate	98 ± 12	115 ± 15

Hep G2 cells were incubated with different butyrate analogues (2 mM) for 24 h, after a 24-h preincubation with the same compound at the same concentration. The concentration of apo A-I and apo B100 in the medium was measured as described in the section "Materials and Methods" and normalized for the amount of cell protein. Results are expressed as percentage of control and are means ± range for two experiments; for butyrate, valerate and propionate results are means ± S.D. for five experiments. Compounds indicated with * were dissolved in DMSO, final concentration DMSO in the medium 0.1% (v/v), and are expressed as a percentage of control medium containing 0.1% DMSO. Apo A-I and apo B100 secretion by Hep G2 in control medium was 2.9 ± 1.2 and 1.9 ± 0.3 μg/mg cell protein/24h, respectively (means ± S.D. for five experiments).
• indicates a significant difference ($p < 0.05$) between control and treated cells.

DISCUSSION

In this paper we show that butyrate increases the secretion of apo A-I and apo B100 by the human hepatoma cell line Hep G2, in a time- and dose-dependent way. This is not due to a reduced uptake and degradation of HDL (apo A-I) and LDL (apo B100) in the presence of butyrate nor to differences in the secretion from possible intracellular pools of apo A-I

and apo B100. It indicates that butyrate stimulates the synthesis and/or the secretion of newly-synthesized apolipoproteins. This was confirmed by the [^{35}S]-methionine incorporation experiment (Fig. 3).

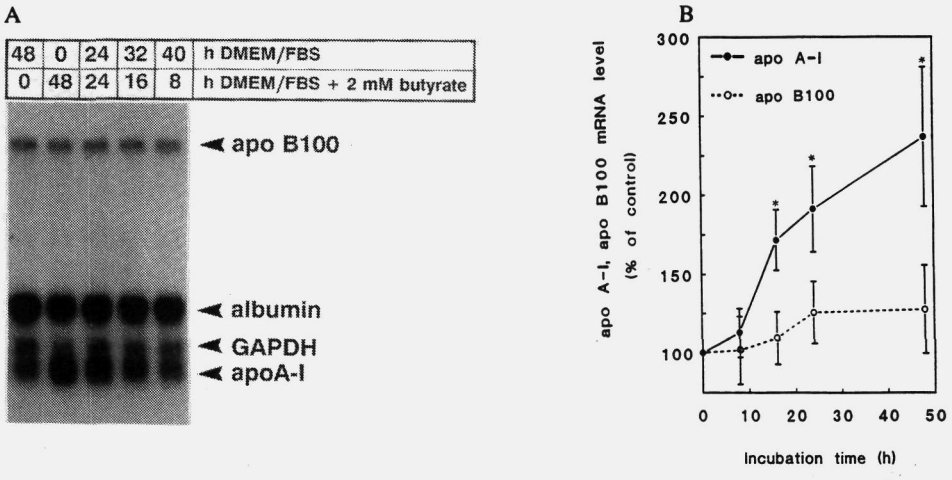

Figure 4. Effect of butyrate on apo A-I and apo B100 mRNA levels in Hep G2 cells. (*a*) Autoradiograph of a Northern blot hybridized with ^{32}P-labelled probes for apo A-I, apo B100, albumin and GAPDH mRNA in a single-step hybridization, with 3 μg of total RNA applied to the agarose/formaldehyde gel. The hybridized blot was exposed for 16 h, at -80°C, to Hyperfilm, by using an intensifying screen. Total RNA was isolated from Hep G2 cultured for various times (indicated on top of the autoradiograph) in culture medium containing 2 mM sodium butyrate. (*b*) Time course of the butyrate effect on apo A-I (•) and apo B100 (○) mRNAs. The intensity of the bands on autoradiographs (as shown, for example, in *a*) was assessed by densitometric scanning. Albumin was used as an internal standard to correct for differences in the amount of total RNA applied to the gel. The results are expressed as percentages of those in the incubation without butyrate and are means ± S.D. for three independent experiments, with analysis of three RNA concentrations in each experiment. * indicates a significant difference ($P < 0.05$) between control and treated cells. Similar results were found when corrected for the GAPDH mRNA signal (results not shown).

As illustrated by the secretion of albumin, fibrinogen and [^{35}S]-methionine labelled proteins, the effect of butyrate on apo A-I and apo B100 was not part of a general effect on proteins secreted by Hep G2. In addition, the stimulatory effect was specific for butyrate. Only propionate and valerate, the C_3- and C_5-straight chain carboxylic acids, respectively, induced a significant increase in the secretion of apo A-I and apo B100, although less markedly than butyrate. Similar quantitative differences were found with butyrate and a limited set of other straight-chain carboxylic acids tested on various functions in different cell types [17]. Kooistra et al. [37] studied a large set of butyrate analogues for their effect on the t-PA (tissue plasminogen activator) synthesis by cultured human endothelial cells. The stimulatory effect on the synthesis of t-PA was in the order of

butyrate > valerate > propionate. Our results on structure-function relationship are comparable with the reports mentioned above, suggesting that a common mechanism may be responsible for the effects described.

Such a mechanism may be acetylation and deacetylation of histones, which play a role in the regulation of the expression of specific genes by introduction of changes in the chromatin structure. Butyrate treatment of HeLa cells and Friend erythroleukemia cells [18] resulted in a hyperacetylation of the histones. Similar findings were described for a rat hepatoma cell line (HTC) by Sealy and Chalkley [19]. These authors showed that this hyperacetylation was due to inhibition of the enzyme histon deacetylase by butyrate. An effect of butyrate on gene expression may also be an explanation for the enhanced secretion apo A-I by Hep G2 cells.

Another possibility may be interference of butyrate with lipid synthesis and secretion. It has been reported that butyrate stimulates synthesis of fatty acids and triglycerides in rat hepatocytes [44,45] and hepatoma cells [46], with only a marginal effect on sterol synthesis [44,45]. Supply of triglycerides or fatty acids leading to an increased triglyceride synthesis did, however, not effect apo A-I secretion [13,47,48] by hepatocytes or the apo A-I mRNA level [14] in Hep G2 cells. Furthermore, apo A-I secretion was variably affected by cholesterol-rich liposomes and only slightly increased upon incubation with β-VLDL [11], indicating that supply of cholesterol has no or little effect on apo A-I secretion by Hep G2 cells. This is supported by the absence of an effect of the HMG-CoA reductase inhibitor CS-514 on apo A-I secretion [49] and the only 20% increase in the apo A-I mRNA level during supplementation of the medium with LDL [12]. In our experiments with butyrate we found a 2.3-fold increase in the apo A-I mRNA level after 48 h, which is comparable with the 2.4-fold increase in the secretion. In addition, the accelerated accumulation of apo A-I, as shown in Fig. 1, is in agreement with the time-course of the effect found on the mRNA level (Fig. 4B). These findings, together with the above-mentioned observations, do not favor the hypothesis that butyrate stimulates apo A-I secretion via a possibly altered lipid secretion and suggest an effect of butyrate on gene expression. However, an increase in the stability of the apo A-I mRNA cannot be excluded.

Although the overall effects of butyrate and butyrate-analogues on the secretion of apo A-I and apo B100 were similar, this needs not to result from the same regulatory mechanism. In contrast to apo A-I, butyrate did not affect the apo B100 mRNA level. Synthesis of apo B100 and/or secretion of newly-synthesized apo B100 was increased in butyrate-treated Hep G2 cells. After separating the culture medium, by density centrifugation, into lipoproteins (d < 1.20 g/ml) and a lipid-poor or lipid-free fraction (d > 1.20 g/ml), we found that cells incubated with or without butyrate secreted apo B100 only as a constituent of lipoproteins. Apo A-I was found both in the lipoprotein-rich and in the lipid-poor or lipid-free fraction (data not shown). The latter finding may be explained

by dissociation of apo A-I from the native discoidal lipoprotein particle after secretion, due to a reduced affinity of apo A-I for this particle in the absence of LCAT activity or as the result of direct secretion of lipid-poor apo A-I in the medium per se [8]. The increase in the secretion of apo A-I upon butyrate treatment was seen in both fractions. The observed distribution of apo B100 over lipoproteins and of apo A-I over the lipoprotein-rich and the lipid-poor fraction, secreted by Hep G2 cells, was in accordance with Thrift et al. [8]. The increased secretion of apo B100 may be regulated translationally (increased synthesis) or by the co- or post-translational processing of apo B100 or assembly of apo B100 into the lipoprotein. This co- or post-translational regulation has been described to be important in determining the amount of apo B100 secreted by Hep G2 and hepatocytes [50-53]. Lipid supply, i.e. fatty acid, triglycerides and cholesterol, was found to be of particular importance in this process by increasing apo B100 secretion [9,15,54]. Although we did not measure lipid synthesis, the finding that butyrate stimulates fatty acid and triglyceride synthesis in rat hepatocytes and hepatoma cells may provide a plausible explanation for the enhanced secretion of apo B100. However, as we show in the structure-specificity studies, propionate also increased the apo B100 secretion significantly and acetate had no effect. Nishina et al. [44] and Wright et al. [55] reported that propionate inhibited fatty acid synthesis and had no or an inhibitory effect on sterol synthesis, whereas acetate stimulated fatty acid synthesis and inhibited sterol synthesis [44,56]. These conflicting data suggest that alterations in lipid synthesis need not to be the only explanation for the increased apo B100 secretion.

Butyrate is present in human nutrition, e.g. dairy products, but it is unlikely that it is a suitable agent to affect apo A-I and apo B100 secretion by hepatocytes *in vivo*. Plasma concentrations of butyrate remain low in general because it is metabolized rapidly. However, the experiments with butyrate provide interesting data which may lead to a better understanding of the mechanism which controls the synthesis and secretion of apo A-I and apo B100 by hepatocytes.

ACKNOWLEDGEMENTS

We wish to thank Dr. Louis M. Havekes for the antisera against human apo A-I and apo B100, Dr. Willem Nieuwenhuizen for his helpful support in the measurement of fibrinogen, and Mrs. C. Horsting-Been and Miss M. Horsting for typing the manuscript.

This work was made possible by financial support from the Netherlands Heart Foundation (Grant 87.035).

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CHAPTER 3

BUTYRATE STIMULATES THE SECRETION OF APOLIPOPROTEIN B100-CONTAINING LIPOPROTEINS FROM Hep G2 CELLS BY INHIBITING THE INTRACELLULAR DEGRADATION.

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Submitted for publication.

ABSTRACT

We have shown previously that sodium butyrate induces a two-fold increase in the secretion of apo B100 by Hep G2 cells. The apo B100 mRNA level was not changed in butyrate-treated cells, indicating regulation at the translational or co- or post-translational level (Biochem. J. 1991; 278: 557-564). In this paper the mechanism by which butyrate increases apo B100 secretion was further investigated.

Pulse-chase analysis showed that the intracellular degradation of apo B100 in butyrate-treated Hep G2 cells decreased 1.8-fold with respect to control cells. In control incubations only 18 ± 4% of the total amount of labelled apo B100 present intracellularly after the 10 min pulse period, was secreted after a 90 min chase period. After addition of butyrate this amount increased to 32 ± 6%. Treatment of Hep G2 cells with butyrate resulted in an enhanced intracellular concentration of triglycerides (+ 30%), with no or only a marginal effect on the cellular content of cholesterol and cholesteryl esters. Secretion of triglycerides (+ 90%) and cholesteryl esters (+ 78%), but not of cholesterol, was increased to the same extent as apo B100 secretion (+ 102%). The total mass of triglycerides, i.e. the sum of triglycerides present intracellularly and secreted by Hep G2 cells, was significantly increased upon incubation with butyrate, whereas the total mass of cholesteryl esters was not affected. Butyrate did not affect the buoyant density of apo B100-containing lipoproteins secreted by Hep G2 cells. These results suggest that an increased availability of triglycerides, formed after the addition of butyrate regulates the amount of apo B100 degraded intracellularly and consequently apo B100 secretion.

INTRODUCTION

Plasma levels of low density lipoprotein cholesterol are positively related to the development of atherosclerosis [1,2]. Apolipoprotein B100 (apo B100), the major protein of LDL, has been reported to be even more discriminatory in determining the risk of developing coronary heart disease than the cholesterol concentration in LDL [3-5].

A vast number of reports describes regulation of the synthesis of apo B100, as a way of altering the amounts and composition of apo B100-containing lipoproteins. Many of these studies were performed using the human hepatoma cell line Hep G2 as a model to study the apolipoprotein and lipoprotein synthesis and catabolism in human hepatocytes. This cell line has been shown to synthesize a number of apolipoproteins [6] and to secrete discrete lipoprotein particles [7]. Before secretion apo B100 must be assembled with lipids, i.e. triglycerides, phospholipids, cholesterol and cholesteryl esters to form apo B100-containing lipoproteins. The availability of these lipids has been shown to be important in the

regulation of the apo B100 secretion [8-15].

In a previous paper [16] we have reported that treatment of Hep G2 cells with the short chain fatty acid butyrate results in an increased secretion of apo B100 and apo A-I. In contrast to apo A-I, the elevated secretion of apo B100 was not accompanied by an increase in the mRNA level. This observation indicates that butyrate stimulates the secretion of apo B100 at the translational or co- or post-translational level. In this study we have assessed the mechanism of regulation of the apo B100 secretion by butyrate. Since butyrate has been reported to stimulate the synthesis of fatty acids and possibly of cholesterol in rat hepatocytes [17,18], the potential involvement of lipids was also investigated.

We found that butyrate diminished the intracellular degradation of apo B100 and that this may be the result of an increased intracellular supply of triglycerides.

METHODS

Materials

Sodium butyrate was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Tran ^{35}S-label (> 1000 Ci/mmol), containing both [^{35}S]-methionine and [^{35}S]-cysteine, was obtained from ICN Biomedicals (ICN Biomedicals Inc., Irvine, Ca, U.S.A.) and [2-^{14}C]-acetate was purchased from Amersham International (Amersham, Bucks., U.K.). Dulbecco's modified Eagle's medium (DMEM) and L-glutamine were purchased from Flow Laboratories (Irvine, Scotland, U.K.). Foetal bovine serum (FBS), penicillin and streptomycin were from Boehringer Mannheim (Mannheim, Germany).

Cell culture

The established Hep G2 cell line, derived from a human liver tumour, was obtained from Dr. B.B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA, U.S.A.). The cells were cultured as described [16,19].

Incubations in the presence of sodium butyrate were performed in DMEM containing 10% (v/v) heat-inactivated FBS (30 min at 65°C) or 10% (v/v) lipoprotein depleted serum (LPDS). LPDS was prepared from heat-inactivated FBS by density ultracentrifugation as described [20]. The effect of sodium butyrate was studied over a 24-h period, after a 24 h preincubation period with the same medium. At the end of the incubation period, medium was collected and centrifuged for 20 sec (8000 x g) in a Heraeus centrifuge (Biofuge A) to remove detached cells and debris. The supernatant was frozen immediately in dry-ice and stored at -20°C until measurement of apo A-I and apo B100 concentrations. Cells were washed three times with cold (4°C) phosphate-buffered saline (PBS, sodium/potassium phosphate buffer: 11 mM, pH 7.5, containing 150 mM NaCl), were harvested by scraping in water, and the cellular protein was determined.

Pulse-chase analysis of apo A-I, apo B100 and albumin production

After two 24-h preincubation periods in DMEM containing 10% (v/v) FBS with or without 2 mM sodium butyrate, Hep G2 cells were used for pulse-chase studies using Tran [^{35}S]-label, as described

[21] with minor modifications. After a 30-min incubation on methionine and cysteine-free MEM (ICN Biomedicals Inc., Irvine, CA, USA), cells were pulsed with Tran [^{35}S]-label (100 μCi/ml) for 10 min. After the pulse period, the medium was removed, cells were washed three times with (chase medium) DMEM at 37°C and incubated with DMEM with or without 2 mM butyrate for 0, 10, 20, 35, 60 and 90 min. At the end of the chase-time cells and medium were harvested separately.

Cells were washed, at 4°C, three times with PBS, homogenized in 1 ml NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.0625 M sucrose, 0.5% Triton X-100, and 0.5% sodium deoxycholate) and protease inhibitors were added to the following final concentrations: 2.5 mM EDTA, 0.5 mM phenyl-methylsulfonyl fluoride, 25 μM p-hydroxymercuriphenyl sulfonic acid, 10 U/ml heparin, 10 U/ml trasylol, 50 μg/ml leupeptin, 50 μg/ml pepstatin). The medium was centrifuged for 20 sec (8000 x g) to remove detached cells and debris. The supernatant was mixed (1:1) with 2 x NET-buffer and protease inhibitors were added. Aliquots of 500 μl of cell extracts or 900 μl of the culture medium were combined with an excess of monospecific affinopurified goat anti-human apo A-I, rabbit anti-human apo B100 [16] and rabbit anti-albumin antiserum (Dakopatts, Glostrup, Denmark) and incubated for 16 h at 4°C. Then 75 μl of 50% solution of protein G-Sepharose (Pharmacia) in NET buffer was added, and incubated for 45 min at 4°C and 30 min at room temperature. The antigen-antibody protein G-Sepharose complex was pelleted by centrifugation (2 min, 4000 rpm), the supernatant was aspirated, and the pellet was resuspended in 500 μl NET buffer and washed by centrifugation through a 30% sucrose solution in NET-buffer (500 μl). After two additional washes with NET-buffer, 50 μl sample buffer was added, the samples were boiled for 5 min and subjected to SDS/PAGE under reducing conditions by the method of Laemmli [22], with resolving gels containing a gradient of 4-20% (w/v) acrylamide and stacking gels of 3.5% (w/v) acrylamide. Protein molecular mass standards (Bio-Rad, Richmond, CA, U.S.A.) were used for calibration of the gel. For autoradiography the gel was treated with an autoradiography enhancer (En-^{3}Hance, NEN DuPont, Boston, MA, U.S.A.) in accordance with the manufacturers' instructions, dried and placed on X-ray film (Kodak X-Omat AR films; Eastman-Kodak Co., Rochester, NY, U.S.A.), and stored at -80°C for the appropriate time. Apo B100, apo A-I and albumin were localized on the dried gel after autoradiography. Bands containing apo B100, apo A-I and albumin were sliced from the gel and the gel fragments were solubilized in 1 ml of a NH_3/H_2O_2 solution (0.94% (w/v) NH_3, 7.5% (v/v) H_2O_2) for 16 h at 55°C and counted for radioactivity.

Measurement of the mass of intracellular and extracellular triglycerides, cholesterol and cholesteryl esters

After two 24-h incubation periods in the presence or absence of butyrate, Hep G2 cells were washed five times with cold phosphate buffer saline (pH 7.4). Thereafter, the cells were harvested by scraping and homogenized by sonication (Branson, 60 W, 20 S). Samples were taken for measurement of protein content. Lipids were extracted from the cell suspension as described by Bligh & Dyer [23], after addition of cholesterol acetate (2 μg) as an internal standard. The neutral lipids were separated by high-performance t.l.c. on silica-gel-60 precoated plates as described [19].

When DMEM containing 10% (v/v) LPDS was used as the culture medium, the mass of triglycerides, cholesterol and cholesteryl esters secreted in the medium was also measured. At the end of the incubation the medium was centrifuged (12000 rpm for 30 min at 4°C) to remove detached cells and cell debris. Extraction and analysis of lipids were identical as for the cell suspension. Blank

culture medium was also analyzed to correct for low amounts of lipids present in LPDS (0.10, 0.20 and 0.45 μg/ml culture medium for triglycerides, cholesterol and cholesteryl esters, respectively).

Analysis of apo A-I and apo B100 containing lipoproteins secreted by Hep G2 cell cultures
After a 24 h incubation of Hep G2 cell cultures in DMEM/10% (v/v) LPDS, containing 0.8 μCi/ml [2-^{14}C]-acetate and 1mM sodium acetate, with or without sodium butyrate (2 mM), after a 24 h preincubation on the same medium without labelled and unlabelled acetate, culture medium was collected and protease inhibitors were added as described above. 2 ml culture medium was used for a density-gradient ultracentrifugation as described by Redgrave et al. [24]. After ultracentrifugation for 16 h at 4°C the gradient was fractionated in 0.6 ml aliquots. The density of the fractions was measured (Density Measuring Cell DMA 602M, Mettler/Paar, Graz, Austria) and samples were taken for a Bligh & Dyer extraction [23]. Incorporation of radioactivity in lipids was determined by liquid scintillation in the residue of the chloroform layer after evaporation. The remainder of the fractions were dialyzed (16 h) against PBS containing 0.1% (w/v) casein and 2.5 mM EDTA, and the apo A-I and apo B100 concentrations in the fractions were measured by Elisa procedure as described previously [16].

Statistical analysis
Statistical significance of differences was calculated by Student's t-test for paired data with the level of significance as $P < 0.05$.

RESULTS

Pulse-chase analysis of the synthesis and secretion of apo B100, apo A-I and albumin
In a previous study [16] we have shown that butyrate stimulates the secretion of apo B100 by Hep G2 cells 2-fold and that the regulation takes place at the translational or co- or post-translational level. To investigate the level of regulation in more detail, pulse-chase experiments were performed. Next to apo B100 the synthesis and secretion of newly synthesized apo A-I and albumin was studied. Data from a representative of three independent experiments is shown in Fig. 1. After the 90 min chase period only 18 ± 4% (n = 3) of the total amount of labelled apo B100 present intracellularly after the 10 min pulse period, was secreted in the control incubation. The total amount (intracellular and secreted) of [^{35}S]-labelled apo B100 recovered after the 90 min chase period was 27 ±5% (n = 3). In Hep G2 cells treated with butyrate the percentage of labelled apo B100 secreted and total amount recovered (in cells and medium) increased to 32 ± 6% and 48 ± 8% respectively (1.8-fold increase for both parameters). For comparison, recovery of labelled apo A-I and albumin (in cells and medium) was 85-90% of initial apo A-I and albumin synthesized both in control and butyrate-treated cultures. By comparing the ratio between [^{35}S]-labelled apo B100 (in cells and medium) of butyrate-treated and control cultures during

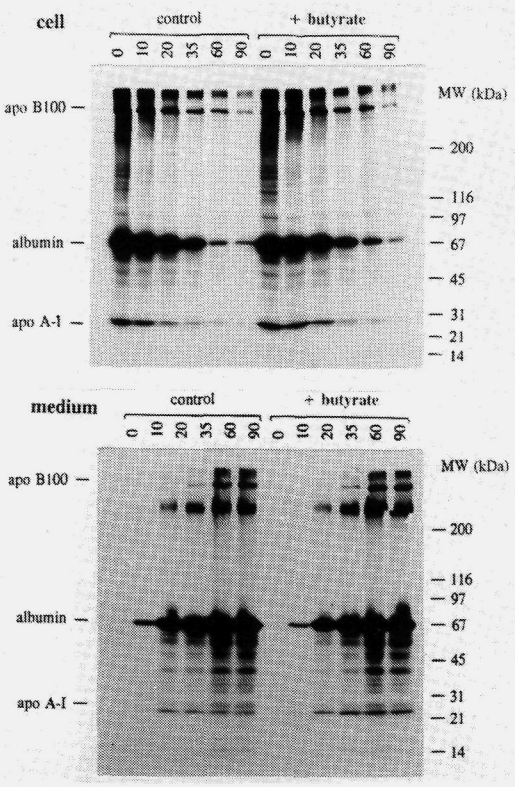

Figure 1. Pulse-chase analysis of the effect of butyrate on de novo synthesized apo B100, apo A-I and albumin.

Fig. 1A. Radiolabelled apo B100, apo A-I and albumin from pulse-chase experiments were immunoprecipitated from cells and media of control and butyrate-treated cells as described in the section Experimental. The immunoprecipitate was separated by gel electrophoresis (4-20% SDS-PAGE) and autoradiographed. Upper and lower autoradiograph show the results of the immunoprecipitation of cells and medium respectively. At the top of the autoradiographs the incubation condition and chase time are indicated. Bands corresponding to apo B100, apo A-I and albumin were sliced from the dried gels and after solubilization of the gel, counted for radioactivity. Data from a representative of three independent experiments are shown.

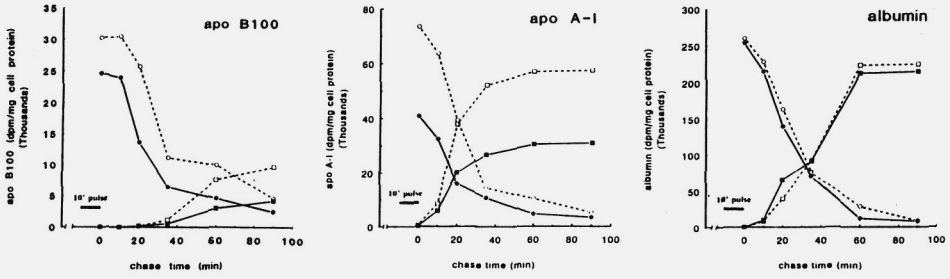

Fig. 1B. Amounts of radiolabelled apo B100, apo A-I and albumin during chase-time present intracellularly in Hep G2 cells treated with (○) or without (●) butyrate, and in the culture medium of these cells treated with (□) or without (■) butyrate.

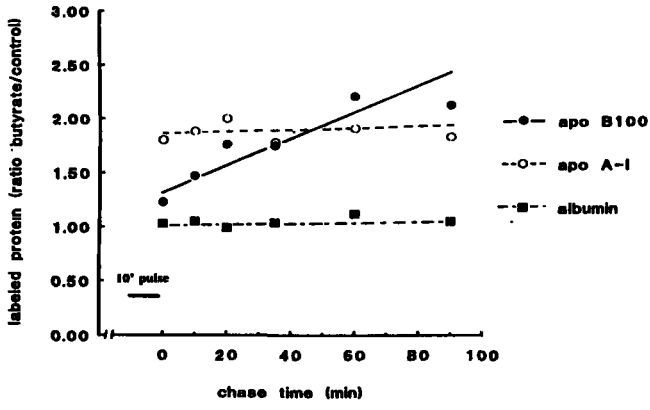

Fig. 1C. Proportion between total radiolabelled (in cells and medium) apo B100, apo A-I and albumin in Hep G2 cell cultures treated with 2 mM sodium butyrate and in control cultures during the chase period.

the chase period (Fig. 1C), it is shown that this ratio increased with the chase-time. This indicates that less apo B100 is degraded intracellularly in butyrate-treated cells than in control cells. The ratio for labelled apo A-I in butyrate-treated versus control Hep G2 cells was 1.8 and remained constant throughout the chase period, indicating that the synthesis of apo A-I was stimulated with a factor 1.8 and that there was no effect of butyrate on intracellular transfer, assembly and secretion of apo A-I and apo A-I-containing lipoproteins. For albumin the ratio remained 1, showing that butyrate neither affected the synthesis nor the intracellular transfer and secretion of albumin in Hep G2 cells.

Effect of butyrate on intracellular and extracellular lipid levels and on buoyant density of lipoproteins secreted by Hep G2 cells

Since apo B100 is secreted by hepatocytes only when assembled with lipids into a lipoprotein (for reviews see [25,26]), the availability of these lipids is important for the secretion of apo B100. To investigate whether butyrate affects the intracellular amounts of lipids the levels of triglycerides, free cholesterol, and cholesteryl esters were measured. The results are shown in Table 1A. Both in lipoprotein-containing (DMEM/FBS) and lipoprotein free medium (DMEM/LPDS) a significant increase in the intracellular amount of triglycerides (+31% and +30% respectively) was found after the addition of butyrate. The cellular content of cholesteryl esters was not affected by butyrate and a small but significant increase in free cholesterol (+18%) was observed in DMEM/FBS. Incubation of Hep G2 cells with butyrate in DMEM/FBS or DMEM/LPDS resulted in a comparable increase in the secretion of both apo B100 and apo A-I in both media (Table 1B).

Table 1. Effect of butyrate on the amount of intracellular and secreted lipids. Hep G2 cells were incubated for 24 h in DMEM/10% (v/v) FBS or DMEM/10% (v/v) LPDS with or without 2 mM Na-butyrate after a 24-h preincubation on the same medium.

1A. Results of the effect of butyrate on triglycerides, free cholesterol and cholesteryl esters, measured as described in the Experimental section. Values were normalized for cell protein and are expressed as a percentage of control, and are means ± S.D. for four independent experiments for DMEM/FBS and for three independent experiments for DMEM/LPDS. Cellular contents of triglycerides, free cholesterol, and cholesteryl esters in control experiments were 87.4 ± 31.3, 13.1 ± 3.4 and 16.3 ± 2.5 μg per mg of cell protein, respectively in DMEM/FBS and 88.1 ± 23.2, 15.1 ± 3.1 and 12.7 ± 2.4 μg per mg of cell protein, respectively in DMEM/LPDS. The secretion of triglycerides, free cholesterol and cholesteryl esters, in control experiments in DMEM/LPDS, was 3.3 ± 0.3, 2.2 ± 0.2 and 0.51 ± 0.02 μg/24 h per mg of cell protein, respectively. * Indicates a significant difference between control and treated cells.

	lipid concentration (% of control)	
	DMEM/FBS + 2 mM Na-butyrate	DMEM/LPDS + 2 mM Na-butyrate
triglycerides (intracellular)	131 ± 14%*	130 ± 15%*
free cholesterol (intracellular)	118 ± 2%*	101 ± 8%
cholesteryl ester (intracellular)	91 ± 9%	100 ± 16%
triglycerides (secreted)	—	190 ± 30%*
free cholesterol (secreted)	—	114 ± 9%
cholesteryl ester (secreted)	—	178 ± 13%*

1B. The effect of butyrate on the apo B100 and apo A-I secretion by Hep G2 cells in the experiments described above. Apo B100 and apo A-I were measured by Elisa procedure as described [16] and normalized for cell protein. * Indicates a significant difference between control and treated cells.

	DMEM/FBS	DMEM/FBS + 2 mM Na-butyrate	DMEM/LPDS	DMEM/LPDS + 2 mM Na-butyrate
apo B100 (μg/mg cell protein/24 h)	5.6 ± 1.1	11.3 ± 2.8*	5.1 ± 0.6	10.3 ± 2.3*
apo A-I (μg/mg cell protein/24 h)	4.8 ± 1.0	8.9 ± 1.7*	6.6 ± 1.2	11.7 ± 2.1*

In incubations in lipoprotein-deficient medium the amount of triglycerides, cholesterol and cholesteryl esters secreted by Hep G2 cells was also determined. Concomitantly with an enhanced apo B100 secretion (Table 1B) an increase in the secretion of triglycerides (+90%) and cholesteryl esters (+78%) from butyrate-treated cells was found. The elevated secretion of apo B100, triglycerides and cholesteryl esters was comparable in magnitude. No effect was seen on the secretion of free cholesterol. The amount of the lipids secreted by Hep G2 cells is only a small fraction of the cellular content of the lipids (legend Table 1). Addition of the secreted and the cellular amounts of the lipids showed that only the total mass of triglycerides was increased significantly (+32%) after butyrate treatment.

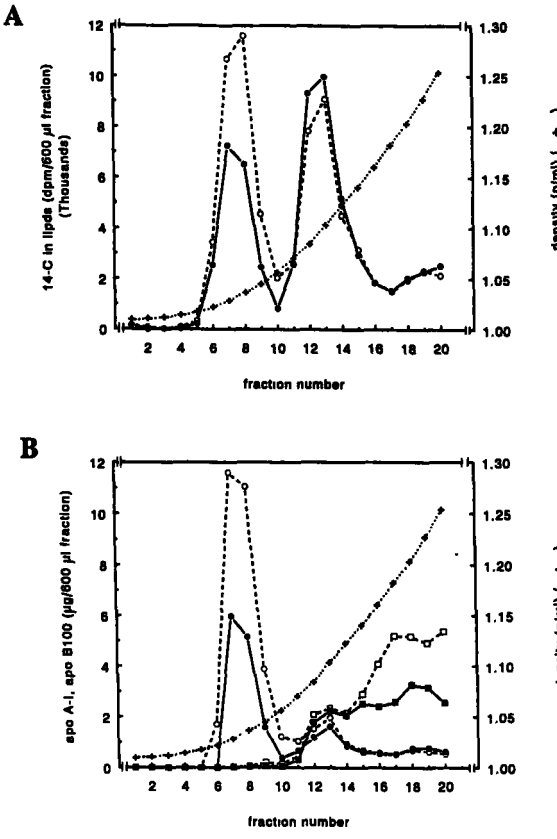

Figure 2. Effect of butyrate on the density gradient pattern of lipoproteins secreted by Hep G2 cell cultures. Hep G2 cells were incubated for 24 h with DMEM/LPDS, containing 0.8 µCi/ml [2-^{14}C]-acetate, with or without 2 mM sodium butyrate, after a 24-h preincubation period on the same medium without labelled acetate. 2 ml culture medium was used for density gradient ultracentrifugation as described in the Experimental section. The gradient was fractionated in 600 µl fractions and ^{14}C incorporation in lipids, and apo A-I and apo B100 concentration in each fraction were determined. Data from a representative of three independent experiments are shown.

A) ^{14}C incorporation in lipids in culture medium of Hep G2 cells after incubation with (○) or without (●) sodium butyrate.

B) Apo A-I in the medium with (□) or without (■) butyrate and apo B100 in the medium with (○) or without (●) butyrate, measured by Elisa procedure after dialysis of the fractions against PBS, containing 0.1% casein and 2.5 mM EDTA.

The differential effect of butyrate on the secretion of lipids did not affect the density of apo B100- and apo A-I-containing lipoproteins secreted by Hep G2 cells (Fig. 2). When [^{14}C]-labelled lipids were extracted from the different fractions of the density gradient two lipoprotein particles could be distinguished (Fig. 2A). The first with a buoyant density

between 1.02-1.06 g/ml contained only apo B100 (Fig. 2B). The second with a density between 1.07-1.12 contained apo A-I but also small amounts of apo B100. The increase in apo B100 and ^{14}C-labelled lipids secreted by butyrate treated Hep G2 cells was observed only for the lipoprotein with the density of LDL (1.02-1.06 g/ml). The increase in the secretion of apo A-I was observed in the buoyant density range between 1.14 and 1.25 g/ml, representing mostly lipid-poor and lipid-free apo A-I.

DISCUSSION

In this study we have shown that butyrate stimulates the secretion of apo B100 by Hep G2 cells at the post-translational level by inhibiting the intracellular degradation of apo B100. The majority of newly-synthesized apo B100 is degraded intracellularly in Hep G2 cells. In control incubations the total amount of newly-synthesized apo B100 recovered (in cells and medium) after the 90 min chase period was found to be 27 $\pm$ 5% of the labelled apo B100 present intracellularly after the 10 min pulse period. This figure is in accordance with other reports on the regulation of the secretion of apo B100 in Hep G2 cells [27,28] and of large apo B in rat hepatocytes [21]. Treatment of Hep G2 cells with butyrate stimulated the secretion and recovery of the total amount of newly-synthesized apo B100 (both with a factor 1.8), as compared to control cells, after the 90 min chase period. This rise is not due to an increased synthesis, since the amount of apo B100 was only marginally elevated after the pulse period, but to an inhibition of the intracellular degradation of apo B100. The proportion between the total amount of radiolabelled apo B100 in butyrate-treated and control cells gradually increased during the chase period. For comparison the synthesis and the secretion of newly synthesized apo A-I and albumin was investigated. The recovery of these proteins was 85-90% of the initially synthesized apo A-I and albumin, indicating (almost) no intracellular degradation of these two proteins. Albumin synthesis and secretion was not affected by butyrate. For apo A-I we observed a 80% increase in the [^{35}S]-labelled apo A-I in the Hep G2 cells, already after the 10 min pulse period. The ratio between total labelled (in cells and medium) apo A-I of butyrate-treated and control cultures remained 1.8 during the whole chase period, indicating that the synthesis of apo A-I is stimulated, and that there is no effect on intracellular transfer, assembly and secretion of apo A-I and/or apo A-I-containing particles. This is in accordance with our previous finding that butyrate increases the apo A-I mRNA level in Hep G2 cells to the same extent as the apo A-I secretion [16], indicating that the increase in the synthesis is regulated at the level of transcription or mRNA stability.

To investigate the mechanism responsible for the decreased intracellular degradation of apo B100 in Hep G2 cells treated with butyrate, the availability of lipids (triglycerides,

free cholesterol, cholesteryl esters) was measured. Cellular amounts of triglycerides in Hep G2 cells increased upon treatment with butyrate. There was no or only a small effect of butyrate on the intracellular amounts of cholesterol and cholesteryl esters.

Furthermore, butyrate stimulated the mass secretion of triglycerides (+90%) and cholesteryl esters (+78%), and had no effect on secretion of cholesterol. The amount of the lipids secreted by Hep G2 cells, however, is only a small fraction of the cellular content of the lipids (see legend Table 1). Addition of the secreted and the cellular amounts of the lipids showed that only the mass, and consequently the synthesis of triglycerides was increased significantly after butyrate-treatment. These results are in line with reports on the increase of the fatty acid synthesis in butyrate-treated rat hepatocytes and the marginal effects of butyrate on sterol synthesis [17,18].

The differential effects of butyrate on the secretion of lipids did not change the buoyant density of lipoproteins secreted by Hep G2 cells. Apo B100 is secreted predominantly as a constituent of an LDL-like particle (density 1.02-1.06 g/ml) and apo A-I as an HDL-like particle but is also found in a lipid-poor or lipid-free form (density 1.14-1.25 g/ml), which is in agreement with other reports [7,9]. This finding suggests that most cholesterol is secreted in apo A-I-containing HDL-like particles, the formation of which remained unchanged with butyrate, and that the triglycerides and cholesteryl esters are secreted in apo B100-containing lipoprotein particles, which increased after butyrate-treatment (Fig. 2). This contention is supported by two reports showing that triglycerides are the most abundant lipids in apo B100-containing lipoproteins, whereas the majority of cholesterol is secreted in apo A-I-containing particles by Hep G2 cells [9,29]. The rise in apo A-I secretion was found only in the lipid-poor and lipid-free fractions of the density gradient.

We suggest that the enhanced secretion of apo B100 by Hep G2 cells treated with butyrate results from an increased availability of triglycerides for the assembly of apo B100-containing lipoproteins and that the elevated secretion of cholesteryl esters may be secondary to this. A similar increase in the apo B100 secretion was found when Hep G2 cells were incubated in the presence of long-chain fatty acids [8,9,11,13,15]. Dixon et al. [28] reported that the mechanism of the increased apo B100 secretion by oleate also resulted from a decreased intracellular degradation. Cholesterol and cholesteryl esters have also been shown to be involved in the regulation of the apo B100 secretion by Hep G2 cells [12,14] and *in vivo* in rats [30,31] and in humans [32]. Sato et al. [27] reported that the increased apo B100 secretion by cholesterol resulted from a similar mechanism as reported here and for oleate. On the other hand, other groups found no effects of the availability of cholesterol and cholesteryl esters on apo B100 secretion in Hep G2 cells [11,33,34]. The absence of changes in the total mass of cholesterol and cholesteryl esters does not support a possible role of these lipids in regulation of apo B100 secretion by butyrate.

ACKNOWLEDGEMENTS

We gratefully thank Silvia van der Plas for excellent technical assistance and Marisa Horsting for typing the manuscript. This work was supported by the Netherlands Heart Foundation (grant 87.035)

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CHAPTER 4

SELECTIVE INHIBITION OF APOLIPOPROTEIN B100 SECRETION BY CYCLOSPORIN A IN THE HUMAN HEPATOMA CELL LINE Hep G2.

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Submitted for publication.

ABSTRACT

Treatment of patients with cyclosporin A results in an increase in the LDL-cholesterol level. In this study we investigated whether an elevated hepatic secretion of apo B100-containing lipoproteins is responsible for the increase of LDL, using the human hepatoma cell line Hep G2.

Addition of cyclosporin A (CsA) to the culture medium of Hep G2 cells resulted in a dose- and time-dependent decrease in the secretion of apo B100. Maximal inhibition (-50%) was obtained at 5 μM CsA and was already achieved within 8 h. The secretion of apo A-I, albumin and [^{35}S]-methionine labelled proteins was not affected by CsA. The reduced accumulation of apo B100 in the culture medium could not be explained by changes in the uptake and degradation of LDL by Hep G2 cells treated with CsA. In addition, [^{35}S]-methionine incorporation studies indicated that synthesis and/or secretion of newly synthesized apo B100 is decreased in the presence of CsA. CsA did not affect the apo B100 mRNA level, indicating that CsA regulates the secretion of apo B100 at the (co- or post-) translational level. The decreased secretion of apo B100 was accompanied by a diminished secretion of triglycerides (-47%), cholesterol (-18%) and cholesteryl esters (-27%) in the presence of CsA. In contrast, the intracellular concentrations and the amount (present in the culture medium and cells) of these lipids were not changed. This indicates that a possible limited availability of one of these lipids was not responsible for the decreased secretion of apo B100 by CsA. Pulse-chase experiments showed that the amount of intracellular apo B100 was already decreased by 50% after the 10 min pulse period, indicating that the inhibition of the apo B100 secretion by CsA takes place at the translational or co-translational level. These results show that the elevated plasma LDL levels, observed in patients treated with CsA, are not caused by hepatic overproduction of apo B100-containing lipoproteins.

INTRODUCTION

Cyclosporin A (CsA) is one of the most effective immunosuppressive drugs available and is widely used in organ transplantation and in an increasing number of autoimmune diseases. The compound, a cyclic undecapeptide, selectively inhibits the production of interleukin-2 by activated T-lymphocytes and prevents activation of resting T-cells by this lymphokine (1,2). Several adverse reactions to CsA have been reported (for a review see Kahan (3)). Among these it has been shown that the drug elevates the serum cholesterol level in patients, primarily by an increase in LDL-cholesterol level (4,5). Next to the LDL-cholesterol level, CsA enhances the concentration of apolipoprotein B100 (5), the sole

protein of LDL. Increased levels of LDL-cholesterol and apo B100 are strongly associated with the development of atherosclerosis (6-8).

The mechanism of the increase in LDL-cholesterol and apo B100 levels in patients treated with CsA is not understood. It has been suggested that the increase may be caused by an enhanced secretion of apo B-containing lipoproteins by the liver (9). In humans apo B100 is synthesized and secreted only by the liver (10). Owing to the limited availability of human liver the human hepatoma cell line Hep G2 has been used frequently as a model to study the apolipoprotein and lipoprotein synthesis in human hepatocytes. This cell line has been shown to synthesize a number of apolipoproteins (11), and has been found to be a suitable model for investigating the regulation of the synthesis and/or secretion of apo B100 in response to lipoproteins (12), fatty acids (13-17), butyric acid (18), hormones (14,16,19-21), and drugs, currently applied in clinical practice (16,22,23).

In this paper we describe the effect of CsA on the secretion of apo B100 in Hep G2 cells. This study shows that CsA inhibits apo B100 secretion at the translational or co-translational level. These findings show that the increased serum LDL-cholesterol levels in patients after long-term CsA therapy, can not be explained by an increased secretion of apo B100-containing lipoproteins by the liver.

METHODS

Materials

Cyclosporin A powder was a gift from Sandoz Ltd., Uden, the Netherlands. Tran ^{35}S-label (> 1000 Ci/mmol), containing both [^{35}S]-methionine and [^{35}S]-cysteine, was obtained from ICN Biomedicals (ICN Biomedicals Inc., Irvine, Ca, U.S.A.). Na ^{125}I (15 mCi/μg iodine) and [2-^{14}C]-acetate (55 mCi/mmol) were purchased from Amersham International (Amersham, Bucks, U.K.). Dulbecco's modified Eagle's medium (DMEM) and L-glutamine were purchased from Flow Laboratories (Irvine, Scotland, U.K.). Fetal bovine serum (FBS), penicillin and streptomycin were from Boehringer Mannheim (Mannheim, Germany).

Cell culture

The established Hep G2 cell line, derived from a human liver tumour, was obtained from Dr. B.B. Knowles (Wistar Institute of Anatomy and Biology, Philadelphia, PA, U.S.A.). The cells were cultured as described (18,24).

Incubations in the presence of CsA were performed in DMEM medium containing 10% (v/v) FBS or 10% (v/v) lipoprotein depleted serum (LPDS). LPDS was prepared from heat-inactivated FBS by density gradient ultracentrifugation (25). A 50 mM stock solution of CsA was prepared in DMSO and stored at -20°C. Immediately before use CsA was diluted in culture medium, such that the DMSO concentration did not exceed 0.01% (v/v). All incubation media were adjusted for the same DMSO concentration. Except when otherwise stated, the effect of CsA was studied over a 24-h

period. At the end of the incubation period, medium was collected and centrifuged for 20 seconds (8000 x g) in a Heraeus centrifuge (Biofuge A) to remove detached cells and debris. The supernatant was frozen immediately in dry-ice and stored at -20°C until measurement of apo A-I and apo B100 concentrations. Cells were washed three times with cold (4°C) phosphate-buffered saline (PBS, sodium/potassium phosphate buffer: 11 mM, pH 7.5, containing 150 mM NaCl) and were harvested by scraping in water and cellular protein was determined.

Measurement of the secretion of apo A-I, apo B100 and albumin and analysis of apo A-I and apo B100 containing lipoproteins secreted by Hep G2 cell cultures

Accumulation of apo A-I and apo B100 in the medium of Hep G2 cell cultures was measured in triplicate using a sandwich Elisa procedure as described previously (18). Accumulation of albumin in the medium was measured by rocket immunoelectrophoresis by the method described by Laurell (26), using rabbit-anti-(human albumin) antiserum (Dakopatts, Glostrup, Denmark).

For analysis of apo A-I and apo B100 containing lipoproteins culture medium was collected after a 24 h incubation of Hep G2 cells with or without 5 μM CsA. After harvesting, protease inhibitors were added to the following final concentrations: 2.5 mM EDTA, 0.5 mM phenyl-methylsulfonyl fluoride, 25 μM p-hydroxymercuriphenylsulfonic acid, 10 U/ml heparin, 10 U/ml trasylol, 50 μg/ml leupeptin, 50 μg/ml pepstatin). 4 ml culture medium was used for a density-gradient ultracentrifugation as described by Redgrave et al. (27). After ultracentrifugation for 16 h at 4°C the gradient was fractionated in 0.6 ml aliquots. The density of the fractions was measured (Density Measuring Cell DMA 602M, Mettler/Paar, Graz, Austria), the fractions were dialyzed (16 h) against PBS containing 0.1% (w/v) casein and 2.5 mM EDTA, and the apo A-I and apo B100 concentrations in the fractions were measured by Elisa procedure.

Protein synthesis

De novo synthesis of proteins was determined by measuring the incorporation of Tran ^{35}S-label into the 10% (w/v) trichloroacetic acid precipitable fraction of the radiolabelled culture medium and cells. The metabolically radiolabelled proteins secreted in the medium were analysed by SDS/PAGE under reducing conditions by the method of Laemmli (28), with resolving gels containing a gradient of 4-20% (w/v) acrylamide and stacking gels of 3.5% (w/v) acrylamide. Protein molecular-mass standards (Bio-Rad, Richmond, CA, U.S.A.) were used for calibration of the gel. For autoradiography the gel was treated with an autoradiography enhancer (EN^{3}Hance, NEN DuPont, Boston, MA, U.S.A.) in accordance with the manufacturers' instructions, dried and placed on X-ray film (Kodak X-Omat AR films; Eastman-Kodak Co., Rochester, NY, U.S.A.) and stored at -80°C for the appropriate time.

RNA hydridization

Total RNA was isolated from Hep G2 cells by the method of Chomczynski & Sacchi (29). Apo B100, apo A-I and albumin mRNA levels were analyzed by Northern blot analysis, exactly as described previously (18).

Measurement of the receptor-mediated binding, internalization and degradation of [^{125}I]-LDL

Binding, internalization and degradation of LDL by Hep G2 cells was measured over a 3 h period with or without 5 μM CsA, following a 18 h preincubation period with or without 5 μM CsA. In

incubations where [^{125}I]-LDL binding, uptake and degradation was studied in the presence of CsA, the [^{125}I]-LDL was preincubated with CsA before addition to the Hep G2 cell culture.

The LDL-receptor mediated binding, internalization and degradation of LDL was measured at 37°C exactly as described previously (24,25). Binding, internalization and degradation were corrected for nonspecific binding, internalization and degradation by performing the same experiments in the presence of an excess of unlabelled LDL (200 μg/ml). Next to the binding at 37°C, the binding of [^{125}I]-LDL was measured at 4°C. At this temperature the LDL is not internalized.

Measurement of the mass of intracellular and extracellular triglycerides, cholesterol and cholesteryl esters

After a 24-h incubation in the presence or absence of CsA, Hep G2 cells were washed five times with cold PBS. Thereafter, the cells were harvested by scraping and homogenized by sonication (Branson, 60 W, 20 S). Samples were taken for measurement of protein content. Lipids were extracted from the cell suspension as described by Bligh & Dyer (30), after addition of cholesterol acetate (2 μg) as an internal standard. The neutral lipids were separated by high-performance t.l.c. on silica-gel-60 precoated plates as described (24).

When DMEM/LPDS was used as the culture medium, the mass of triglycerides, cholesterol and cholesteryl esters secreted in the medium was also measured. At the end of the incubation the medium was centrifuged (12000 rpm for 30 min at 4°C) to remove detached cells and cell debris. Extraction and analysis of lipids were the same as for the cell suspension. Blank culture medium was also analyzed to correct for low amounts of lipids present in LPDS (0.10, 0.20 and 0.45 μg triglycerides, cholesterol and cholesteryl esters, respectively, were found per ml culture medium).

Pulse-chase analysis of apo A-I, apo B100 and albumin production

After a 16-h preincubation in DMEM/LPDS with or without 5 μM CsA, Hep G2 cells were used for pulse-chase studies using Tran [^{35}S]-label, as described (31) with minor modifications. After a 30-min incubation on methionine and cysteine free MEM (ICN Biomedicals Inc., Irvine, CA, USA), cells were pulsed with Tran [^{35}S]-label (100 μCi/ml) for 10 min. After the pulse period, the medium was removed, cells were washed three times with (chase medium) DMEM at 37°C and incubated with DMEM with or without 5 μM CsA for 0, 10, 20, 35, 60 and 90 min. At the end of the chase-time cells and media were harvested separately.

Cells were washed, at 4°C, three times with PBS, homogenized in 1 ml NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4, 0.0625 M sucrose, 0.5% Triton X-100, and 0.5% sodium deoxycholate) and protease inhibitors were added as described above. The medium was centrifuged for 20 seconds (8000 x g, Heraeus centrifuge, Biofuge A) to remove detached cells and debris. The supernatant was mixed (1:1) with 2 x NET-buffer and protease inhibitors were added. Aliquots of 500 μl cell extracts or 900 μl of the culture medium were combined with an excess of monospecific affinopurified goat anti-human apo A-I, rabbit anti-human apo B100 (18) and rabbit anti-albumin antiserum (Dakopatts, Glostrup, Denmark) and incubated for 16 h at 4°C. Then 75 μl of a 50% solution of protein G-Sepharose (Pharmacia) in NET buffer was added, and incubated for 45 min at 4°C and 30 min at room temperature. The antigen-antibody- protein G-Sepharose complex was pelleted by centrifugation (2 min, 4000 rpm), the supernatant was aspirated and the pellet was resuspended in 500 μl NET buffer and washed by centrifugation through a 30% sucrose solution in

NET-buffer (500 μl). After two additional washes with NET-buffer, 50 μl sample buffer was added, the samples were boiled for 5 min and subjected to electrophoresis as described above. Apo B100, apo A-I and albumin were localized on the dried gels after autoradiography. Bands containing apo B100, apo A-I and albumin were sliced from the gel and the gel fragments were solubilized in 1 ml of a NH_3/H_2O_2 solution (0.94% (w/v) NH_3, 7.5% (v/v) H_2O_2) for 16 h at 55°C and counted for radioactivity.

Statistical analysis

Statistical significance of differences was calculated by Student's t-test for paired data with the level of significance as $P < 0.05$.

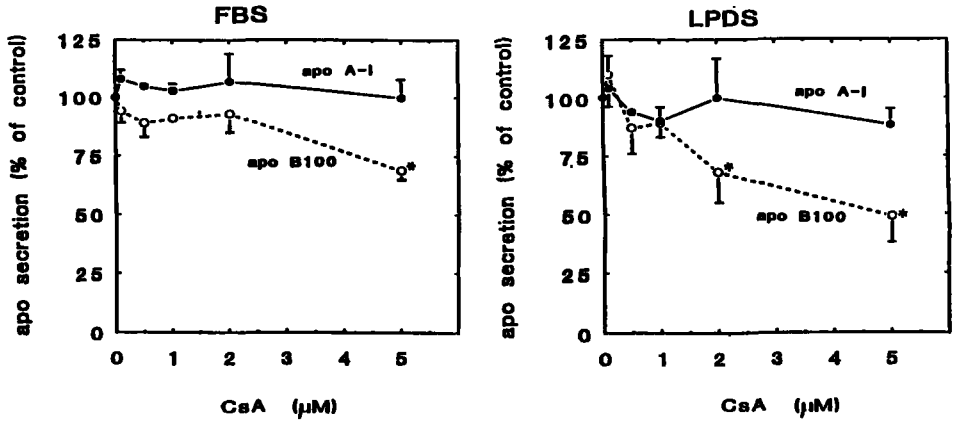

Figure 1. Effect of the cyclosporin A concentration on the secretion of apo A-I and apo B100 in the medium of Hep G2 cell cultures. Hep G2 cells were incubated for 24 h with various concentrations of CsA in DMEM medium containing 10% (v/v) FBS or 10% (v/v) LPDS. Apo A-I and apo B100 concentrations in the culture medium were measured as described in the Methods section. Results were normalized for cell protein in culture dishes and are expressed as a percentage of control, and are means ± S.D. for three independent experiments. Apo A-I (●) production by Hep G2 cells on DMEM/FBS and DMEM/LPDS was 4.5 ± 0.4 and 6.0 ± 0.4 μg/24 h per mg of cell protein, respectively (means ± S.D. for three experiments). Apo B100 (○) production by Hep G2 cells on DMEM/FBS and DMEM/LPDS was 5.6 ± 0.4 and 5.4 ± 0.8 μg/24 h per mg of cell protein, respectively (means ± S.D. for three experiments).

* Indicates a significant difference ($p < 0.05$) between control and treated cells.

RESULTS

Dose-dependency and time-course of the effect of cyclosporin A on the secretion of apo B100 and apo A-I in the medium of cultured Hep G2 cells

As shown in Fig. 1, addition of increasing amounts of CsA resulted in a decrease in the secretion of apo B100 in the medium of Hep G2 cell cultures, without affecting the apo A-I

secretion. The decrease in the apo B100 secretion was observed both in DMEM/FBS and DMEM/LPDS, but was more pronounced in DMEM/LPDS. In both media a significant decline of the apo B100 secretion was observed with 5 μM CsA. With 2 μM CsA the decrease reached significance only when DMEM/LPDS was used. Judging from the changes in cell morphology and loss of cells from the culture dishes, CsA concentrations $\geq$ 10 μM were cytotoxic to Hep G2 cell cultures. Therefore, further experiments were performed at a concentration of 5 μM CsA. At this concentration no adverse effect was observed on the cell morphology, on the amount of cell protein present in the culture dishes at the end of the incubation, and on apo A-I, albumin and total protein synthesis (see below).

In Fig. 2 the time course of the effect of CsA on the secretion of apo B100 and apo A-I by Hep G2 cells is shown. With control medium, apo B100 and apo A-I accumulation increased linearly with time over the 24-h period of the experiment. A significant decrease of the apo B100 secretion was already observed after a 4-h incubation with 5 μM CsA present in the medium. Maximal inhibition of the apo B100 secretion was reached after incubation for 8 h in the presence of 5 μM CsA. In subsequent experiments the effect of CsA on Hep G2 cell cultures was studied over a 24-h period.

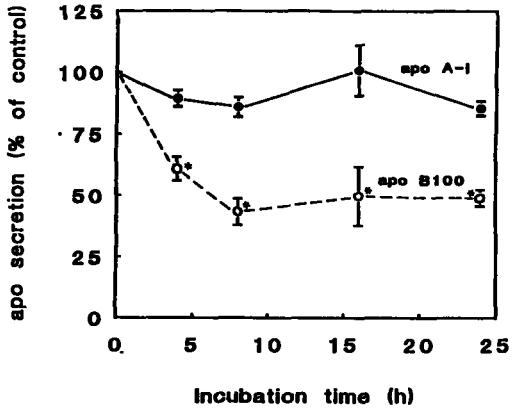

Figure 2. Time course of the effect of cyclosporin A on apo A-I and apo B100 secretion in the medium of Hep G2 cells. Hep G2 cells were cultured on DMEM/LPDS with and without 5 μM CsA. At various times, medium and cells were collected. Apo A-I and apo B100 concentrations were measured as described in the Methods section. Results were normalized for cell protein in culture dishes and are expressed as a percentage of control in the same incubation period, and are means $\pm$ S.D. for three independent experiments. Apo A-I (•) and apo B100 (○) production by Hep G2 cells in control medium was 0.29 $\pm$ 0.06 and 0.21 $\pm$ 0.05 μg/mg cell protein/h.
* Indicates a significant difference ($P < 0.05$) between control and treated cells.

Specificity of the inhibition of apo B100 secretion by cyclosporin A

The finding that CsA does not affect the secretion of apo A-I by Hep G2 cells, indicates that the inhibitory effect of CsA on the secretion of apo B100 is not part of a general effect of CsA on protein synthesis. To further exclude this possibility we measured the secretion

of albumin and total protein. No effects were found on the secretion of albumin and total, *de novo* synthesized, protein (Table 1), and on the cellular content of *de novo* synthesized proteins (data not shown) upon incubation in DMEM/FBS or DMEM/LPDS with 5 μM CsA. To explore the possibility that CsA may alter the pattern of the proteins secreted in the culture medium, the medium of a metabolic labelling with Tran [^{35}S]-label was separated by SDS/PAGE and autoradiographed (Fig. 3). Hep G2 cells incubated with CsA secreted a similar pattern of proteins in the culture medium as control cells. By specific immunoprecipitation the protein with molecular mass of approximately 500 kDa, which showed a lower intensity on the autoradiograph, was identified as apo B100.

Table 1. Effect of cyclosporin A on the secretion of albumin and [^{35}S]-labelled proteins. Hep G2 cells were incubated for 24 h in the presence or absence of 5 μM CsA. Results are means ± S.D. for three independent experiments. No statistical difference between control and CsA-treated cells was observed.

	DMEM/FBS	DMEM/FBS + 5 μM CsA	DMEM/LPDS	DMEM/LPDS + 5 μM CsA
albumin secretion (μg/mg cell protein/24 h)	42.6 ± 4.5	43.1 ± 0.8	38.3 ± 8.7	33.0 ± 4.1
[^{35}S] incorporation in secreted proteins (% of control)	100%	89 ± 11%	100%	98 ± 14%

Since Hep G2 cells are capable of binding and internalizing LDL (25), a stimulation of the uptake and degradation might have caused the decreased accumulation of apo B100 in the culture medium. However CsA inhibited the binding (-31% (4°C), -38% (37°C)), internalization (-22%) and degradation (-23%) of LDL by Hep G2 cells (Table 2).

These results indicate that the inhibitory effect of CsA on the apo B100 secretion by Hep G2 cells is specific and that the secretion of newly synthesized apo B100 is affected.

Effect of cyclosporin A on apo B100, apo A-I and albumin mRNA level

To investigate whether the decreased secretion of newly synthesized apo B100 is regulated at the mRNA level, the effect of CsA on the apo B100 mRNA level was determined, together with the apo A-I and albumin mRNA level. As shown in Fig. 4, CsA did not affect the mRNA level of apo B100, apo A-I or albumin, indicating that the decrease in apo B100 secretion by CsA must be regulated at the translational level or at the co- or post-translational level.

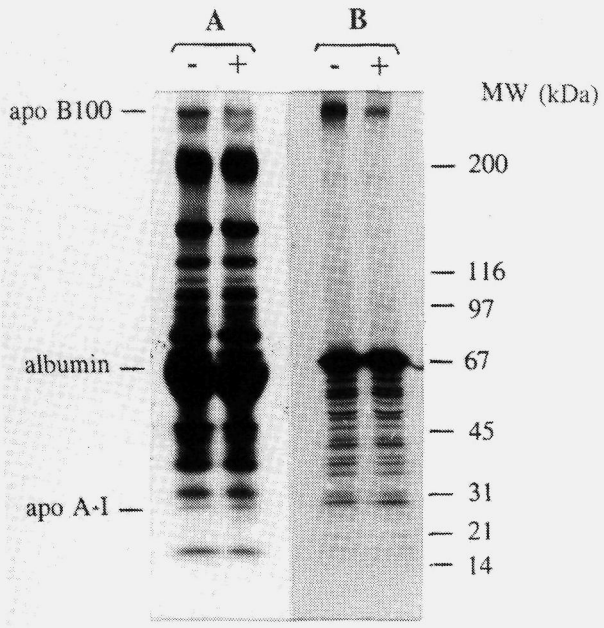

Figure 3. Effect of cyclosporin A on the pattern of metabolically radiolabelled proteins secreted in the medium of Hep G2 cell cultures. Hep G2 cells were incubated for 24 h in DMEM/LPDS with or without 5 μM CsA, containing 20 μCi of Tran [^{35}S]-label/ml. Both culture medium (A) and the immunoprecipitate of the culture medium (B) obtained by using anti-human apo B100, anti-human apo A-I and anti-human albumin were applied to the gel. + or - indicates incubation with or without CsA, respectively. The position of the molecular-mass standards are indicated on the right of the autoradiograph. At the left side of the autoradiograph the positions of apo B100, apo A-I and albumin are indicated.

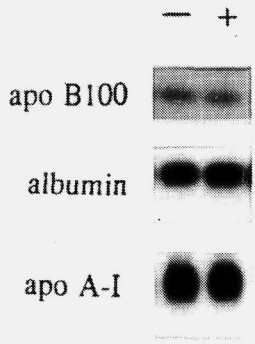

Figure 4. Effect of cyclosporin A on apo B100, apo A-I and albumin mRNA level. Autoradiograph of a Northern blot hybridization with ^{32}P-labelled probes for apo B100, apo A-I and albumin mRNA, with 6 μg of total RNA applied to the gel. The hybridized blot was exposed for the appropriate time to detect the different mRNAs. Total RNA was isolated from Hep G2 cells incubated 24 h in the presence or absence of 5 μM CsA (indicated by + and -, respectively, at the top of the autoradiograph).

Table 2. Effect of cyclosporin A on the binding, internalization and degradation of LDL by Hep G2 cell cultures. Hep G2 cells were preincubated for 18 h in DMEM/FBS with or without 5 μM CsA, followed by a 3-h incubation with [^{125}I]-labelled LDL (10 μg/ml) with or without CsA (final concentration in the binding assay 5 μM CsA). Comparable findings were made in experiments using DMEM/LPDS. The data are corrected for nonspecific binding, internalization and degradation. Each value represents the mean ± S.D. of quadruplicate determinations. *Indicates a significant difference ($P < 0.05$) from control incubations.

	DMEM/FBS	DMEM/FBS + 5 μM CsA
LDL binding (4°C) (ng LDL protein/mg cell protein)	30.2 ± 2.1	20.9 ± 1.4*
LDL binding (37°C) (ng LDL protein/mg cell protein)	30.4 ± 5.6	18.9 ± 3.5*
LDL internalized (ng LDL protein/mg cell protein/3 h)	230.0 ± 14.1	178.3 ± 6.2*
LDL degraded (ng LDL protein/mg cell protein/3 h)	101.4 ± 10.0	78.0 ± 5.2*

Table 3. Effect of cyclosporin A on the amount of intracellular and secreted lipids. Hep G2 were incubated for 24 h in DMEM/FBS or DMEM/LPDS with or without 5 μM CsA. Mass of triglycerides, free cholesterol and cholesteryl esters was measured as described in the Methods section. Results were normalized for cell protein and are expressed as a percentage of control, and are means ± S.D. for four independent experiments. Cellular contents of triglycerides, free cholesterol and cholesteryl esters for these four experiments were 87.4 ± 31.3, 13.1 ± 3.4 and 16.3 ± 2.5 μg per mg of cell protein, respectively in DMEM/FBS and 88.1 ± 23.2, 15.1 ± 3.1 and 12.7 ± 2.4, respectively in DMEM/LPDS. The secretion rate of triglycerides, free cholesterol, and cholesteryl esters, measured only in DMEM/LPDS, was 3.4 ± 0.3, 2.2 ± 0.2 and 0.51 ± 0.02 μg/24 h per mg of cell protein, respectively. * Indicates a significant difference between control and CsA-treated cells.

	lipid concentration (% of control)	
	DMEM/FBS + 5 μM CsA	DMEM/LPDS + 5 μM CsA
triglycerides (intracellular)	108 ± 4%	106 ± 10%
free cholesterol (intracellular)	109 ± 7%	103 ± 13%
cholesteryl ester (intracellular)	96 ± 8%	95 ± 7%
triglycerides (secreted)	—	53 ± 12%*
free cholesterol (secreted)	—	82 ± 9%*
cholesteryl ester (secreted)	—	73 ± 8%*

Effect of cyclosporin A on intracellular and extracellular lipid levels

Since apo B100 is secreted by hepatocytes only when assembled with lipids into a lipoprotein (for reviews see (32,33)), the availability of these lipids is important for the secretion of apo B100. To investigate whether CsA affects the intracellular amounts of lipids the levels of triglycerides, free cholesterol, and cholesteryl esters were measured. The results are shown in Table 3. No effect of CsA was seen on the cellular content of triglycerides, free cholesterol, and cholesteryl esters. In incubations in the presence of LPDS the amount of these lipids secreted by the Hep G2 cells was also measured. Concomitantly with the diminished secretion of apo B100, a decrease in the secretion of triglycerides, free cholesterol, and cholesteryl esters was found. The amount of the lipids secreted by Hep G2 cells, however, is only a small fraction of the cellular content of the lipids (see legend Table 3). The total mass of lipids (secreted and cellular amounts) was not affected by CsA.

The fall in the secretion of triglycerides is comparable in magnitude to the effect of CsA on the apo B100 secretion, whereas the secretion of free cholesterol and cholesteryl esters was less inhibited by CsA. This may indicate that the composition of the apo B100-containing lipoproteins secreted by the Hep G2 cells is affected. To investigate this we separated Hep G2 cell culture medium after a 24-h incubation with or without 5 μM CsA by density gradient ultracentrifugation (Fig. 5). The majority of apo B100 is present in a lipoprotein particle with a buoyant density of LDL (1.02-1.06 g/ml). When Hep G2 cells were incubated in the presence of CsA (5 μM) the distribution of apo B100 in the density gradient was similar to that of the control incubation, except that a 50% lower amount of apo B100 was found.

Pulse-chase analysis of the synthesis and secretion of apo A-I and apo B100

The above experiments suggest that the decrease in the secreted amount of triglycerides, cholesterol and cholesteryl esters results from a decline in apo B100 secretion. To investigate the level of regulation of the apo B100 secretion in more detail pulse-chase experiments were performed. Hep G2 cells, preincubated with or without 5 μM CsA, were pulse-labelled for 10 min with Tran [^{35}S]-label and chased for 0, 10, 20, 35, 60, and 90 min in medium without label. Cells and media were analysed for [^{35}S]-incorporation in apo A-I, apo B100 and albumin (Fig. 6). The synthesis of [^{35}S]-labelled albumin and apo A-I by Hep G2 cells and the secretion of these proteins in the culture medium were not affected by CsA. CsA decreased the amount of [^{35}S]-labelled apo B100 secreted by the Hep G2 cells. After a 90 min chase period in control medium 21% of the pulse-labelled apo B100 was found in the medium. In the presence of 5 μM CsA the apo B100 secretion was 2-fold lower. In addition, the experiments show that the intracellular amount of [^{35}S]-labelled apo B100 was already decreased by 50% after the 10 min pulse period, upon incubation with

CsA. By comparing the ratio between total labelled (in cells and medium) apo B100 of CsA treated and control cells during the chase period, it was shown that the later steps in the intracellular assembly or processing of apo B100 containing lipoproteins were not affected by CsA (Fig. 6C). This indicates that CsA inhibits the synthesis of apo B100 directly, or indirectly by a process which is closely linked to the synthesis of apo B100.

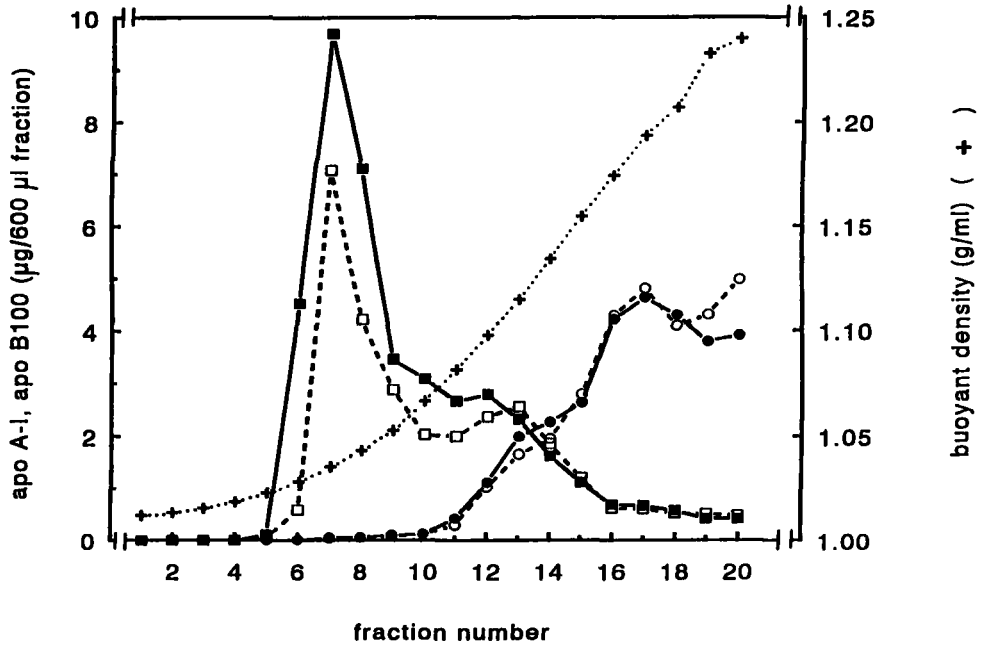

Figure 5. Effect of cyclosporin A on the density gradient pattern of lipoproteins secreted by Hep G2 cell cultures. Hep G2 cells were incubated for 24 h in culture medium in the presence or absence of 5 µM CsA. 4 ml culture medium was used for density gradient ultracentrifugation as described in the Methods section. Apo A-I concentration in gradient fractions of incubations with (○) or without (●) CsA and apo B100 concentration in gradient fractions of incubations with (□) or without (■) CsA were measured by Elisa procedure after dialysis against PBS, containing 0.1 % casein and 2.5 mM EDTA.

DISCUSSION

In this paper we have shown that cyclosporin A inhibits the secretion of apo B100 by the human hepatoma cell line Hep G2 in a dose- and time-dependent way. The inhibition of the apo B100 secretion by CsA occurred rapidly. Maximal inhibition was achieved after a 8 h incubation period and a significant decrease was already observed after 4 h of incubation at 5 µM CsA. The secretion of apo A-I and albumin, and total protein synthesis were not

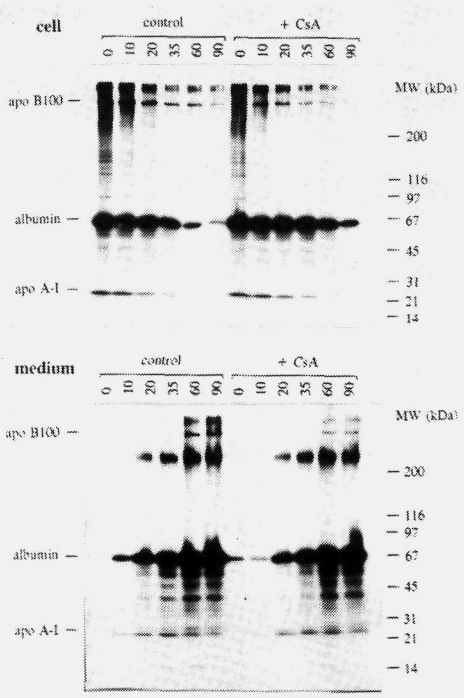

Figure 6. Pulse-chase analysis of the effect of cyclosporin A on *de novo* synthesized apo B100, apo A-I and albumin in cells and medium.

Fig. 6A. Apo B100, apo A-I and albumin were immunoprecipitated from cells and media of pulsed-chased control and CsA (5 μM) -treated cells, separated by gel electrophoresis (4-20% SDS-PAGE) and autoradiographed. Upper and lower autoradiograph show the results of the immunoprecipitation of cells and media respectively. At the top of the autoradiographs the incubation condition and chase time are indicated.

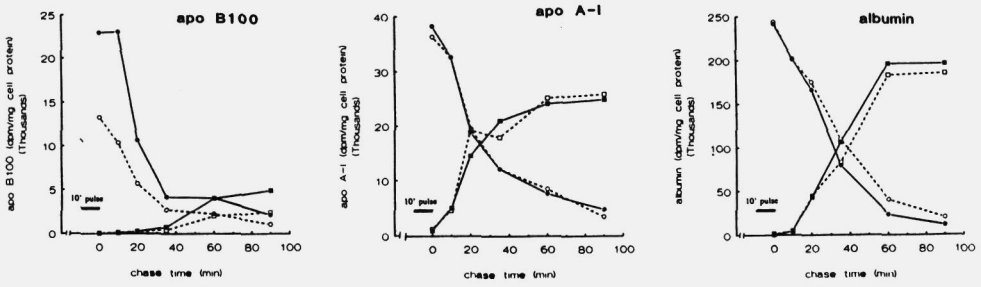

Fig. 6B. The apo B100, apo A-I and albumin bands were sliced from the dried gel and counted after solubilization of the gel. ○ and ● in the figures for apo B100, apo A-I and albumin are the data of immunoprecipitated [^{35}S]-labelled apo B100, apo A-I and albumin present intracellularly in Hep G2 cells, with or without CsA (5 μM) treatment, respectively. □ and ■ in the same figures depict the data for the secreted amounts of [^{35}S]-labelled apo B100, apo A-I and albumin by Hep G2 cells treated with or without CsA, respectively.

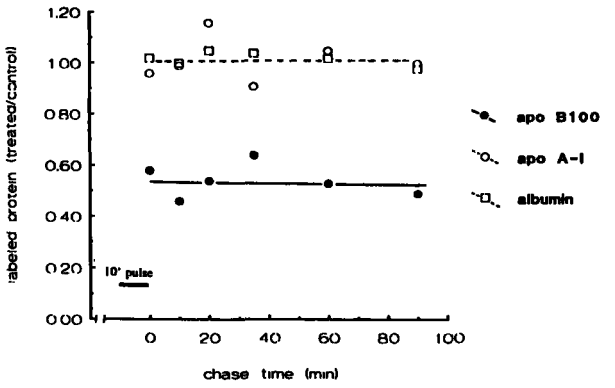

Fig. 6C. Proportion between total radiolabeled (in cells and medium) apo B100, apo A-I and albumin in Hep G2 cell cultures treated with 5 μM CsA and in control cultures not incubated with CsA.

affected by CsA, indicating that the effect of CsA on apo B100 secretion is not due to a general effect of the drug on protein synthesis and secretion by Hep G2 cells. Apo B100 secretion was inhibited significantly at concentrations (2-5 μM) which are close to plasma levels of the drug commonly observed in patients (0.5-5 μM, (34-36)). The decreased accumulation of apo B100 in the culture medium of Hep G2 cells treated with CsA can not be explained by an effect of CsA on binding and uptake of LDL by Hep G2 cells. Treatment of Hep G2 cells with CsA resulted in an inhibition of the binding, internalization and degradation of LDL by Hep G2 cells (Table 2). Analysis of metabolically labelled and secreted proteins by Hep G2 cells in the presence or absence of CsA (Fig. 3) showed that CsA inhibits the synthesis and/or the secretion of newly synthesized apo B100.

We observed no differences in the apo B100, apo A-I and albumin mRNA levels in Hep G2 cells upon incubation with CsA (Fig. 4). This indicates that the decreased *de novo* synthesis and secretion of apo B100 is regulated at the translational or co- or post-translational level. Before secretion from hepatocytes, apo B100 must be assembled with lipids into a lipoprotein. Triglycerides, cholesterol and cholesteryl esters are required for the assembly of apo B-containing lipoproteins in hepatocytes (for reviews see (32,33)) and have been shown to be involved in the regulation of apo B100 secretion by Hep G2 cells (12-17,22,37). To investigate the possibility that CsA inhibits the secretion of apo B100 by decreasing the availability of one or more of these lipids, we measured the intracellular amounts of cholesterol, cholesteryl esters and triglycerides (Table 3). No changes in the intracellular levels of these three lipids were found. However, treatment with CsA resulted in an impaired secretion of all three lipids. Since only small amounts of triglycerides, cholesterol and cholesteryl esters were secreted, the total mass of these lipids (present in

cells and medium) was not affected by CsA. This indicates that the inhibitory effect of CsA on the apo B100 secretion is not caused by a decrease in the availability of cholesterol, cholesteryl esters and/or triglycerides for the assembly of apo B100-containing lipoproteins.

The decrease of the triglyceride secretion in Hep G2 cells treated with CsA, was comparable in magnitude to the inhibitory effect of CsA on the apo B100 secretion, whereas the reduction in cholesterol and cholesteryl esters secretion was less pronounced. The differences in lipid secretion were not reflected in changes in buoyant density of the apo B100-containing lipoproteins secreted. This finding suggests that the observed changes in lipid composition do not notably affect the density of the lipoproteins secreted by Hep G2 cells and/or that more cholesterol and cholesteryl esters are secreted in apo A-I-containing HDL-like particles. The latter contention is conceivable, since triglycerides are the most abundant lipids in apo B100-containing lipoproteins and cholesterol and cholesteryl esters in apo A-I-containing particles secreted by Hep G2 cells (14,38). The observed distribution of apo B100 (predominantly as a constituent of a LDL-like particle) and apo A-I (both in a dense HDL-like particle and in a lipid-poor or lipid-free form) in the density gradient was in accordance with earlier reports (14,39).

To explore whether the decreased lipid secretion was caused by inhibition of apo B100 synthesis and/or secretion, pulse-chase experiments were conducted. After a 10-min pulse and a 90-min chase period with control cells, total [^{35}S]-labelled apo B100 recovered from cells and medium was 30% and from the culture medium alone 21% of initial apo B100 synthesized (Fig. 6). These data show that the major part of synthesized apo B100 is not secreted but intracellularly degraded, which is in agreement with other studies in Hep G2 cells (15,40,41) and rat hepatocytes (31,42). The recovery of labelled apo A-I and albumin (cells and medium) was 85-90% of initial apo A-I and albumin synthesized, indicating there is (almost) no intracellular degradation of these two proteins, in accordance with (31,40,43). Treatment of Hep G2 cells with CsA resulted in a 50% decrease in secretion of [^{35}S]-labelled apo B100 after the 90-min chase period, without an effect on secretion of newly synthesized apo A-I and albumin. The decrease in radiolabelled apo B100 was already observed after the 10-min pulse period. Since the synthesis of apo B100 in Hep G2 takes 14 min (44), our data indicate that CsA either directly inhibits the translation of apo B100 or affects a process involved in the co-translational processing of apo B100. It has been shown that CsA binds with high affinity to cyclophilin (45), a peptidyl-prolyl cis-trans isomerase (46,47), and inhibits this enzyme, that catalyzes the folding of nascent proteins transferred into the endoplasmic reticulum (43,48,49). We suggest that inhibition of cyclophilin by CsA may inhibit the correct folding of apo B100 during biosynthesis, leading to increased intracellular degradation. This will result in a reduced apo B100 and consequently lipid secretion from Hep G2 cells by CsA. A similar effect of CsA on protein folding has recently been described to take place during the intracellular maturation of

transferrin in Hep G2 (43). It appears that CsA only affects proteins with relative long maturation times such as apo B100 (this study) and transferrin (43) and not with rapidly transported proteins as apo A-I and albumin.

The suggestion by others (9) that the increased plasma LDL-levels in patients treated with CsA may be caused by hepatic overproduction of apo B100-containing lipoproteins is not supported by the data presented in this paper. We also found a decreased binding, internalization and degradation of LDL in Hep G2 cells incubated in the presence of CsA (Table 2). Whether the latter observation and our previous finding that CsA specifically inhibits the alternative pathway in bile acid synthesis (50) are related to the elevated levels of apo B100-containing lipoproteins *in vivo* remains to be elucidated.

ACKNOWLEDGEMENTS

We gratefully thank Elly de Wit and Sylvia van der Plas for excellent technical assistance and Marisa Horsting for typing the manuscript. This work was supported by the Netherlands Heart Foundation (grant 87.035).

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CHAPTER 5

RETINOIDS STIMULATE APOLIPOPROTEIN A-I SYNTHESIS BY INDUCTION OF GENE TRANSCRIPTION IN PRIMARY HEPTOCYTE CULTURES FROM CYNOMOLGUS MONKEY (*MACACA FASCICULARIS*)

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Submitted for publication.

ABSTRACT

The influence of different retinoids on apolipoprotein A-I (apo A-I) synthesis and secretion was investigated in primary monolayer cultures of hepatocytes from cynomolgus monkey. Addition of retinol and retinoic acid to the culture medium resulted in a time- and dose-dependent increase in the secretion of apo A-I. No effect was observed during the first 24 h incubation period, however, apo A-I secretion was enhanced 1.5-fold in the following 24 h period in the presence of 10 μM retinoic acid. Maximal stimulation (2.7-fold) was obtained at 10 μM retinoic acid during a third 24 h incubation. In these experiments apo B100 secretion was unaffected. Performing [^{35}S]-methionine incorporation studies it was assessed that the *de novo* synthesis of apo A-I was increased, whereas total protein synthesis remained constant. The observations indicate that the induction of apo A-I synthesis is not part of a general effect of retinoic acid on hepatic protein synthesis. Among different natural and synthetic retinoids, retinoic acid and its 9-cis and 13-cis isomers were equally active and the most potent inducers of apo A-I synthesis, whereas the maximal stimulation induced by retinol (vitamin A) was lower (1.6-fold). Apo A-I mRNA abundance was increased 3-fold in hepatocytes exposed for 72 h to 10 μM retinoic acid, which was associated with a 2-fold increase in transcriptional rate of the apo A-I gene. In contrast, no changes were found in apo B100 mRNA level and transcriptional activity of the apo B100 gene. We conclude that retinoids enhance apo A-I synthesis in simian hepatocytes by transcriptional regulation.

INTRODUCTION

Apolipoprotein A-I (apo A-I) is the major protein constituent of plasma high density lipoprotein (HDL) (1). In mammals, the protein is mainly synthesized in the liver and the small intestine (1,2). Decreased plasma levels of HDL cholesterol are associated with an accelerated development of atherosclerotic lesions, which is one of the main causes of coronary artery disease (3-5). The plasma level of apo A-I has been reported to be even more discriminatory in determining the risk of cardiovascular disease than the cholesterol concentration of HDL (6,7). It is assumed that the protective effect of HDL is based on its role in the so-called reverse cholesterol transport (8) (for a review see (9)). Experimental evidence strongly suggests that in the first stage of this process, HDL facilitates the efflux of cholesterol from peripheral tissues, such as cells of the vascular wall, thereby preventing accumulation of cholesterylesters and formation of fatty streaks and plaques (10,11). The thus removed cholesterol is esterified by the enzyme lecithin: cholesterol acyltransferase, and transported, either directly or via transfer to other lipoproteins to the liver, where

cholesterol is excreted as such or after conversion to bile acids. This hypothesis is supported by the finding that genetic deficiencies in apo A-I and HDL are associated with excessive intracellular cholesterol accumulation and premature atherosclerosis (4).

Apo A-I synthesis has been reported to be modulated by a variety of factors including hormones, e.g. insulin (12), thyroid hormone (13) and estrogen (14), diet (15) and pharmacologic substances (16). We have previously shown that sodium butyrate stimulates apo A-I synthesis by induction of its mRNA (17). The effects of butyrate on mammalian cells have been extensively studied, since this agent exhibits potent effects on cell growth and differentation and can induce specific gene expression (18). Many of the effects of butyrate can be mimicked by retinoids (19-22). An effect of retinoids, including retinol (vitamin A), retinoic acid, and its natural occurring isomers (23-25) on apo A-I synthesis would be physiologically more significant, since these compounds are well known physiologically active modulators (26-29, and references therein). We, therefore, assessed the ability of retinoids to alter apo A-I synthesis in primary cultures of hepatocytes.

Owing to the limited availability of human liver, experiments were conducted using primary monolayer cultures of hepatocytes from cynomolgus monkey (*Macaca fascicularis*), a nonhuman primate. This species has been shown to be a suitable model to study lipid and lipoprotein metabolism (30-32) and development of atherosclerosis (33). The primary hepatocyte model has a potential advantage over hepatoma cell lines in that the cells in primary culture might reflect more accurately what is occurring in the liver.

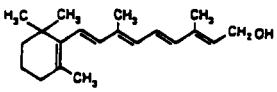
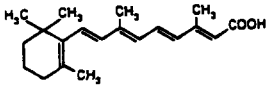
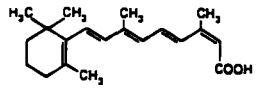
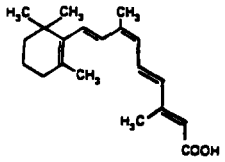
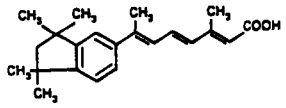
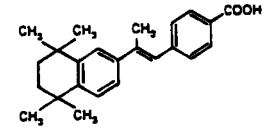
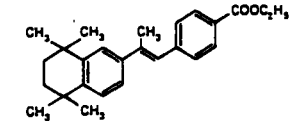
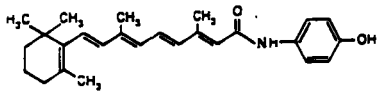
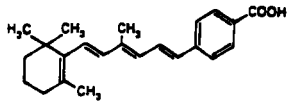
In this paper we report that retinoids enhance apo A-I synthesis in hepatocyte cultures from cynomolgus monkey by induction of apo A-I gene transcription. Among various natural and synthetic analogues retinoic acid and its natural isomers showed to be the most potent stimulators. In contrast to apo A-I, synthesis of apo B100 was found to be unchanged by retinoids.

METHODS

Materials

Retinol (vitamin A), retinoic acid, and 13-cis-retinoic acid were purchased from Sigma Chemical Co., U.S.A. 9-Cis-retinoic acid, RO 13-4306, RO 13-7410, and RO 13-6298 were generous gifts from Mr. P. Weber and Dr. F. Schneider, Hoffmann-La Roche Ltd., Basel, Switzerland. Fenretinide was a generous gift from Dr. M. Rosenthale, the R.W. Johnson Pharmaceutical Research Institute, Raritan, N.J. USA. Pelretin was a generous gift from Dr. B. Janssen, BASF, Ludwigshafen, Germany. The structures of the various retinoids are listed in Table 1. All retinoids were stored at -20°C in sealed containers. Stock solutions of 10 mM were prepared in 100% DMSO (freshly prepared just before each culture experiment) and preserved at -20°C for no longer than 5 days. Immediately before use the retinoids were diluted in culture medium, such that the DMSO

Table 1. Structure of retinoids

	Code No./chemical name
	(all-E)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraen-1-ol, Retinol (vitamin A)
	(all-E)-3,7-Dimethyl-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid, Retinoic acid (vitamin A acid; Tretinoin)
	13-cis-retinoic acid (Isotretinoin; Accutane)
	9-cis-retinoic acid
	RO 13-4306 (all-*E*)-3-Methyl-7-(1,1,3,3-tetramethyl-5-indanyl)-2,4,6-octatrienoic acid
	RO 13-7410 p-[(E)-2-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoic acid
	RO 13-6298 Ethyl p-[(E)-2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)-1-propenyl]benzoate
	N-[4-hydroxyphenyl]retinamide, Fenretinide
	(all E)-1-(4-carboxyphenyl)-4-methyl-6-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1,3,5-hexatriene, Pelretin

concentration did not exceed 0.1% (v/v). Since the compounds are light sensitive, all experiments were performed in subdued light. Materials used for the isolation and culturing of simian hepatocytes were obtained from sources described previously (34), except for fetal bovine serum which was purchased from Boehringer Mannheim (Mannheim, Germany). [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P] UTP (400 Ci/mmol), and [^{35}S]-methionine (> 1000 Ci/mmol) were obtained from Amersham International (Amersham, Buckinghamshire, U.K.).

Simian hepatocyte isolation and culture

Simian hepatocytes were isolated from livers of both male and female cynomolgus monkeys (*Macaca fascicularis*, 1.5-3 years old), which were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The animals were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitis vaccine at this institute. The isolation procedure was essentially as described for human hepatocytes (34-36), with a few modifications. The simian liver was perfused with 1.5 litre of a HEPES buffer pH 7.4 containing 10 mM HEPES, 132 mM NaCl, 6.7 mM KCl and 20 mM glucose at a rate of 100 ml per min. After the preperfusion the liver was perfused successively with 500 ml of a HEPES buffer, pH 7.6, containing 100 mM HEPES, 67 mM NaCl, 6.7 mM KCl, and 5 mM $CaCl_2$ without circulation and with 200 ml of this buffer containing 0.05% collagenase with recirculation for 30 min. Liver tissue was dissociated in Hank's buffer containing 2% bovine serum albumin, cells were filtered through a 250 μm filter, centrifuged (60 g, 5 min), and washed three times in cold culture medium to remove damaged and nonparenchymal cells. Total cell yields varied from 0.74 to 2.3 x 10^9 viable cells. Viability, based on the ability of hepatocytes to exclude tryptan blue dye (0.11%) was 66-96%. The cells were seeded on culture dishes at a density of 2 x 10^5 viable cells per cm^2 and were maintained in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum (30 min at 56°C), 2 mM L-glutamine, 20 mU insulin per ml (135 nM), 50 nM dexamethasone, 100 IU penicillin per ml, 100 μg streptomycin per ml, and 100 μg kanamycin per ml at 37°C in a 5% CO_2/95% air atmosphere. After 14-16 hours the non-adherent cells were washed from the plates, using the same culture medium as above. After 24 hours from seeding the incubations with the various retinoids were started in the same culture medium but with a lower insulin concentration, 10 nM instead of 135 nM, using two or three separate wells per culture condition. Since the retinoids were added to the culture medium as a stock solution in dimethyl sulphoxide (DMSO), all incubations, control and with retinoids at various concentrations were performed with medium containing 0.1% (v/v) DMSO. The medium was renewed every 24 h thereafter. At the end of an incubation period, medium was collected and centrifuged for 20 sec in an Eppendorf centrifuge (type 5414) to remove detached cells and debris. The supernatant was frozen immediately in dry-ice and stored at -20°C, until measurement of apo A-I and apo B100 concentrations. After the last incubation period cells were washed three times with cold phosphate-buffered saline (sodium/potassium phosphate buffer, 11 mM, pH 7.5, containing 150 mM NaCl). The cellular protein was determined as described by Lowry et al. (37).

ELISA of apo A-I and apo B100

Apo A-I and apo B100 concentrations in culture medium were measured in triplicate using a sandwich Elisa procedure with polyclonal antibodies to human apo A-I or human apo B100,

respectively, both as catching and detecting antibodies as described (17). The standard curves for apo A-I and apo B100 in human serum and serum from the cynomolgus monkey and in culture medium of the cynomolgus hepatocytes were parallel indicating that similar epitopes on apo A-I and apo B100 of the two species are recognized.

Protein synthesis

Overall secretion of newly synthesized proteins was determined by measuring the incorporation of [^{35}S]-methionine into the 10% (w/v) trichloroacetic acid precipitable fraction of radiolabelled culture medium. The metabolically radiolabelled proteins secreted in the medium were analysed by SDS/PAGE under reducing conditions by the method of Laemmli (38), with resolving gels containing a gradient of 4-20% (w/v) acrylamide and stacking gels of 3.5% (w/v) acrylamide. Protein molecular-mass standards (Bio-Rad, Richmond CA, U.S.A.) were used for calibration of the gel. For autoradiography the gel was treated with an autoradiography enhancer (EN^{3}hance; NEN DuPont, Boston MA, U.S.A.) in accordance with manufacturers' instructions, dried and placed on X-ray film (Kodak X-Omat AR films; Eastman-Kodak Co., Rochester NY, U.S.A.) and stored at -80°C for the appropriate time.

RNA hybridization

Total RNA was isolated from cynomolgus hepatocytes by the method of Chomczynski & Sacchi (39). After washing the RNA pellets with 70% (v/v) ethanol, RNA samples were dissolved in water. The RNA concentration in each sample was determined spectrophotometrically, assuming that one A_{260} unit corresponds with 40 μg RNA/ml.

Equal amounts of total RNA from different incubations were fractionated by electrophoresis in a 0.8% (w/v) agarose gel containing 1 M formaldehyde, and transferred to Hybond N (Amersham) in accordance with the manufacturer's instructions. RNA blots were hybridized with different probes at 65°C in a sodium phosphate buffer (0.5 M, pH 7.5), containing 7% (w/v) SDS and 1 mM EDTA. DNA fragments used as probes were isolated from low-melting-point agarose (40). One blot was hybridized with 25 ng of probe, labelled by the random-primer method (Multi-prime, Amersham) to approximately 2 x 10^8-10^9 c.p.m./μg of DNA.

After hybridization, the blots were washed twice with 2 x SSC/0.1% SDS (30 min at 65°C) (1 x SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0), twice with 1 x SSC/0.1% SDS (30 min at 65°C), and twice with 0.1 SSC/0.1% SDS (30 min at 65°C) successively). The blots were exposed to Hyperfilm MP (Amersham), together with an intensifying screen (Eastman-Kodak Co.) for 15-96 h at -80°C. For quantification of the relative amounts of mRNA, the autoradiographs were scanned with a Shimadzu CS 910 chromatograph scanner, and areas under the peaks were integrated and plotted with the aid of a data processor (Shimadzu Corp., Kyoto, Japan). The mRNA levels were quantified by using different amounts of total RNA loaded on the gel, giving a linear relation between the specific mRNA signal and the amount of mRNA applied. For apo A-I and actin, linearity was observed between 1.5 and 6 μg of total RNA. For apo B100 mRNA there was linearity between 3 and 12 μg of total RNA. The following fragments were used as probes in the hybridization experiments: a 2.2 kb Pst I fragment of the human apo A-I genomic DNA (kindly provided by Dr. S.E. Humphries (41)), a 2.7 kb Hind III fragment located at the 3' end of human apo B100 cDNA (kindly provided by Dr. J. Scott (42)), and a 1.2 kb Pst I fragment of hamster actin cDNA (kindly

provided by Dr. W. Quax (43)).

Nuclear run-on studies

Nuclear run-on studies were conducted essentially as described by Groudine et al. (44), with minor modifications (45).

Isolation of nuclei. Cells were washed (twice), and scraped, in 0.9% (w/v) saline solution, using a rubber policeman, and collected by centrifugation at 500 x g at 4°C for 5 min. The cells were resuspended in NP40-lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40, 1 mM PMSF, 1 mM DTT), and after being left on ice for 5 min, homogenized in a Potter Elvehjem tube with pestle B for 25 strokes at 4°C. This homogenate was left on ice for 5 min and again homogenized for 25 strokes at 4°C. Resulting nuclei were again centrifuged at 500 x g and resuspended in NP40-lysis buffer. This procedure was repeated until the nuclei were free of cellular debris. Nuclei were then taken up in glycerol storage buffer (50 mM Tris-HCl, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 5 mM DTT), counted, and aliquoted at approximately 4.10^7 nuclei per ml before being frozen at -80°C.

RNA labelling and isolation. An aliquot of frozen nuclei (2×10^7) was added to 200 µl of transcription buffer (10 mM Tris-HCl, pH 7.9, 140 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM $MnCl_2$, 1 mM of dGTP, dATP, dCTP, 0.1 mM s-adenosyl-l-methionine, 14 mM β-mercaptoethanol, 1 mg/ml heparin sulphate, 1.7 mM spermidine, 10 mM creatine phosphate, 40 µg/ml creatine kinase, 25% glycerol and 100 µCi of [α-^{32}P]UTP), and incubated while shaking at 30°C for 30 min. 600 µl of a buffer containing 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.4, and 200 U/ml DNAse I (Bethesda Research Laboratories) was added and the mixture was incubated for an additional 5 min at 30°C. 200 µl of SDS/Tris (5% (w/v) SDS, 0.5 M Tris-HCl, pH 7.4, 0.125 M EDTA) with 200 µg/ml Proteinase K (Boehringer Mannheim) was added, and the mixture was incubated for 30 min at 42°C. RNA was extracted with 1 volume of phenol/chloroform/isoamyl-alcohol (50:49:1), precipitated with 2.5 volumes of ethanol and 10 µg tRNA/ml, washed, and taken up in 50 µl of Tris/EDTA (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). Labeled RNA was separated from free nucleotides by passage over a Sephadex G50 (fine)-column (Boehringer Mannheim). The RNA was mildly degraded by a 10 min incubation on ice in 0.25 M NaOH, and the mixture was neutralized by addition of a half volume of 1 M HEPES (free acid) and precipitated with 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate. Incorporation of label was measured by liquid scintillation counting, and equal amounts of labelled RNA were added to the filters.

Hybridization. Target DNA, being 5 µg of plasmid material containing DNA sequences of human apo A-I, human apo B100, hamster actin or empty plasmid DNA as negative control (for details see the section "RNA hybridization") were slot blotted onto strips of Hybond N^+ filter (Amersham), and crosslinked with 0.4 M NaOH for 30 min. The filters were preincubated for 30 min at 65°C in a sodium phosphate buffer as described before, and hybridized with the labelled RNA for 36 hours in the same buffer. The various filters were washed once for 5 min and twice for 30 min in 2 x SSC/1% SDS at 65°C, and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of relative amounts of mRNA was conducted using actin mRNA signal as an internal standard.

Statistical analysis

Statistical significance of differences was calculated by Student's t-test for paired data with the level

of significance selected as $P < 0.05$. Values are expressed as means ± S.D..

RESULTS

Time course and dose-dependency of the effect of retinol and retinoic acid on the apo A-I and apo B100 secretion

In Fig. 1 the time course is shown of the effect of retinol and retinoic acid on apo A-I and apo B100 secretion by cynomolgus hepatocytes. Effects of retinol and retinoic acid are expressed as percentages of control during the same incubation period. Both the secretion of apo A-I and apo B100 remained constant or almost constant during the three consecutive 24 hour incubation periods in all four independent hepatocyte cultures.

With 10 μM retinoic acid, a significantly accelerated accumulation of apo A-I (1.5-fold) was observed during a 24 h incubation after a 24 h preincubation with the same concentration of retinoic acid. A further significant increase in the accumulation of apo A-I in the culture medium was observed in the third 24 h incubation in the presence of 10 μM retinol (1.6-fold) or 10 μM retinoic acid (2.7-fold). The increase of the apo A-I secretion caused by retinoic acid was significantly higher than with retinol. Both with retinol and retinoic acid the secretion of apo B100 was not affected. In subsequent experiments the effect of retinol, retinoic acid or other retinoids was studied over a 24 h period, after two 24 h preincubation periods in the presence of the same retinoid concentration.

As shown in Fig. 2, addition of increasing amounts of retinol or retinoic acid resulted in an increase in the secretion of apo A-I, without affecting the apo B100 secretion. The effect of retinoic acid on the apo A-I secretion is more pronounced than that of retinol. A significant increase of the apo A-I secretion was observed with 1 and 10 μM retinoic acid and 10 μM retinol.

Structural specificity studies

Because of the difference in the induction of the apo A-I secretion caused by retinoic acid and retinol we studied the effect of other natural and synthetic retinoids. Table 1 shows the structure of the various retinoids, whereas in Table 2 the effects are listed of these retinoids, all tested at a concentration of 10 μM, on the secretion of apo A-I and apo B100 by the hepatocytes from the cynomolgus monkey. The natural retinoids, retinoic acid and its 9- and 13-cis-isomers and the three synthetic RO-compounds have comparable effects on the apo A-I secretion. Next to retinol, the synthetic retinoids fenretinide and pelretin were found to be less effective. None of the retinoids had an effect on the secretion of apo B100.

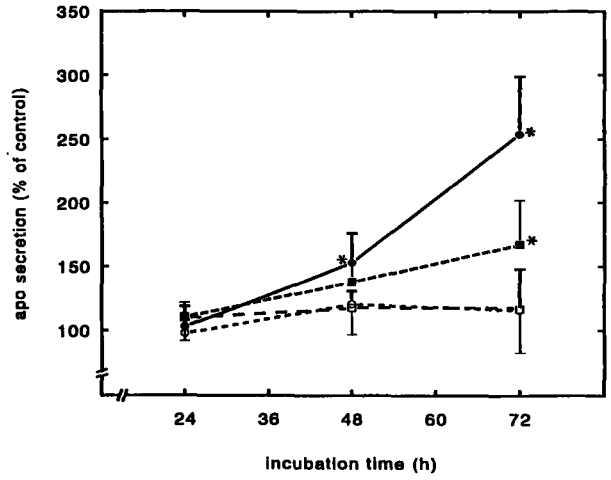


Figure 1. Time course of the effect of retinol and retinoic acid on apo A-I and apo B100 accumulation in the medium of cynomolgus hepatocytes. After a 24-h attachment and recovery period hepatocytes were cultured for 24, 48 or 72 h on standard medium with or without retinol or retinoic acid (10 μM). At the end of each incubation period medium was collected and analysed for apo A-I and apo B100, as described in the Methods section. Values were normalized for cell protein in culture dishes and are expressed as a percentage of control in the same incubation period. Values are means ± S.D. of duplicate incubations of hepatocytes from four independent hepatocyte isolations. Apo A-I and apo B100 production in control medium during the first 24-h incubation period for these four hepatocyte cultures was 794 ± 455 (range 390-1449) and 631 ± 308 (range 278-1029) ng/24 h per mg of cell protein, respectively (means ± S.D.). Apo A-I and apo B100 secretion remained constant or almost constant during the three consecutive periods. (●) Apo A-I and (○) apo B100 production in the presence of 10 μM retinoic acid. (■) Apo A-I and (□) apo B100 production in the presence of 10 μM retinol. *Indicates a significant difference ($P < 0.05$) between control and treated cells.

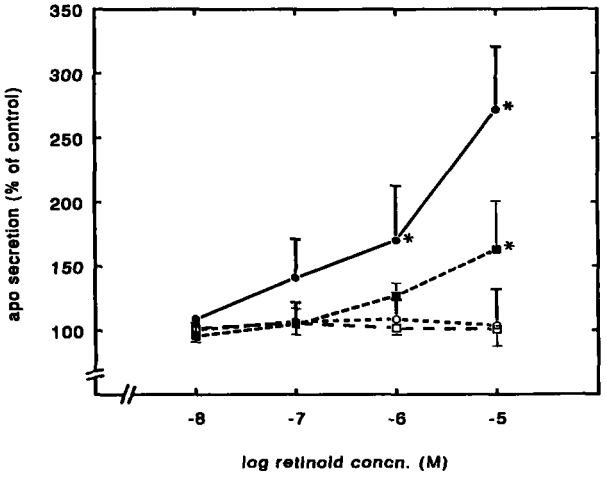


Figure 2. Dose-dependency of the effect of retinol and retinoic acid on the secretion of apo A-I and apo B100 in the medium of primary cultures of cynomolgus hepatocytes. Hepatocytes were incubated for 24 h with various concentrations of retinol or retinoic acid, after two 24 h preincubation periods with the same concentration of retinol or retinoic acid, respectively. Apo A-I and apo B100 concentrations in the culture medium were measured as described in the Methods section. Results were normalized for cell protein and are expressed as a percentage of control, and are means ± S.D. for four different hepatocyte isolations. Apo A-I and apo B100 production in control medium for these four hepatocyte cultures was 824 ± 357 (range 383-1170) and 545 ± 173 (range 384-769) ng/24 h per mg cell protein; respectively (means ± S.D.). (●) Apo A-I and (○) apo B100 secretion in the presence of retinoic acid. (■) Apo A-I and (□) apo B100 secretion in the presence of retinol. *Indicates a significant difference ($P < 0.05$) between control and treated cells.

Table 2. Structural specificity of retinoids

Primary hepatocytes from the cynomolgus monkey were incubated with different retinoids (10 μM) for 24 h, after two 24 h preincubation periods with the same retinoid at the same concentration. The concentrations of apo A-I and apo B100 in the medium were measured as described in the Methods section and normalized for the amount of cell protein on the culture dishes. Results are expressed as percentages of control, and are means ± S.D. for three to five independent experiments. Apo A-I and apo B100 secretion in control medium was 709 ± 334 and 599 ± 167 ng/24 h per mg cell protein respectively (means ± S.D.). Apo A-I, but not apo B100 secretion in incubations with all retinoids was significantly different ($P < 0.05$) from control. *Indicates a significance ($P < 0.05$) between retinol and other retinoids.

Compound added to the medium	Secretion (% of control)	
	apo A-I	apo B100
Retinol	163 ± 38	101 ± 13
Retinoic Acid	272 ± 49*	104 ± 28
13-cis Retinoic Acid	238 ± 37	117 ± 7
9-cis Retinoic Acid	247 ± 14*	124 ± 17
RO-13-4306	233 ± 41	99 ± 14
RO-13-7410	262 ± 13*	122 ± 24
RO-13-6298	267 ± 28*	110 ± 23
Fenretinide	191 ± 32	125 ± 12
Pelretin	185 ± 23	107 ± 5

Specificity of the stimulation of the apo A-I secretion by retinoic acid

Our finding that the various retinoids do not affect the apo B100 secretion by cynomolgus hepatocytes indicates that the increase of apo A-I is not part of a general effect of retinoids on the hepatocytes. In addition the effect of retinoic acid on the total protein secretion by the hepatocytes was studied by using [^{35}S]-methionine incorporation in newly synthesized and secreted proteins. Retinoic acid had no effect on the total amount of [^{35}S]-methionine labelled proteins secreted by the simian hepatocytes. To explore the possibility that retinoic acid may alter the pattern of the proteins secreted into the culture medium, the proteins in the medium of the [^{35}S]-methionine incorporation experiment were separated by SDS/PAGE and autoradiographed (Fig. 3). The autoradiograph shows that retinoic acid increases the secretion of newly synthesized apo A-I. The effect on apo A-I appears to be rather specific although there is a limited number of proteins that are also affected by retinoic acid. Both increases and decreases of some of the secreted labelled proteins are observed, indicating that both positive and negative regulation of synthesis of proteins by retinoic acid may occur.

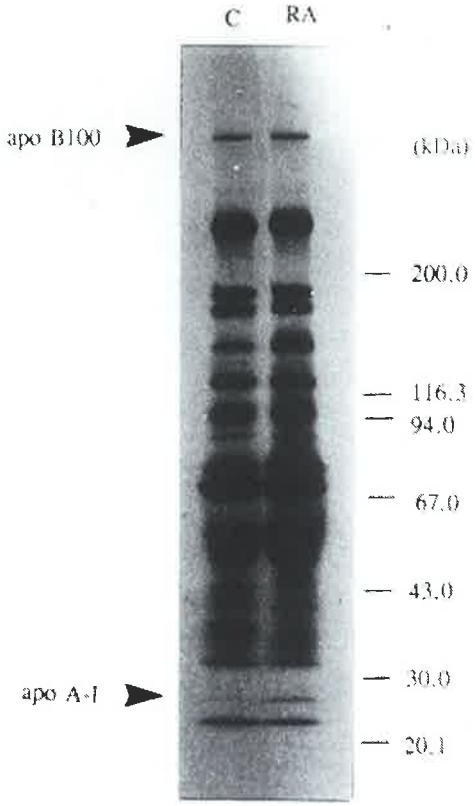


Figure 3. SDS/PAGE and autoradiography of metabolically radiolabelled proteins in the medium of cynomolgus monkey hepatocytes. Hepatocytes were incubated for 24 h in standard medium with or without 10 μM retinoic acid, containing 20 μCi of [^{35}S]-methionine/ml, after two 24-h preincubation periods in the same medium without labelled methionine. Molecular-mass standards are indicated on the right of the autoradiograph. At the left side of the autoradiograph the positions of apo A-I and apo B100 are depicted. RA and C indicates incubation with or without retinoic acid, respectively.

Effect of retinoic acid on apo A-I and apo B100 mRNA levels and transcriptional activity

To investigate the mechanism of induction of apo A-I secretion, the effect of retinoic acid on the mRNA level of apo A-I, together with the apo B100 mRNA level, was assessed by Northern-blot hybridization (Fig. 4). The apo A-I and apo B100 mRNA levels were compared with the actin mRNA level, which was not affected by retinoic acid. A 3-fold increase in the apo A-I mRNA level was observed when cynomolgus monkey hepatocytes were treated with 10 μM retinoic acid for 72 h. In contrast to the apo A-I mRNA level, the apo B100 mRNA level was not changed by retinoic acid.

To further investigate the mechanism of regulation of the apo A-I mRNA induction, nuclear run-on studies were performed. Nuclear run-on transcripts were analyzed after 72 h of incubation of the simian hepatocytes with or without 10 μM retinoic acid (see Fig. 5). The transcriptional activity of the actin gene was used as an internal standard. The empty vector pUC 18 shows that there is no aspecific hybridization of the gene-transcripts.

A 2-fold increase in the transcriptional activity of the apo A-I gene was observed without changes in the expression of the apo B100 gene, indicating that the elevated apo A-I mRNA level resulted from an increased transcriptional activity.

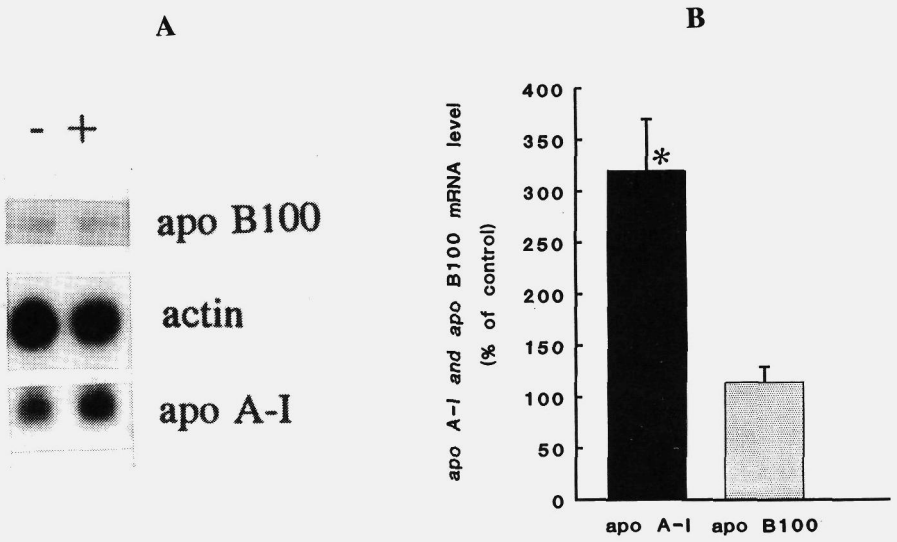


Figure 4. Effect of retinoic acid on apo A-I and apo B100 mRNA levels in cynomolgus monkey hepatocytes. A) Autoradiograph of a Northern-blot hybridized with [^{32}P]-labeled probes for apo A-I, apo B100 and actin mRNA. The hybridized blot was exposed for the appropriate time, at -80°C, to Hyperfilm, by using an intensifying screen. + and - indicates incubation for 72 h with or without 10 μM retinoic acid, respectively. B) The effect of a 72 h treatment of simian hepatocytes with 10 μM retinoic acid on apo A-I (closed bar) and apo B100 (open bar) mRNA levels. The intensity of the bands on autoradiographs (as illustrated, for example, in Fig. 4A) was assessed by densitometric scanning. Actin mRNA level was used as an internal standard to correct for differences in the amount of total RNA applied to the gel. The results are expressed as percentages of those in the incubation without retinoic acid and are means $\pm$ S.D. for three independent isolations, with analysis of three RNA concentrations in each experiment. *Indicates a significant difference ($p < 0.05$) between control and treated cells.

DISCUSSION

In this paper we have shown that retinoids increase the secretion of apo A-I in primary hepatocyte cultures from cynomolgus monkey in a time- and dose-dependent way, without affecting apo B100 secretion. [^{35}S]-Methionine incorporation experiments demonstrate that retinoic acid stimulates the secretion of newly synthesized apo A-I, without changing total protein synthesis. Although there were changes in a few other proteins synthesized by the hepatocytes, these findings indicate that the effect of retinoids on apo A-I synthesis is not

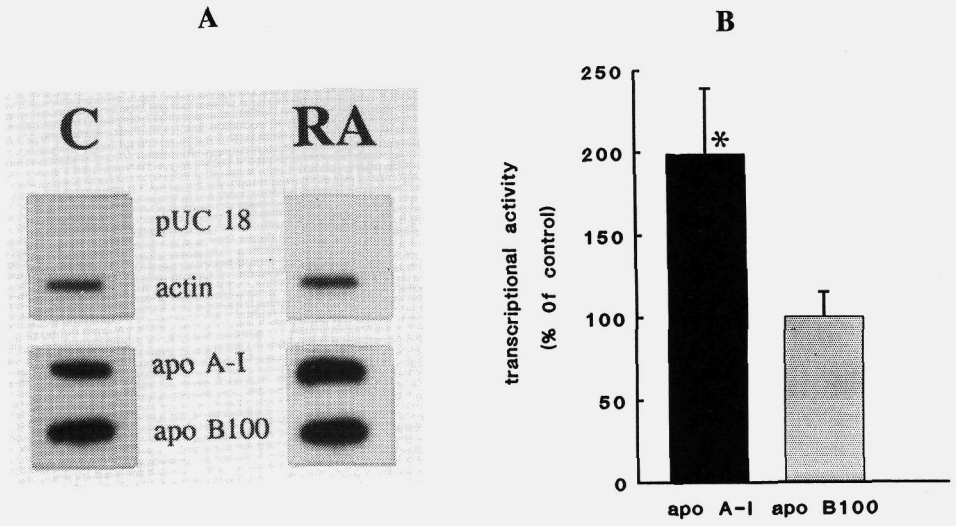


Figure 5. Transcriptional activation of the apo A-I gene in response to retinoic acid.
A) Autoradiograph of the run-on transcripts for apo A-I, apo B100 and actin of nuclei from simian hepatocytes treated with (RA) or without (C) 10 μM retinoic acid for 72 h. The hybridized blot was exposed for 75 h, at -80°C, to Hyperfilm by using an intensifying screen. Nonspecific hybridization was checked by using the vector pUC 18. For details see the Methods section.
B) The effect of a 72 h treatment of cynomolgus hepatocytes with 10 μM retinoic acid on the transcriptional activity of the apo A-I gene (closed bar) and the apo B100 gene (open bar). The density of the bands on the autoradiograph (as illustrated, for example, in Fig. 5A) was assessed by densitometric scanning. Transcriptional activity of the actin gene was used as an internal standard. The results are expressed as percentages of those in the incubation without retinoic acid and are means $\pm$ S.D. for three independent isolations. *Indicates a significant difference ($p < 0.05$) between control and treated cells.

part of a general increase in protein synthesis by simian hepatocytes. The enhanced apo A-I synthesis was accompanied by a comparable increase of the apo A-I mRNA level and of the transcriptional activity of the apo A-I gene in the retinoic acid-treated hepatocytes. The latter data demonstrate that retinoids regulate apo A-I synthesis in simian hepatocytes by induction of gene expression. In contrast with apo A-I, retinoic acid did not change apo B100 mRNA level and transcriptional activity.

We have found, to our knowledge for the first time, that retinoids stimulate apo A-I synthesis and secretion in a physiological system. This may not seem surprising, as the presence of a retinoic acid responsive element in the 5'-flanking region of the A-I gene has recently been reported, that responds preferentially to the retinoic acid-responsive receptor RXRα (46). However, addition of retinoic acid to the human hepatoma cell line Hep G2 did not lead to induction of the apo A-I gene in these cells (47) or secretion of apo A-I in the culture medium (Kaptein and Princen, unpublished results), also not after prolonged

exposure to the retinoid. In contrast to retinoids apo A-I synthesis in Hep G2 cells is stimulated by butyrate (17), indicating that regulation of apo A-I is possible. The lack of responsiveness could only be overcome by transfection of a high amount of RXRα, leading to overexpression of this receptor in Hep G2 and subsequent transcriptional activation in the presence of retinoic acid (47). These data may indicate that Hep G2 cells do not contain and do not have the capability to induce significant amounts of RXRα to stimulate apo A-I gene expression and secretion upon addition retinoids. In contrast, primary simian hepatocytes are responsive to retinoids, as we show herein. We suggest that this difference in responsiveness between hepatoma cells and primary hepatocytes results from the well-known phenomenon of dedifferentiation in hepatoma cells, which is accompanied by loss of certain functions, e.g. as discussed above the loss of RXRα. Therefore, hepatocytes in primary culture may give a more accurate reflection of the actual physiology of the liver.

Retinoic acid is known to be an active metabolite of retinol, which exhibits a variety of potent effects on cell growth and differentiation, such as suppression of carcinogenesis *in vivo* (26) and regulation of pattern formation in developing and regenerating limbs (27,28). Retinoic acid in turn can be converted into physiologically active compounds as its 9-cis- and 13-cis-isomers (23-25). We have compared the potency of these naturally occurring retinoids to induce apo A-I synthesis with several synthetic compounds.

Our results suggest that a terminal carboxylic acid moiety in the retinoids is of importance in the modulation of apo A-I synthesis in the hepatocytes. Replacement of this group by a hydroxy group (as in retinol) or amidation of the carboxylic function (as in fenretinide) diminishes the activity of the compounds as compared to the carboxyl-group-containing analogue, retinoic acid. On the other hand esterification of the carboxylic acid moiety (RO 13-6298) did not lead to a decrease in activity as compared with the free carboxylic acid-containing analogue RO 13-7410, suggesting that this compound is active as such or that hydrolysis of the ester proceeds rapidly in the hepatocytes, in contrast with hydrolysis of the amide-bond in fenretinide. Structural modification of the side-chain such that a free carboxylic acid moiety is retained as in RO-13-4306, RO-13-7410 and in the 9- and 13-cis isomers of retinoic acid resulted in comparable effectivity in stimulation of the apo A-I synthesis as found with retinoic acid itself. Pelretin which also contains a free carboxylic acid appeared to be less effective than retinoic acid, although the difference between retinoic acid and pelretin was not statistically significant. The importance of a terminal carboxylic acid group in retinoids has also been shown by other investigators using different types of cultured cells (48,49). This may point to a common mechanism responsible for all these different effects. However, the actual molecular mechanism of regulation of gene expression by retinoids may be more complicated since two different families of nuclear retinoid receptors have been characterized now (RARs and RXRs), each consisting of three receptor subtypes.

Our finding that induction of apo A-I secretion is regulated by transcriptional activation in primary hepatocytes is in line with two recent reports, showing that apo A-I promotor constructs can be regulated by incubation of cells transfected with retinoid receptors with retinoic acid and its 9-cis isomer (47,50). However, the exact molecular mechanism of induction of apo A-I gene expression by retinoids is not fully understood. Whereas Widom et al. (47) have reported that formation of RXRα homodimers in the presence of retinoic acid may abolish the inhibition of transcription of the apo A-I gene by the nuclear factor ARP-I, Zhang et al. (50) have recently shown that the heterodimer RARα-RXRα is more efficient in activation of the apo A-I retinoic acid-responsive element and most efficiently activated in the presence of 9-cis-retinoic acid. This intracellularly generated stereoisomeric metabolite of retinoic acid is believed to be the natural ligand for the retinoid receptor RXRα (24,25). We did not observe a significant difference in the magnitude of stimulation of apo A-I secretion in simian hepatocytes with both retinoids. This is not only the case for the 10 μM concentration listed in Table 2 but also for lower concentrations, i.e. 0.01, 0.1 and 1 μM (data not shown). A possible explanation for the comparable potency of retinoic acid and its 9-cis-isomer may be intracellular isomerization of the retinoids to each other in metabolically active hepatocytes. Our finding that the A-I synthesis is stimulated after a lag phase of at least 24 hours, i.e. during the second and third day of culture, suggests that one or more of the receptors have to be induced to sufficient levels to activate apo A-I gene expression.

The results in the present study showing that retinoids activate apo A-I gene expression and apo A-I secretion raise the possibility that physiological signals relevant to vitamin A metabolism play an important role in the regulation of plasma apo A-I and HDL levels and consequently the atherosclerotic process. In this context it is interesting that ingestion of β-carotene, which is considered to be a precursor of retinol and retinoic acid (51) has been reported to increase HDL levels (52). Similarly, feeding of retinoic acid to rabbits appears to enhance plasma HDL cholesterol and apo A-I levels (47). On the other hand, it should be noted that therapeutical use of retinoids, e.g. isotretinoin and etretinate in dermatological applications (53-55) has been associated with increases in concentration of serum triglycerides and cholesterol, contained predominantly in very low density lipoproteins (VLDL) and low density lipoproteins (LDL), respectively. No change in apo A-I levels and no or a slight decrease in HDL cholesterol was found. The mechanism of these changes has recently been suggested to involve reduced VLDL uptake by the liver after isotretinoin treatment in rat (56), possibly as a consequence of a decreased lipoprotein lipase activity (57). The reduction of HDL cholesterol may be secondary to the increase of VLDL, i.e. decreased production of surface components because of decreased catabolism of VLDL, or a reflection of the greater cholesterol ester-triglyceride exchange between HDL and VLDL, IDL (intermediate density lipoprotein) and LDL. Thus, it is still conceivable that retinoids

in vivo enhance apo A-I synthesis, which is, however, not reflected in increased plasma apo A-I levels as a result of the concomitant elevation of the triglyceride level. In view of the reported stimulation of apo A-I synthesis by retinoids in hepatocytes, we suggest that development of retinoid analogues which do not cause hypertriglyceridemia may be suitable in regulating HDL levels.

ACKNOWLEDGEMENTS

We gratefully thank Mr. Arie Timmerman (RIVM, Bilthoven) for his enthousiastic cooperation and excellent zootechnical assistance, Dr. Jaap Twisk for performing nuclear run-on assays and Mrs. Marisa Horsting for typing the manuscript. We thank Dr. Teake Kooistra for fruitful discussion.

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CHAPTER 6

DELAYED RESPONSE OF APO A-I mRNA TO RETINOIDS IN PRIMARY HEPATOCYTE CULTURES FROM CYNOMOLGUS MONKEY (*MACACA FASCICULARIS*)

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ABSTRACT

We have shown previously that retinoids stimulate the apolipoprotein A-I (apo A-I) synthesis by induction of gene transcription in primary hepatocyte cultures from cynomolgus monkey. The response to retinoids occured at supra-physiological concentrations, and was observed only after a lag phase of 24 to 48 h. In this paper the biochemical background of these effects of retinoids on the apo A-I secretion was elaborated.

Retinoids are strongly metabolized by the hepatocytes, explaining the high starting concentrations needed to induce apo A-I secretion. The time course of the effect of retinoids on the apo A-I mRNA level is comparable with the effect on the apo A-I secretion. Only minor changes in the apo A-I mRNA level were observed after 24 h of incubation in the presence of retinoids. However, after 48 h of incubation an enhanced apo A-I mRNA level was observed with all-trans-retinoic acid (+115%) and 9-cis-retinoic acid (+126%). A further increase was found after 72 h of incubation in the presence of all-trans-retinoic acid (+254%) and 9-cis-retinoic acid (+279%). Nuclear run-on studies showed that the transcriptional rate of the apo A-I gene was already increased after a 4 h incubation with all-trans-retinoic acid (+60%) or 9-cis-retinoic acid (+50%). These results indicate that the delayed effect of retinoids on the apo A-I secretion by cynomolgus hepatocytes is caused by a slow time-dependent increase of the apo A-I mRNA level.

INTRODUCTION

Decreased plasma levels of high density lipoprotein (HDL) cholesterol are associated with an increased risk for the development of coronary heart disease [1-3]. Apolipoprotein A-I (apo A-I), the major constituent of HDL, has been reported to be even more predictive than the cholesterol concentration of HDL [4,5].

A changed apo A-I synthesis has been reported to be positively associated the HDL-cholesterol level in the circulation *in vivo* [6,7]. This indicates that regulation of the synthesis may be useful in increasing the HDL level and subsequently decrease the risk on the development of atherosclerosis. In a previous report [8] we have shown that physiologically important modulators as retinoids, including retinol (vitamin A), retinoic acid and its natural occuring isomers stimulated the apo A-I synthesis by induction of the apo A-I gene transcription in primary hepatocyte cultures from cynomolgus monkey. However, the stimulatory effect was observed only after prolonged incubation with retinoids. In this report we have assessed the biochemical background for the delayed stimulation of the apo A-I secretion.

We found an enhanced apo A-I mRNA level in cynomolgus hepatocytes treated with all-trans-retinoic acid and 9-cis-retinoic acid, after a 24 h preincubation with the same retinoid. An increase in the transcription of the apo A-I gene was already observed after a 4 h incubation period in the presence of these retinoids.

METHODS

Materials

All-trans-retinoic acid was purchased from Sigma Chemical Co.(St. Louis, MO, U.S.A.). 9-Cis-retinoic acid was a generous gift from Mr. P. Weber and Dr. F. Schneider, Hoffmann-La Roche Ltd. (Basel, Switzerland). Stock solutions of 10 mM were prepared in 100% DMSO (freshly prepared just before each culture experiment) and preserved at -20°C for no longer than 5 days. Immediately before use the retinoids were diluted in culture medium, such that the DMSO concentration did not exceed 0.2% (v/v). Since the compounds are light sensitive, all experiments were performed in subdued light. Materials used for the isolation and culturing of simian hepatocytes were obtained from sources described previously [8]. [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P] UTP (400 Ci/mmol) were obtained from Amersham International (Amersham, Buckinghamshire, U.K.).

Simian hepatocyte isolation and culture

Simian hepatocytes were isolated from livers of both male and female cynomolgus monkeys (*Macaca fascicularis*, 1.5-3 years old), which were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The animals were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitis vaccine at this institute. The isolation procedure was exactly as described [8]. Total cell yields varied from 0.74 to 2.3 x 10^9 viable cells. Viability, based on the ability of hepatocytes to exclude tryptan blue dye (0.11%) was 66-96%. The cells were seeded on culture dishes at a density of 2 x 10^5 viable cells per cm^2 and were maintained in Williams E medium supplemented with 10% heat-inactivated fetal bovine serum (30 min at 56°C), 2 mM L-glutamine, 20 mU insulin per ml (135 nM), 50 nM dexamethasone, 100 IU penicillin per ml, 100 μg streptomycin per ml, and 100 μg kanamycin per ml at 37°C in a 5% CO_2/95% air atmosphere. After 14-16 hours the non-adherent cells were washed from the plates, using the same culture medium as above. After 24 hours from seeding the incubations with all-trans-retinoic acid and or 9-cis-retinoic acid were started in the same culture medium but with a lower insulin concentration, 10 nM instead of 135 nM. Since the retinoids were added to the culture medium as a stock solution in dimethyl sulphoxide (DMSO), all incubations were performed with medium containing 0.2% (v/v) DMSO. The medium was renewed every 24 h thereafter.

Retinoid analysis

Retinoids were extracted from both hepatocytes and culture medium using the method described by Barua et al. [9]. Retinoids were analyzed by reverse phase high performance liquid chromatography (HPLC) using a Spherisorb (Phase Separations) S5ODS2 C_{18}analytical column (25x0.46 cm) developed by a non-linear gradient modified from Eckhoff and Nau [10] using two eluents, i.e. 60

mM ammonium acetate, pH 5.75 (A) and methanol (B). The gradient program with a flow rate of 1 ml/min was as follows: 5 min isocratic at 75% B, followed by a linear gradient to 85% B in 5 min and a further linear gradient to 99% B in 20 min. Absorbance was monitored at 340 nm using a 759A Absorbence Detector (Separations).

RNA hybridization

Total RNA was isolated from cynomolgus hepatocytes by the method of Chomczynski & Sacchi [11]. After washing the RNA pellets with 70% (v/v) ethanol, RNA samples were dissolved in water. The RNA concentration in each sample was determined spectrophotometrically, assuming that one A_{260} unit corresponds with 40 μg RNA/ml.

Equal amounts of total RNA (3 and 6 μg, giving linear relation between the specific apo A-I and actin mRNA signal, and the amount of mRNA applied) from different incubations were slot-blotted to Hybond N+ (Amersham) in accordance with the manufacturer's instructions. RNA blots were hybridized and quantitated as described previously [8].

The following fragments were used as probes in the hybridization experiments: a 2.2 kb Pst I fragment of the human apo A-I genomic DNA (kindly provided by Dr. S.E. Humphries [12]) and a 1.2 kb Pst I fragment of hamster actin cDNA (kindly provided by Dr. W. Quax [13]).

Nuclear run-on studies

Nuclear run-on studies were conducted as described [8,14]. The [^{32}P]UTP-labeled RNAs from the nuclear run-on studies were hybridized with target DNA, being 5 μg of plasmid material containing DNA sequences of human apo A-I or hamster actin (for details see the section "RNA hybridization"), slot blotted onto strips of Hybond N^+ filter (Amersham), and crosslinked with 0.4 M NaOH for 30 min. The filters were preincubated for 30 min at 65°C in a sodium phosphate buffer as described before, and hybridized with the labelled RNA for 36 hours in the same buffer. The various filters were washed once for 5 min and twice for 30 min in 2 x SSC/1% SDS at 65°C, and exposed to Hyperfilm MP (Amersham) for 2-5 days. Quantitation of relative amounts of mRNA was conducted using actin mRNA signal as an internal standard.

RESULTS AND DISCUSSION

Previously we have reported that retinoids stimulate the secretion of apo A-I in primary hepatocyte cultures from cynomolgus monkey [8]. An increase of the apo A-I secretion by retinoids was observed only after a lag phase of 24 to 48 h. To further investigate the biochemical background for this delayed effect we measured the time course of the effect of all-trans- and 9-cis-retinoic acid on the apo A-I mRNA level (Fig. 1 and Table 1). In accordance with the effect on the secretion of apo A-I only minor changes were observed in the first 24 h incubation with all-trans- and 9-cis-retinoic acid. In the second 24 h incubation period an enhanced apo A-I mRNA level was observed with all-trans-retinoic acid (+115%) and 9-cis-retinoic acid (+126%). In the third 24 h incubation period a

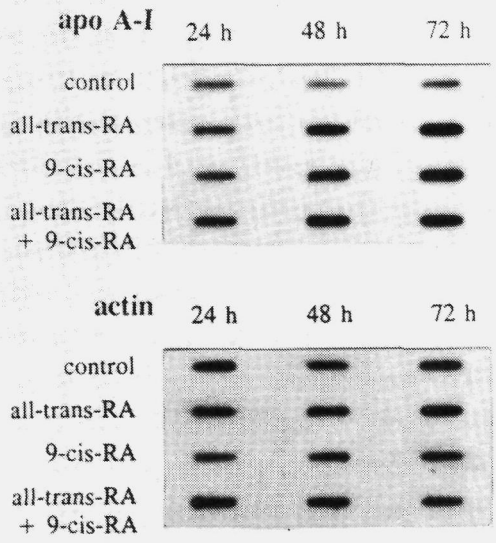

Figure 1. Time course of the effect of all-trans-retinoic acid and 9-cis-retinoic acid on apo A-I mRNA level in cynomolgus hepatocytes. Autoradiographs of slot-blot hybridization with [^{32}P]-labeled probes for apo A-I and actin, with 6 μg total RNA per slot. After a 24 h attachment period total RNA was isolated from hepatocytes after incubation for 24, 48, or 72 h on standard medium with or without 10 μM all-trans-retinoic acid (all-trans-RA) and/or 10 μM 9-cis-retinoic acid (9-cis-RA). Upper and lower autoradiograph show the results of the hybridization of apo A-I mRNA and actin mRNA, respectively. At the top of the autoradiograph the incubation time in the presence or absence of retinoids is indicated. At the right side of the autoradiographs the incubation conditions are indicated: control, standard medium; all-trans-RA, 10 μM all-trans-retinoic acid; 9-cis-RA, 10 μM 9-cis-retinoic acid; all-trans-RA + 9-cis-RA, 10 μM all-trans-retinoic acid and 10 μM 9-cis-retinoic acid.

further stimulation of the apo A-I mRNA level was observed with all-trans-retinoic acid (+254%) and 9-cis-retinoic acid (+279%). When cynomolgus hepatocytes were incubated with all-trans-retinoic acid (10 μM) and 9-cis-retinoic acid (10 μM) simultaneously the induction of the apo A-I mRNA level was lower than the separate incubation with one of the two retinoids at a concentration of 10 μM. This may be caused by the high retinoid concentration, reaching cytoxic levels, to which the hepatocytes are exposed when both retinoids are added to the culture medium.

The prolonged incubation needed to observe an effect of the retinoids on the apo A-I mRNA level does not seem to result from a delayed induction of the apo A-I gene expression. As shown in Fig. 2 the transcription of the apo A-I gene was already increased by 60% after a 4 h incubation with 10 μM all-trans-retinoic acid (+60%). A comparable induction of the apo A-I gene expression was observed with 10 μM 9-cis-retinoic acid (+50%) or in an incubation in which both 10 μM all-trans-retinoic acid and 10 μM 9-cis-retinoic acid (+70%) were added to the culture medium. We suggest that the delayed stimulation of the apo A-I mRNA level by retinoids may be caused by a high stability of the apo A-I mRNA. Stimulation of the transcriptional rate of the apo A-I gene will increase the apo A-I mRNA level, but may give a detectable stimulation of the mRNA level only

Table 1. Time course of the effect of all-trans-retinoic acid and/or 9-cis-retinoic acid on apo A-I mRNA level in cynomolgus hepatocytes. Cynomolgus hepatocytes were cultured for 24, 48 or 72 h on standard medium with or without all-trans-retinoic acid (10 μM) and/or 9-cis-retinoic acid (10 μM).

1A. The effect of the retinoids on apo A-I and actin mRNA level. Apo A-I and actin mRNA levels were analyzed as shown in Fig. 1. The intensity of the bands on the autoradiograph was assessed by densitometric scanning. Actin mRNA level was used as an internal standard to correct for differences in the amount of total RNA applied to the slot-blot. The results are expressed as percentages of the 24 h incubation on medium without retinoids, with analysis of 3 and 6 μg total RNA.

	apo A-I mRNA (% of control)		
	24 h	48 h	72 h
standard medium	100	83 ± 12	126 ± 9
10 μM all-trans-retinoic acid	146 ± 23	215 ± 18	354 ± 33
10 μM 9-cis-retinoic acid	140 ± 10	226 ± 13	379 ± 25
10 μM all-trans-retinoic acid + 10 μM 9-cis-retinoic acid	129 ± 4	163 ± 4	254 ± 13

1B. The effect of all-trans-retinoic acid (10 μM) or 9-cis-retinoic acid (10 μM) on the apo A-I secretion in the experiment described above. Apo A-I was measured by Elisa procedure as described previously [15], normalized for cell protein and is expressed as a percentage of control incubation without retinoids.

	apo A-I secretion (% of control)		
	24 h	48 h	72 h
10 μM all-trans-retinoic acid	123 ± 17	188 ± 27	334 ± 33
10 μM 9-cis-retinoic acid	140 ± 10	219 ± 30	304 ± 30

after prolonged incubation. Previously we reported that after a 72 h incubation with 10 μM all-trans-retinoic acid a 2-fold increase in the transcriptional rate of the apo A-I gene was found. This suggests that their may also be a time-dependent increase of the apo A-I gene expression and that after a 4 h incubation the stimulation of the apo A-I gene transcription may not be maximal.

Our results that retinoids stimulate the apo A-I mRNA level in cynomolgus hepatocytes by induction of the transcription of the apo A-I gene is in accordance with the transcriptional activation by retinoic acid of a apo A-I promotor construct in Hep G2 cells

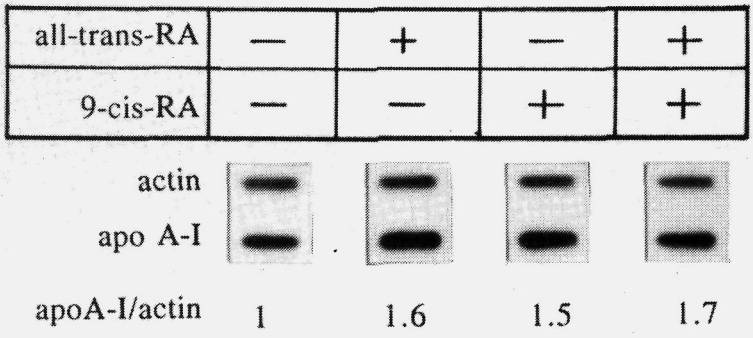

Figure 2. Transcriptional activation of the apo A-I gene in response to all-trans-retinoic acid and 9-cis-retinoic acid. Cynomolgus hepatocytes were treated for 4 h with or without 10 μM all-trans-retinoic acid (all-trans-RA) and/or 10 μM 9-cis-retinoic acid (9-cis-RA). At the top of the autoradiographs of the run-on transcripts the incubation condition is indicated. At the bottom of the autoradiographs the ratio of the apo A-I/actin run-on transcripts is listed. This ratio was normalized for the ratio of the control incubation. For details of the nuclear run-on studies see the Materials and methods section.

[16]. This activation was observed when the retinoid receptor RXRα was also transfected to the Hep G2 cells. The natural ligand for this retinoid receptor is believed to be 9-cis-retinoic acid [17,18]. Zhang et al. [19] have recently shown that the heterodimer of the retinoid receptors RARα and RXRα is involved in the activation of the apo A-I retinoic acid-responsive element, which is most efficiently activated by 9-cis-retinoic acid. This indicates that 9-cis-retinoic acid might be a more potent activator of the apo A-I synthesis. Nevertheless, we did not observe any difference in the stimulatory effect of all-trans- and 9-cis-retinoic acid on the transcription of the apo A-I gene, the apo A-I mRNA level. All-trans- and 9-cis-retinoic acid also had the same effect on the apo A-I secretion by cynomolgus hepatocytes [8]. The comparable potency of all-trans-retinoic acid and its 9-cis isomer may result from the isomerization of the retinoids to eachother. We observed in standard medium with a starting concentration of 10 μM all-trans-retinoic acid that after a 24 h incubation at 37°C (comparable to the incubation conditions of the hepatocytes) 60% of the added all-trans-retinoic acid was still present in the medium. Part of the all-trans-retinoic acid was converted into 9-cis-retinoic acid (0.5 μM) and 13-cis-retinoic acid (1.7 μM). Similar results were found for the spontaneous isomerization of 9-cis-retinoic acid into all-trans- and 13-cis-retinoic acid. These results indicate that addition of all-trans-retinoic acid or 9-cis-retinoic acid leads to the isomerization to eachother, such that the ligands for the retinoid receptors RAR (all-trans-retinoic acid [20]) and RXR (9-cis-retinoic acid [17,18]) are present in the culture medium. When the isomerization of all-trans-retinoic acid was investigated in an incubation in the presence of cynomolgus hepatocytes all-trans-,

9-cis- and 13-cis-retinoic acid in cells and medium was found. The amounts of the different retinoids present in cells and medium after the 24 h incubation were low. Of the added all-trans-retinoic acid (10 μM, 15 nmol per mg of cell protein) less than 2% was recovered after the incubation. Taking the amounts of 9-cis- and 13-cis-retinoic acid into account no more than 5% of the added all-trans-retinoic acid was recovered. These data show that cynomolgus hepatocytes metabolize retinoic acid, suggesting that the high starting concentrations of the retinoids are needed to maintain a sufficiently high concentration necessary for the induction of the apo A-I synthesis.

ACKNOWLEDGEMENTS

We gratefully thank Mr. Arie Timmerman (RIVM, Bilthoven) for his enthousiastic cooperation and excellent zootechnical assistance and Dr. Teake Kooistra for fruitful discussion.

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CHAPTER 7

STIMULATION OF APOLIPOPROTEIN (a) AND B100 SECRETION BY EXOGENOUS CHOLESTEROL-RICH LIPOPROTEINS IN PRIMARY HEPATOCYTE CULTURES FROM CYNOMOLGUS MONKEY (*MACACA FASCICULARIS*)

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ABSTRACT

Primary hepatocyte cultures from cynomolgus monkey accumulate lipoprotein(a) (Lp(a)) when cultured in lipoprotein-deficient medium. Lp(a) in the culture medium has a similar buoyant density as Lp(a) present in human or simian serum (d = 1.075 g/ml). Next to Lp(a), lipoprotein-free apo(a) was secreted into the culture medium. No Lp(a) could be detected intracellularly, whereas substantial amounts of apo(a) were observed in the hepatocytes. This suggests that cynomolgus hepatocytes synthesize and secrete apo(a) and not the complete lipoprotein particle Lp(a) and that apo(a) may associate with LDL extracellularly to form lipoprotein(a). The accumulation of total apo(a), both present in lipoprotein(a) and as free apo(a), in the culture medium of cynomolgus hepatocytes did not significantly change during a 7-day culture period. Similarly, the secretion of apo B100, which is predominantly present in a lipoprotein particle with a buoyant density of LDL (d = 1.02-1.06 g/ml) remained constant throughout the culture period. However, an enhanced accumulation of Lp(a) was observed in the medium after the third day of culture.

Incubation of cynomolgus hepatocytes in the presence of rabbit β-VLDL or LDL caused opposing effects on the production of Lp(a) and total apo(a). The production of total apo(a) increased upon incubation with β-VLDL (+26%) and LDL (+76%), whereas less lipoprotein(a) was found in medium containing rabbit β-VLDL (-49%) or rabbit LDL (-38%). The latter finding may be explained by association of apo(a) with the excess of rabbit lipoproteins, leading to formation of lipoprotein complexes which are not detected by our assays as such, but in which apo(a) can still be measured. The secretion of apo B100 was enhanced 2.2-fold and 2.6-fold by β-VLDL and LDL, respectively, whereas the apo A-I production did not change. We conclude that rabbit β-VLDL and LDL stimulate the total apo(a) and apo B100 secretion in primary cultures of cynomolgus hepatocytes.

INTRODUCTION

A high plasma level of lipoprotein(a) (Lp(a)) is positively associated with the development of coronary heart disease and cerebrovascular disease in men and women, especially when plasma levels exceed 0.20-0.30 g/l [1-4]. Lp(a) levels are under strong genetic control [5-7]. However, this does not mean that the production or catabolization of Lp(a) can not be affected. Reduced Lp(a) levels were observed in humans after treatment with hormones [8-10] or hypolipidemic drugs [11-14].

Whether the *in vivo* observed changes are the result of a decreased synthesis or of an enhanced catabolization remains to be elucidated. However, from *in vivo* turnover studies

of Lp(a) in man, it was concluded that Lp(a) levels are mainly determined by synthesis of the lipoprotein particle [15,16]. Since the liver is the major and maybe the sole site for the synthesis of Lp(a) or apo(a) [17,18], the regulation of Lp(a) or apo(a) synthesis may be investigated using hepatocyte cultures as an *in vitro* model system. Because there are no reports that the human hepatoma cell line Hep G2 secretes Lp(a) and since the availability of human livers is restricted, we looked for hepatocytes from other species that may produce Lp(a). The number of species in which Lp(a) is present in plasma or serum is restricted. Until now Lp(a) is detected only in old world monkeys [19-21], the new world monkey marmoset [22], the hedgehog [23], and guinea pigs [24]. Cynomolgus monkey (*Macaca fascicularis*) is an old-world monkey in which Lp(a) is found in the serum [20]. The animal has been shown to be a suitable model to study the development of atherosclerosis [25] and the lipid and lipoprotein metabolism [26,27], including regulation of plasma Lp(a) levels [28].

In this paper we report that primary hepatocyte cultures from cynomolgus monkey accumulate apo(a) and Lp(a) in the culture medium and that the secretion of apo(a) is stimulated by the addition of the cholesterol-rich lipoproteins β-VLDL and LDL to the culture medium.

METHODS

Materials

Materials used for the isolation and culturing of simian hepatocytes were obtained from sources described previously [29,30], except for fetal bovine serum which was purchased from Boehringer Mannheim (Mannheim, Germany).

Preparation of lipoprotein depleted serum and lipoproteins

Lipoprotein depleted serum (LPDS) was prepared from heat inactivated (30 min at 65°C) fetal bovine serum (FBS) by density gradient ultracentrifugation as described previously [31]. β-VLDL and LDL were isolated from serum of a rabbit fed a diet containing 1% cholesterol (Hope-Farms, Woerden, The Netherlands) essentially as described [31]. The concentration of β-VLDL and LDL used in the incubations was 40 and 200 μg protein/ml respectively.

Simian hepatocyte isolation and culture

Simian hepatocytes were isolated from livers of both male and female cynomolgus monkeys (*Macaca fascicularis*, 1.5-3 years old), which were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The animals were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitis vaccine at this institute. The isolation procedure was essentially as described for human hepatocytes [29,30], with a few modifications. The simian liver was perfused with 1.5 litre of a HEPES buffer pH 7.4 containing 10

mM HEPES, 132 mM NaCl, 6.7 mM KCl and 20 mM glucose at a rate of 100 ml per min. After the preperfusion the liver was perfused successively with 500 ml of a HEPES buffer, pH 7.6, containing 100 mM HEPES, 67 mM NaCl, 6.7 mM KCl, and 5 mM $CaCl_2$ without circulation and with 200 ml of this buffer containing 0.05% collagenase with recirculation for 30 min. Liver tissue was dissociated in Hank's buffer containing 2% bovine serum albumin, cells were filtered through a 250 μm filter, centrifuged (60 g, 5 min), and washed three times in cold culture medium to remove damaged and nonparenchymal cells. Total cell yields varied from 0.74 to 2.3 x 10^9 viable cells. Viability, based on the ability of hepatocytes to exclude tryptan blue dye (0.11%) was 66-96%. The cells were seeded on culture dishes at a density of 2 x 10^5 viable cells per cm^2 and were maintained in Williams E medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 20 mU insulin per ml (135 nM), 50 nM dexamethasone, 100 IU penicillin per ml, 100 μg streptomycin per ml, and 100 μg kanamycin per ml, at 37°C in a 5% CO_2/95% air atmosphere. After 14-16 hours the non-adherent cells were washed from the plates, using the same culture medium as above. After 24 hours from seeding the incubations were started on standard medium (same medium as described above, except that the medium was supplemented with 10% LPDS instead of FBS and with a lower insulin concentration, 10 nM instead of 135 nM). The medium was changed every 48 h thereafter. At the end of an incubation period, medium was collected and centrifuged for 20 sec (8000 x g) in a Heraeus centrifuge (Biofuge A) to remove detached cells and debris. The supernatant was frozen immediately in dry-ice and stored at -20°C, until measurement of Lp(a), apo(a), apo A-I and apo B100 concentrations. After the last incubation period cells were washed three times with cold phosphate-buffered saline (PBS, sodium/potassium phosphate buffer, 11 mM, pH 7.5, containing 150 mM NaCl). The cellular protein was determined as described by Lowry et al. [32].

Measurement of Lp(a), apo(a), apo B100 and apo A-I and analysis of apo(a), apo B100 and apo A-I containing lipoproteins secreted by cynomolgus hepatocytes

Secretion and cellular amount of Lp(a) and total apo(a) in hepatocyte cultures from cynomolgus monkey were measured with the Innotest Lp(a) (Innogenetics, Antwerp, Belgium) and the TintElize Lp(a) (Biopool AB, Umeå, Sweden) respectively. The Innotest Lp(a) kit (working range 10-500 ng/ml, and lower detection limit 5 ng/ml Lp(a)) uses monolonal antibodies to human apo(a) as catching antibodies and polyclonal antibodies to human apo B100 as detecting antibodies. The TintElize Lp(a) ELISA (working range 10-230 ng/ml, and lower detection limit 5 ng/ml Lp(a)) uses polyclonal antibodies to human apo(a) both as catching and detecting antibodies. Thus the Innotest assay only detects lipoprotein(a) particles, whereas with the TintElize procedure both lipoprotein(a) and free apo(a) glycoprotein can be measured, denoted total apo(a) in the following. In human plasma comparable Lp(a) levels were measured with these two assays (R = 0.98), indicating that no free apo(a) glycoprotein is present in human plasma. The antibodies in both kits showed strong immunological cross-reactivity with Lp(a) from cynomolgus monkeys, indicating a high level of homology between human and cynomolgus Lp(a), in accordance with references 20 and 28. No lipoprotein-free apo(a) could be detected in simian sera and in fractions hereof after density gradient ultracentrifugation. Since the TintElize assay gave consistently a 1.9-fold higher Lp(a) level in sera and isolated Lp(a) from cynomolgus monkeys as compared to the Innotest procedure (TintElize Lp(a) = 1.9 x Innotest Lp(a), R = 0.99), the values obtained by the Innotest Lp(a) assay were corrected by a factor 1.9 to allow comparison between the two procedures. The difference in the Lp(a)

concentration with the two assays may result from the catching antibodies used. As stated above the TintElize Lp(a) uses polyclonal antibodies to human apo(a) as catching antibodies, whereas Innotest Lp(a) uses monoclonal antibodies to human apo(a).

Apo A-I and apo B100 concentrations in culture medium and cells were measured in triplicate using a sandwich Elisa procedure with polyclonal antibodies to human apo A-I or human apo B100, respectively, both as catching and detecting antibodies as described [33]. The standard curves for apo A-I and apo B100 in human serum, serum from the cynomolgus monkey and culture medium of the cynomolgus hepatocytes were parallel indicating that similar epitopes on apo A-I and apo B100 of the two species are recognized.

For analysis of apo(a), apo B100 and apo A-I containing lipoproteins, culture medium was collected at the end of the second 48 h incubation period with cynomolgus monkey hepatocytes on lipoprotein depleted medium. After harvesting protease inhibitors were added to the following final concentrations: 2.5 mM EDTA, 0.5 mM phenyl-methylsulfonyl fluoride, 25 μM p-hydroxymercuriphenyl sulfonic acid, 10 U/ml heparin, 10 U/ml trasylol, 50 μg/ml leupeptin, 50 μg/ml pepstatin). 4 ml culture medium was used for a density-gradient ultracentrifugation as described by Redgrave et al. [34]. After ultracentrifugation for 16 h at 4°C the gradient was fractionated in 0.6 ml aliquots. The density of the fractions was measured (Density Measuring Cell DMA 602M, Mettler/Paar, Graz, Austria), the fractions were dialyzed (16 h) against PBS containing 0.1% (w/v) casein and 2.5 mM EDTA, and the Lp(a), apo(a), apo A-I and apo B100 concentrations in the fractions were measured by Elisa procedure.

Statistical analysis

Statistical significance of differences was calculated by Student's t-test for paired data with the level of significance as $p < 0.05$.

RESULTS

Characterization of lipoprotein(a) and apolipoprotein(a) production by primary hepatocyte cultures from cynomolgus monkey

Primary hepatocytes from cynomolgus monkey accumulate lipoprotein(a) (Lp(a)), apo(a), apo B100 and apo A-I in the culture medium. As shown in Table 1 the accumulation of apo B100 and of total apo(a), which consits of lipoprotein(a) and free apo(a), did not change significantly during the three consecutive 48 h incubation periods investigated, whereas the accumulation of Lp(a) increased significantly in the second and third 48 h periods as compared to the first 48 h incubation period. The accumulation of apo A-I decreased during the third 48 h period of culturing.

The results in Table 1 indicate that a large amount of total apo(a), present in the culture medium (ranging from 60 to 42% during the culture time), consists of free apo(a), not bound to apo B100. To ellaborate the finding that both free apo(a) and Lp(a) accumulate in the culture medium from cynomolgus hepatocytes, medium was separated by density

Table 1. Accumulation of Lp(a), apo(a), apo B100 and apo A-I in the culture medium of cynomolgus hepatocytes during culture time. After a 24 h attachment and recovery period hepatocytes were cultured for 48, 96 or 144 h on standard medium (lipoprotein depleted medium). Medium was changed every 48 h. At the end of each incubation period medium was collected and analyzed for Lp(a), total apo(a) (being Lp(a) + free apo(a)), apo B100 and apo A-I. Values were normalized for cell protein in culture dishes and are means ± S.D. and range for duplicate incubations of hepatocytes from four independent isolations. In brackets the secretion is shown, expressed as a percentage (means ± S.D.) of the secretion rates in the 24-72 h culture period. The values for free apo(a) were obtained by subtraction of values for lipoprotein(a) from total apo(a). *Indicates a significant difference between the culture period 24-72 h and the period indicated (paired t-test, $p < 0.05$).

	Culture time		
	24-72 h	72-120 h	120-168 h
total apo(a) (ng/mg cell protein/48 h)	120 ± 68 (100%) 55-200	153 ± 103 (129 ± 41%) 61-296	163 ± 125 (150 ± 55%) 87-352
free apo(a) (ng/mg cell protein/48 h)	72 ± 30 (100%) 38-10	67 ± 68 (101 ± 78%) 23-188	68 ± 63 (103 ± 95%) 13-213
lipoprotein(a) (ng/mg cell protein/48 h)	48 ± 38 (100%) 15-99	86 ± 67* (186 ± 43%) 27-167	95 ± 44* (240 ± 85%) 44-137
apo B100 (μg/mg cell protein/48 h)	1.70 ± 0.70 (100%) 1.17-2.45	1.53 ± 0.86 (88 ± 13%) 0.89-2.51	1.66 ± 0.88 (96 ± 15%) 1.14-2.68
apo A-I (μg/mg cell protein/48 h)	3.51 ± 2.00 (100%) 1.17-5.55	3.06 ± 1.32 (95 ± 19%) 1.31-4.32	1.57 ± 0.52* (56 ± 27%) 0.91-2.15

gradient ultracentrifugation (Fig. 1). Using the TintElize assay, which measures apo(a) as a constituent of Lp(a) or as a free apoprotein, apo(a) was found in a lipoprotein particle with a buoyant density of Lp(a) (1.04-1.11 g/ml) and at the bottom of the density gradient (> 1.21 g/ml). The lipoprotein particle that contained apo(a) could also be detected with an Elisa assay (Innotest) using anti-apo(a) as catching antibody and anti-apoB as detecting antibody, whereas apo(a) present in the bottom fractions could not. These results show that primary hepatocytes from cynomolgus monkey accumulate Lp(a) and free apo(a) in the culture medium. The majority of apo B100 secreted was found in a lipoprotein particle with a buoyant density of LDL (1.02-1.06 g/ml) and a small amount (± 10%) in a lipoprotein particle with a buoyant density of VLDL (< 1.011 g/ml). Apo A-I was found in the density range of 1.13-1.25 g/ml indicating that apo A-I was present in the culture medium

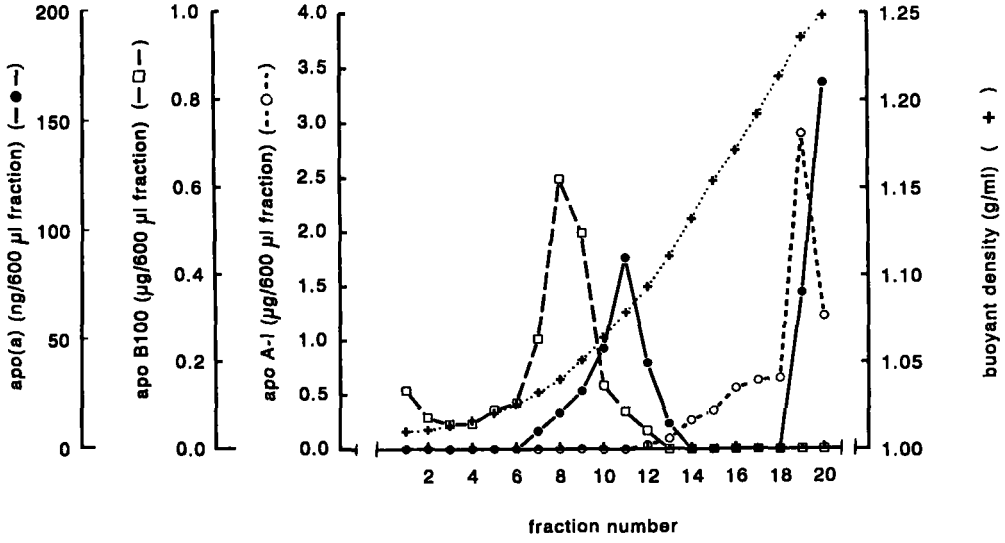

Figure 1. Density gradient pattern of apo(a), apo B100 and apo A-I containing lipoproteins in the culture medium of cynomolgus hepatocytes. Cynomolgus hepatocytes were incubated for 48 h in standard medium (lipoprotein depleted medium) after a 48 h preincubation period on the same medium. 4 ml culture medium was used for density gradient ultracentrifugation as described in the Methods section. Apo(a) (●), apo B100 (□) and apo A-I (○) concentration in gradient fractions were measured by Elisa procedure after dialysis against PBS, containing 0.1% casein and 2.5 mM EDTA.

in lipoprotein particles with the buoyant density of HDL but also in a lipid-poor or lipid-free form. The majority of apo A-I was found in the lipid-poor or lipid-free fractions. The results on apo B100 and apo A-I containing particles present in the culture medium is in accordance with what has been observed for Hep G2 cells [35-38].

In addition to the accumulation in the culture medium the cellular content of Lp(a), apo(a), apo B100 and apo A-I was investigated (Table 2). For apo(a) a relatively high cellular concentration was observed (31 ± 10% of the sum of the secreted and cellular amounts), whereas no Lp(a) could be detected in the hepatocytes. These results suggest that cynomolgus hepatocytes secrete apo(a) as a free protein and that the formation of Lp(a) occurs outside the hepatocytes. For apo A-I and apo B100 we found that 95 ± 2% and 88 ± 4%, respectively, of the total amounts of the apolipoproteins were present in the culture medium.

Table 2. Cellular and extracellular amounts of Lp(a), apo(a), apo B100 and apo A-I in cynomolgus hepatocytes. After a 24 h attachment and recovery period primary cynomolgus hepatocytes were cultured in standard medium (lipoprotein depleted medium) for two 48 h periods. At the end of the second 48 h incubation period medium and cells were collected. The cells were washed three times with PBS and were harvested in PBS containing 0.1% Triton X100. Lp(a), total apo(a) (being Lp(a) + free apo(a), apo B100 and apo A-I in cells and medium were measured as described in the Methods section. The values for free apo(a) were obtained by subtraction of values for lipoprotein(a) from total apo(a). Values are means ± S.D. for duplicate incubations of hepatocytes from five independent isolations. In brackets the amounts of apo(a), apo B100 and apo A-I secreted or present intracellular are expressed as a percentage (mean ± S.D.) of the total amount (sum of secreted and cellular amounts).

	(apo-)lipoprotein secreted (ng/mg cell protein/48 h)	(apo-)lipoprotein cellular (ng/mg cell protein)	(apo-)lipoprotein total (ng/mg cell protein/48 h)
total apo(a)	177 ± 76 (69 ± 10%)	91 ± 76 (31 ± 10%)	268 ± 150
free apo(a)	103 ± 97 (56 ± 17%)	91 ± 76 (44 ± 17%)	194 ± 167
lipoprotein(a)	74 ± 55 (> 95%)	n.d. (< 5%)	74 ± 55
apo B100	1442 ± 622 (88 ± 4%)	176 ± 39 (12 ± 4%)	1618 ± 651
apo A-I	2385 ± 718 (95 ± 2%)	118 ± 26 (5 ± 2%)	2503 ± 734

n.d.; not detectable (< 3 ng/mg cell protein)

Effect of β-VLDL and LDL on Lp(a), apo(a), apo B100 and apo A-I production by primary hepatocyte cultures from cynomolgus monkey

To study whether the production of apo(a) and Lp(a) can be regulated by the addition of cholesterol-rich lipoproteins, the effect of rabbit β-VLDL and LDL was investigated (Table 3). We did not detect Lp(a) or apo(a) in the serum or in the lipoprotein fractions isolated from rabit serum. With β-VLDL a significant increase (+26%) was observed in the production of the total amount of apo(a) (sum of secreted and cellular amounts). Incubation of cynomolgus hepatocytes with LDL resulted in an even more pronounced production of total apo(a) (+76%). In contrast to the effect on total apo(a), the production of Lp(a), decreased when cynomolgus hepatocytes were incubated with β-VLDL (-49%)

Table 3. Effect β-VLDL and LDL on the production of Lp(a), apo(a), apo B100 and apo A-I by hepatocyte cultures of cynomolgus monkey. Cynomolgus hepatocytes were incubated for 48 h in standard medium (lipoprotein depleted medium) in the presence or absence of rabbit β-VLDL (40 μg protein/ml) or of rabbit LDL (200 μg protein/ml), after a 48 h preincubation period on the same medium. The culture medium and cells were analyzed for Lp(a), total apo(a) (being lipoprotein(a) + free apo(a)), apo B100 and apo A-I. Results, normalized for cell protein in culture dishes, are expressed as a percentage of control and are means ± S.D. for duplicate incubations of hepatocytes from four independent isolations. Lp(a) and total apo(a) production (sum of secreted and cellular amounts) in standard medium was 84 ± 59 and 205 ± 74 ng/mg cell protein/48 h respectively. Apo B100 and apo A-I production was 1.11 ± 0.48 and 2.46 ± 0.51 μg/mg cell protein respectively. The values for free apo(a) were obtained by subtraction of values for lipoprotein(a) from total apo(a). *Indicates a significant difference between control and treated cells (paired t-test, $p < 0.05$).

	β-VLDL	LDL
total apo(a) (% of control)	126 ± 6*	176 ± 26*
free apo(a) (% of control)	150 ± 15*	217 ± 59*
lipoprotein(a) (% of control)	51 ± 18*	62 ± 19*
apo B100 (% of control)	217 ± 27*	262 ± 60*
apo A-I (% of control)	98 ± 12	79 ± 24

or LDL (-38%). The secretion of apo B100 was significantly stimulated by β-VLDL (+117%) and LDL (+162%). The secretion of apo A-I by cynomolgus hepatocytes was not significantly affected by β-VLDL or LDL.

DISCUSSION

In this study we have shown that primary hepatocytes from cynomolgus monkey accumulate lipoprotein(a) (Lp(a)) in the culture medium. The buoyant density of this Lp(a) particle is similar to that of Lp(a) found in human and simian serum [19,21,39], and in the culture medium of primary hepatocytes from baboons [40]. Next to Lp(a), free apo(a) was observed in the culture medium of cynomolgus hepatocytes. This free apo(a) was present in the bottom fractions after density gradient ultracentrifugation, indicating that it was present in the culture medium as a protein which was not associated with lipoproteins.

We found a substantial amount of apo(a) to be associated with the cells, indicating that this is intracellular apo(a) or apo(a) associated with the hepatocytes. Arguing against the latter suggestion, is the finding proteinase K treatment of the hepatocytes (0.5 mg/ml proteinase K, 30 min 4°C) at the end of the incubation period did not change the cellular

amount of apo(a). This indicates that apo(a) is present intracellularly. Although substantial amounts of apo(a) were found intracellularly no Lp(a) was observed in the hepatocytes. This suggests that cynomolgus hepatocytes synthesize and secrete apo(a) and that the binding of apo(a) to an apo B100-containing lipoprotein, leading to the formation of Lp(a), occurs outside the hepatocyte. This would also explain the large amounts of free apo(a) in the culture medium. Our results that both free apo(a) and Lp(a) accumulate in the culture medium, but that only apo(a) is present in the hepatocytes is in accordance with what has been reported recently for baboon hepatocytes [41]. The contention that apo(a) associates extracellularly with LDL to form Lp(a) particles is supported by the recent finding that Lp(a) can be reconstituted *in vivo* in transgenic mice expressing human apo(a) by infusion of human LDL [42].

It has been shown that a high plasma Lp(a) level of Lp(a) is particularly atherogenic at elevated levels of LDL [4]. To investigate whether the production of Lp(a) or apo(a) can be regulated by cholesterol-rich lipoproteins, cynomolgus hepatocytes were incubated in the presence of rabbit β-VLDL and LDL. We found a significant increase in the total apo(a) production upon addition of β-VLDL (+26%) and an even stronger elevation with LDL (+76%). In contrast with the increased accumulation of total apo(a), the formation of Lp(a) decreased in the presence of β-VLDL (-49%) and LDL (-38%). We suggest that this difference may be caused by association of apo(a) with the apo B-containing lipoproteins β-VLDL and LDL from rabbits added to the culture medium, which may compete with the apo B100-containing lipoproteins secreted by the cynomolgus hepatocytes in the formation of Lp(a). The thus formed complex of apo(a) with rabbit apo B can not be detected with an assay using anti-human-apo B as a detecting antibody.

We have shown here, to our knowledge for the first time, that cholesterol-rich lipoproteins regulate apo(a) production by a direct effect on hepatocytes. These results raise the possibility that supply of cholesterol to hepatocytes by cholesterol-rich lipoproteins, plays an important role in the regulation of plasma Lp(a) levels and consequently the atherosclerotic process. In this context it is interesting that dietary supplementation of baboons with cholesterol has been reported to increase Lp(a) levels [43]. On the other hand, no such effect was found in humans, in which the dietary cholesterol was increased 4-fold [44]. However, our results do not exclude the possibility that not cholesterol but another compound, present in rabbit β-VLDL and LDL, is responsible for the increased secretion of apo(a) by cynomolgus hepatocytes. The mechanism by which β-VLDL and LDL increase apo(a) production remains to be investigated.

The secretion of apo B100 increased more than 2-fold when β-VLDL (+117%) and LDL (+162%) were added to the culture medium. Similar results have been reported in previous studies in Hep G2 cells [45,46] and rabbit hepatocytes [47], indicating that cholesterol and/or cholesteryl esters are important for the secretion of apo B100. β-VLDL

and LDL did not significantly affect the apo A-I secretion. This is in accordance with the absence of an effect on the apo A-I secretion in Hep G2 cells in which the cellular cholesterol content was increased [45,46].

This report shows that primary hepatocyte cultures from cynomolgus monkey are suitable to get a better understanding of the regulatory mechanism(s) involved in the Lp(a) production. Further research will be necessary to clarify the role of hepatic synthesis of apo(a) and binding of apo(a) to apo B100-containing lipoproteins in determining Lp(a) levels in the circulation *in vivo*.

ACKNOWLEDGEMENTS

We gratefully thank Mr Arie Timmerman (RIVM, Bilthoven, The Netherlands) for his enthousiastic cooperation and excellent zootechnical assistance.

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SUMMARY AND DISCUSSION

Elevated plasma levels of LDL-cholesterol and lipoprotein(a) (Lp(a)) are associated with an increased risk for the development of coronary heart disease, whereas an increased level of HDL-cholesterol is associated with a decreased risk. One way of reducing the risk of developing atherosclerosis is by lowering the cholesterol content of the liver, which will lead to an enhanced hepatic uptake of LDL from the circulation. Both dietary and pharmacological intervention have been used successfully to lower the cholesterol content of the liver.

Another possibility that may reduce the risk for atherosclerosis is by increasing the production of HDL and/or by decreasing the production of LDL. A way to do this is by regulating the synthesis of the proteins (called apolipoproteins), which are part of these lipoproteins. The major protein on HDL is apolipoprotein A-I (apo A-I), whereas apolipoprotein B100 (apo B100) is the sole protein on LDL. In various studies the concentrations of these two apolipoproteins in the circulation were reported to be even better predictors for the risk of developing atherosclerosis than measurement of the cholesterol content of the lipoproteins.

The lipoprotein Lp(a) has been shown to be an independent risk factor for atherosclerosis. The concentration of Lp(a) in the circulation is under strong genetic control. However, several reports show that the Lp(a) level can be regulated by diets and drugs, currently applied in clinical practice. Whether the reported changes are the result of an effect on the synthesis of Lp(a) or on the clearance from the circulation of this lipoprotein remains to be elucidated.

In the studies described in this thesis the synthesis and secretion of apo A-I, apo B100 and Lp(a) was investigated in two hepatocyte culture systems, i.e. the human hepatoma cell line Hep G2 and primary hepatocyte cultures from cynomolgus monkey (*Macaca fascicularis*). Cultured hepatocytes are a relevant model system because the liver is the sole organ where apo B100 and Lp(a) is synthesized. Apo A-I is synthesized both in the liver and intestine in approximately equal amounts. In Table 1 the results of the different regulatory studies of the synthesis and secretion of apo A-I and apo B100, described in this thesis, are listed. In order to study the regulation of the hepatic production of Lp(a) our attention was focused on finding a model system of cultured hepatocytes that produce Lp(a) and that is suitable for regulatory studies.

Underneath, the different studies on the synthesis and secretion of apo A-I, apo B100 and Lp(a) in cultured hepatocytes will be discussed in more detail.

Table 1. Results of the regulatory studies of the synthesis and secretion of apo A-I and apo B100 described in this thesis.

Compound	System	Effect on apo A-I and apo B100
Butyrate	Hep G2 cells	Increased secretion of apo A-I, accompanied by a stimulation of the apo A-I mRNA level Increased secretion of apo B100, resulting from a reduced intracellular degradation
Cyclosporin A	Hep G2 cells	No effect on the secretion of apo A-I Decreased secretion of apo B100, regulated at the translational or co-translational level
Retinoids	Cynomolgus hepatocytes	Increased secretion of apo A-I, caused by an enhanced transcription of the apo A-I gene No effect on the secretion of apo B100

Using the human hepatoma cell line Hep G2 we investigated the effect of sodium butyrate on the secretion of apo A-I and apo B100. Incubation of Hep G2 cells with sodium butyrate resulted in a time- and dose-dependent increase in the secretion of apo A-I and apo B100 (chapter 2). After a 24 h preincubation, a 2.4- and 2.2-fold increase in the secretion of apo A-I and apo B100, respectively, was obtained during the next 24 h incubation period in the presence of 2 mM sodium butyrate. Butyrate specifically stimulated the secretion of apo A-I and apo B100. In addition, the stimulatory effect was specific for butyrate. Only propionate and valerate, induced a significant increase in the secretion of apo A-I and apo B100, although less-markedly than butyrate. Other straight-chain carboxylic acids, or hydroxylated, aminated or otherwise modified butyrate derivatives were inactive.

The enhanced accumulation of apo A-I and apo B100 in the culture medium could not be explained by changes in the uptake of the synthesized apolipoproteins or by alterations in the secretion of possible intracellular pools. [^{35}S]-methionine incorporation studies indicated that synthesis and/or secretion of newly synthesized apo A-I and apo B100 was enhanced.

An effect of butyrate on the acetylation of histones and subsequently an effect on the gene expression may explain the increased synthesis of apo A-I. After treatment of Hep G2 cells with 2 mM butyrate for 48 h a 2.3-fold increase was observed in the apo A-I mRNA level. The data from the structure-function studies with various straight-chain fatty acids support the contention that butyrate regulates the synthesis of apo A-I at the transcriptional

level. Propionate and valerate also induced the apo A-I secretion, but less than butyrate. Similar differences were reported for butyrate, propionate and valerate on the acetylation of histones. However, an increase in the stability of the apo A-I mRNA level in butyrate-treated Hep G2 cells can not be excluded.

In contrast to apo A-I, butyrate did not affect the apo B100 mRNA level. The increased secretion of newly synthesized apo B100 in butyrate-treated Hep G2 cells resulted from a decreased intracellular degradation (chapter 3). Pulse-chase studies showed that in control incubations only 18 ± 4% of the total amount of labelled apo B100 present intracellularly after a 10 min pulse period, was secreted during a 90 min chase period. After addition of butyrate this amount increased to 32 ± 6%.

Since the availability of lipids is important for the intracellular assembly of apo B100-containing lipoproteins the effect of butyrate on the amount of lipids present intracellularly and secreted by Hep G2 cells was studied. The decreased intracellular degradation of apo B100 was associated with an increase in the cellular content of triglycerides (+30%). The cellular content of cholesterol and cholesteryl esters were not changed after treatment with butyrate. Secretion of triglycerides (+90%) and cholesteryl esters (+78%), but not of cholesterol, was increased to the same extent as the apo B100 secretion (+102%) in butyrate-treated Hep G2 cells. Butyrate did not affect the buoyant density of apo B100-containing lipoproteins secreted by Hep G2 cells. The total mass of triglycerides present intracellularly and secreted by Hep G2 cells was the only lipid that was affected after butyrate treatment. This indicates that in butyrate-treated Hep G2 cells the enhanced intracellular concentration of triglycerides prevents degradation of apo B100.

The human hepatoma cell line Hep G2 was used to study the effect of cyclosporin A (CsA) on the apo B100 secretion. CsA is one of the most effective immunosuppressive drugs available and is widely used in organ transplantation and for treating an increasing number of auto-immune diseases. Treatment of patients with CsA leads to an increase in the LDL-cholesterol level. In the study shown in chapter 4 we investigated whether an increased hepatic synthesis of apo B100 might be responsible for the elevated plasma LDL levels.

Addition of CsA to the culture medium of Hep G2 cells resulted in a dose- and time-dependent decrease in the secretion of apo B100. Maximal inhibition (-50%) was obtained at 5 μM CsA and was already achieved within 8 h. The secretion of apo A-I, albumin and [^{35}S]-methionine labelled proteins was not affected by CsA, indicating that the effect of CsA was specific for the secretion of apo B100. The reduced accumulation of apo B100 in the culture medium could not be explained by changes in the uptake of LDL by Hep G2 cells treated with CsA. In addition, [^{35}S]-methionine incorporation studies indicated that the synthesis and/or the secretion of newly synthesized apo B100 is decreased in the presence of CsA.

The apo B100 mRNA level in Hep G2 cells is not influenced by CsA, indicating that a decreased synthesis or secretion of newly-synthesized apo B100 is not regulated at the transcriptional level. As illustrated in chapter 3 changes in the cellular lipid content are important for the intracellular assembly of apo B100-containing lipoproteins and may regulate the secretion of apo B100. With CsA no changes were observed in the intracellular concentration of triglycerides, cholesterol or cholesteryl esters in Hep G2 cells. This indicates that the inhibitory effect of CsA on the secretion of apo B100 is not caused by a decrease in the cellular levels of these lipids. Concomitantly with the inhibition of the apo B100 secretion, the secretion of triglycerides (-47%), cholesterol (-18%) and cholesteryl esters (-27%) was diminished in the presence of CsA. These differences in the effect of CsA on the secreted lipids were not reflected in changes in the buoyant density of the apo B100-containing lipoproteins that were secreted by Hep G2 cells in the presence or absence of CsA.

Pulse-chase experiments showed that the amount of intracellular apo B100 was already decreased by 50% after the 10 min pulse period, indicating that the inhibition of the apo B100 secretion by CsA takes place at the translational or co-translational level. It has been shown that CsA binds with high affinity to cyclophilin, a peptidyl-prolyl cis-trans isomerase, and inhibits this enzyme that catalyses the folding of nascent proteins transferred into the endoplasmic reticulum. We suggest that inhibition of cyclophilin by CsA may inhibit the correct folding of apo B100 during biosynthesis, leading to increased intracellular degradation. This will result in a reduced amount of apo B100 available for lipid secretion from Hep G2 cells. This study shows that the apo B100 secretion can be inhibited via a mechanism, not reported previously.

These results show that the elevated plasma LDL levels, observed in patients treated with CsA, are not caused by hepatic overproduction of apo B100-containing lipoproteins. We also found a decreased binding, internalization and degradation of LDL in Hep G2 cells incubated in the presence of CsA. Whether the latter observation and the finding that CsA specifically inhibits the alternative pathway in bile acid synthesis are related to the elevated levels of apo B100-containing lipoproteins *in vivo* remains to be elucidated.

Next to studies with the human hepatoma cell line Hep G2 we performed experiments with primary hepatocyte cultures from cynomolgus monkey. Primary hepatocyte cultures may have the potential advantage over hepatoma cell lines that no loss of cellular functions, due to dedifferentiation have occurred. One example of this may be the stimulating effect of retinoids on the apo A-I synthesis in primary hepatocyte cultures from cynomolgus monkey (chapter 5). This stimulating effect was not found in Hep G2 cells, indicating that this cell line may not have the factor(s), for instance the retinoid receptors, necessary for the induction of the apo A-I synthesis by retinoids.

In primary cynomolgus hepatocytes addition of retinol (vitamin A) or retinoic acid (vitamin A acid) resulted in a time- and dose-dependent increase in the secretion of apo A-I. Maximal stimulation (2.7-fold) was obtained with 10 μM retinoic acid during a 72 h incubation. The secretion of apo B100 and [^{35}S]-methionine labelled proteins was not affected by retinoic acid, indicating that the stimulating effect is specific for apo A-I. This was confirmed by the absence of an effect on other secreted, metabolically labelled proteins.

The metabolic labelling also showed that retinoic acid stimulates the synthesis or the secretion of newly-synthesized apo A-I. Further investigation of the mechanism of induction by retinoic acid showed that the apo A-I mRNA level was increased 3-fold in cynomolgus hepatocytes exposed for 72 h to 10 μM retinoic acid. The elevated mRNA level was associated with a 2-fold increase in the transcriptional rate of the apo A-I gene. These results show that retinoic acid stimulates the synthesis and consequently the secretion of apo A-I in cynomolgus hepatocytes by transcriptional regulation.

Among different natural retinoids, retinoic acid and its 9-cis and 13-cis isomers were equally active, whereas the maximal stimulation of the apo A-I secretion induced by retinol was lower (1.6-fold). Next to the naturally occurring retinoids, five synthetic retinoids were investigated for their effect on the apo A-I and apo B100 secretion by cynomolgus hepatocytes. The results of the structure function studies with the different natural and synthetic retinoids suggest that a terminal carboxylic acid moiety in the retinoids is of importance in the modulation of apo A-I synthesis in the hepatocytes.

Recently, it has been shown that the retinoic acid receptorα (RARα) and retinoid X receptorα (RXRα) might be involved in the activation of the retinoic acid-responsive element in the promotor of the apo A-I gene. The natural ligands for the retinoid receptors RARα and RXRα are believed to be all-trans-retinoic acid and 9-cis-retinoic acid respectively. The observed comparable effects of all-trans- and 9-cis-retinoic acid may be caused by the isomerization into each other and into 13-cis-retinoic acid (chapter 6). We show that the ligands for the retinoid receptors RARα and RXRα are both present in the culture medium when the incubation is started with either all-trans- or 9-cis-retinoic acid. This interconversion was already observed after incubation in culture medium without hepatocytes. In a 24 h incubation of all-trans-retinoic acid with cynomolgus hepatocytes all-trans, 9-cis- and 13-cis-retinoic acid were found in cells and medium. However, the majority of all-trans-retinoic acid was metabolized. Less than 2% of the added all-trans-retinoic acid (10 μM, 15 nmol per mg cell protein) was found in cells and medium after the 24 h incubation period. When the amounts of 9-cis- and 13-cis-retinoic acid formed by interconversion, in cells and medium, are also taken into account still no more than 5% of the added all-trans-retinoic acid was recovered.

The biochemical mechanism for the delayed effect of the retinoids on the apo A-I

secretion was investigated by studying the time course of the induction of the apo A-I gene transcription and the apo A-I mRNA level (chapter 6). Induction of the transcription of the apo A-I gene was already observed after a 4 h incubation with 10 μM retinoic acid and/or 9-cis retinoic acid. Despite the fast effect on the transcription of the apo A-I gene in cynomolgus hepatocytes treated with retinoids, only minor changes were observed in the apo A-I mRNA level after a 24 h incubation period. An enhanced apo A-I mRNA level was observed after a second 24 h incubation with retinoic acid (+136%) or 9-cis-retinoic acid (+126%). A further increase was found after the third 24 h incubation period with all-trans-retinoic acid (+347%) or 9-cis retinoic acid (+379%). We suggest that the increase of the apo A-I mRNA level, caused by an enhanced transcription of the apo A-I gene, is observed only after prolonged incubation with retinoids because of a high stability of the apo A-I mRNA.

Next to apo A-I and apo B100, primary hepatocyte cultures from cynomolgus monkey accumulate Lp(a) in the culture medium (chapter 7). This Lp(a) had a similar buoyant density as Lp(a) present in human or simian serum. In addition to Lp(a), free apo(a) was found in the culture medium of cynomolgus hepatocytes. No Lp(a) could detected in the hepatocytes, whereas substantial amounts of apo(a) were found intracellularly. These results suggest that apo(a) may be secreted by cynomolgus hepatocytes as a free protein and that apo(a) binds to an apo B100-containing lipoprotein outside the hepatocyte.

To investigate whether the accumulation of Lp(a) can be regulated by cholesterol-rich lipoproteins, cynomolgus hepatocytes were incubated in the presence or absence of rabbit β-VLDL and LDL. Addition of β-VLDL and LDL to the culture medium resulted in a decrease of the accumulation of Lp(a) in the medium with 49% and 38% respectively. These findings could not be explained by an inhibition of the apo(a) production. In contrast to Lp(a) the total amount of apo(a) (free apo(a) and apo(a) present in Lp(a)) increased when cynomolgus hepatocytes were incubated in the presence of β-VLDL (+39%) and LDL (+103%). A possible explanation for the opposing effects of β-VLDL and LDL on the accumulation of Lp(a) and apo(a) in the culture medium may be that apo(a) binds to rabbit β-VLDL and LDL added to the culture medium, which may compete with the apo B100-containing lipoproteins secreted by the cynomolgus hepatocytes. Apo(a) bound to apo B-containing lipoproteins from rabbits will not be detected with an assay using anti-human-apo B as a detecting antibody. This suggests that measurement of total apo(a) in regulatory studies may more accurately reflect a possible effect on the Lp(a) production *in vivo*. Whether the supply of cholesterol (or cholesteryl esters) is responsible for the stimulation of the apo(a) synthesis, remains to be elucidated.

SAMENVATTING EN DISCUSSIE

Atherosclerose van de kransslagaderen in het hart veroorzaakt in Nederland per jaar de dood van 51.500 mensen (rapport vakgroep Epidemiologie en Biostatistiek, Erasmus Universiteit Rotterdam, in opdracht van de Nederlands Hartstichting, 1988). Een veelvoud daarvan wordt, door dit vermoedelijk grotendeels te voorkomen proces, chronisch ziek. Volgens cijfers van het CBS (Statistisch Jaarboek 1990) zijn bij mannen en vrouwen respectievelijk 1,5 en 1,3 miljoen ziekenhuisdagen aan ziekten van het hart- en vaatstelsel toe te schrijven. Uiteindelijk wordt 41% van de totale sterfte in Nederland, zowel bij mannen als (enkele jaren later) bij vrouwen door hart- en vaatziekten veroorzaakt. Het is dan ook niet verbazingwekkend dat er veel aandacht wordt besteed aan het verminderen van het risico op hart- en vaatziekten, waaronder atherosclerose (vaatvernauwing). Belangrijke atherosclerotische risicofactoren zijn ondermeer verhoogde concentraties van cholesterol en triglyceriden in het bloed.

Cholesterol is een essentieel bestanddeel van de membranen van alle dierlijke en menselijke cellen. Daarnaast wordt cholesterol in het lichaam gebruikt voor de aanmaak van hormonen en galzuren. Triglyceriden worden in het spierweefsel gebruikt als energiebron. Het eventuele overschot aan triglyceriden wordt opgeslagen in het vetweefsel. Verhoogde concentraties van cholesterol en triglyceriden in het bloed doen echter de kans op het krijgen van hart- en vaatziekten toenemen.

Cholesterol en triglyceriden zijn onoplosbaar in waterig milieu zoals bloed. Ze worden daarom vervoerd in de bloedbaan in de vorm van lipide-eiwit complexen, die lipoproteïnen worden genoemd. Een lipoproteïne deeltje bestaat uit een kern van apolaire lipiden (triglyceriden en cholesterolesters), omgeven door een schil bestaand uit eiwitten en meer polaire lipiden (cholesterol en fosfolipiden). De eiwitten die deel uitmaken van de lipoproteïnen worden apolipoproteïnen genoemd en zijn o.a. van belang voor de synthese en klaring van lipoproteïnen, of dienen als cofactor voor enzymen die betrokken zijn bij het lipide-metabolisme in het bloed.

De lipoproteïnen kunnen onderverdeeld worden in: chylomicronen, zeer lage dichtheids-lipoproteïne (VLDL; very low density lipoprotein), lage dichtheids-lipoproteïne (LDL; low density lipoprotein), hoge dichtheids-lipoproteïne (HDL; high density lipoprotein) en lipoproteïne(a) (Lp(a)). Zoals de naam van een aantal van de lipoproteïnen al aangeeft verschillen ze onderling in dichtheid, welke verband houdt met een verschil in grootte en samenstelling.

Chylomicronen vervoeren lipiden die via de voeding worden opgenomen, van de darm naar de lever. Deze lipiden tesamen met nieuw gesynthetiseerde lipiden worden in de lever verpakt tot het lipoproteïne VLDL. Nadat VLDL in het bloed is uitgescheiden, wordt een deel van de triglyceriden verwijderd door het enzym lipoproteïn lipase. De gevormde VLDL remnants (ook wel IDL (intermediate density lipoprotein) genoemd), kunnen via het enzym lever lipase worden omgezet tot LDL. Dit LDL kan langzaam door de lever worden verwijderd maar ook door andere weefsels. Verhoogde LDL-concentraties in de circulatie leiden tot een grotere kans dat LDL onder de endotheelcellen (de "bekleding" van bloedvaten) terecht komt in zogenaamde subendotheliale ruimte of intima. Een extra risico op atherosclerose vormt de oxidatieve modificatie van LDL, een proces dat kan optreden onder invloed van cellen in de vaatwand. Dit gemodificeerd LDL kan worden opgenomen door macrofagen wat leidt tot de vorming van zgn. schuimcellen. Met name deze schuimcellen zijn primair betrokken bij de vorming van atherosclerotische plaques (ophopingen van cholesterol) in de bloedvatwand, die leiden tot vernauwing en mogelijke afsluiting van het vat.

HDL is in staat om cholesterol uit extra-hepatische weeefsels te ontrekken. Vervolgens wort dit cholesterol wordt omgezet tot cholesterolesters, die daarna kunnen worden uitgewisseld tegen triglyceriden van VLDL, IDL en LDL. Cholesterolesters die zo in VLDL, IDL en LDL komen, kunnen via deze lipoproteïnen weer door de lever worden opgenomen. Dit proces staat bekend als het zgn. omgekeerde cholesterol transport. Daarnaast kan HDL de cholesterolesters ook direct aan de lever kan afgeven. HDL bindt hierbij aan de lever, maar wordt niet opgenomen en geeft zijn cholesterolesters af om vervolgens weer van de lever te vertrekken. Dit HDL-gemedieerde transport van cholesterol van de bloedvaten naar de lever verklaart de anti-atherosclerotische werking van HDL.

Het lipoproteïne deeltje lipoproteïne(a) (Lp(a)) is samengesteld uit een LDL deeltje, waarbij als extra eiwit het apolipoproteïne(a) (apo(a)) via een disulfide brug aan apo B100 van LDL gekoppeld zit. De functie van Lp(a) is onduidelijk. Het eiwit apo(a) vertoont sterke gelijkenis met plasminogeen, een eiwit dat betrokken is bij het oplossen van bloedstolsels (fibrinolyse). De Lp(a) concentratie in het bloed varieert sterk van persoon tot persoon. Het risico voor atherosclerose neeemt bij verhoogde concentraties Lp(a) (boven 0.2-0.3 g/l) sterk toe. Wanneer de concentratie van Lp(a) in de circulatie en atherosclerotische plaques wordt vergeleken met die van de andere lipoproteïnen, blijkt dat er relatief veel Lp(a) ophoopt in de plaques.

De concentraties van de apolipoproteïnen in het bloed zijn ook onderzocht t.a.v. het risico op atherosclerose. Hierbij is vooral van belang het apolipoproteïne A-I (apo A-I) en apolipoproteïne B100 (apo B100), de twee meest in het bloed voorkomende apolipoproteïnen. Apo A-I is het belangrijkste eiwit op HDL en apo B100 het enige eiwit op LDL.

Uit verschillende studies blijken de concentraties van deze twee apolipoproteïnen een betere voorspellende waarde te hebben voor het risico op coronaire hartziekte dan de cholesterol-concentratie van de lipoproteïnen, waar ze deel van uitmaken. Beïnvloeding van de synthese van apo A-I en apo B100 zou in principe moeten leiden tot een verandering in de concentratie van HDL en LDL, waardoor het risico op atherosclerose zou kunnen worden beïnvloed.

Deze relatie bevat de motivatie voor de experimenten die in dit proefschrift beschreven staan. De studies zijn uitgevoerd met gekweekte hepatocyten (levercellen) en dan in het bijzonder met de humane hepatoma cellijn Hep G2 en primaire hepatocyten van de Java aap (*Macaca fascicularis*). Hepatocyten zijn als model systeem gebruikt, omdat de lever de enige plaats in het lichaam is waar apo B100 en Lp(a) worden aangemaakt, terwijl apo A-I zowel in de lever als de darm worden gesynthetiseerd (in ongeveer gelijke hoeveelheden).

Met de humane hepatoma cellijn Hep G2 hebben we het effect onderzocht van butyraat (boterzuur) op de apo A-I en apo B100 secretie (hoofdstuk 2). Incubatie van Hep G2 cellen met butyraat leidde tot een tijds- en dosis-afhankelijke toename van de apo A-I en apo B100 secretie. Na een preïncubatie van 24 uur met 2 mM butyraat, werd in de volgende 24 uurs incubatie met dezelfde concentratie butyraat een 2.4- en 2.2-voudige toename gevonden van de secretie van, respectievelijk, apo A-I en apo B100. De stimulatie van de apo A-I en apo B100 secretie door butyraat was specifiek voor deze twee eiwitten. Daarnaast was het effect specifiek voor butyraat. Propionaat en valeraat gaven eveneens een verhoging van de apo A-I en apo B100 secretie, echter minder dan butyraat. Andere onvertakte carbonzuren, of gehydroxyleerde, geamineerde of anderszins gemodificeerde butyraat derivaten waren niet actief.

De toegenomen hoeveelheid apo A-I en apo B100 in het cultuurmedium kon niet verklaard worden door veranderingen in de opname van de uitgescheiden apolipoproteïnen. De hoeveelheid apo A-I en apo B100 aanwezig binnen de Hep G2 cellen veranderde niet, wat aangeeft dat de secretie van nieuw gesynthetiseerd apo A-I en apo B100 toeneemt. Dit wordt bevestigd door de paralelle toename in nieuw gesynthetiseerd apo A-I en apo B100 bij de metabole labeling met [^{35}S]-methionine.

Nadat de Hep G2 cellen gedurende 48 uur aan 2 mM butyraat waren blootgesteld, werd een 2.3-voudige toename van het apo A-I mRNA niveau gevonden. Dit wijst op een effect van butyraat op de apo A-I genexpressie, waarbij een veranderde acetylering van de histonen een rol kan spelen. Dat de acetylering van histonen hierbij betrokken is, wordt bevestigd door de bevinding dat naast butyraat alleen propionaat en valeraat een stimulatie van de apo A-I secretie induceren. Uit de literatuur blijkt dat butyraat tot een sterkere acetylering van de histonen leidt dan propionaat en valeraat. Het kan echter niet worden

uitgesloten dat de toename in het apo A-I mRNA niveau door butyraat het gevolg is van een verhoogde stabiliteit van het apo A-I mRNA.

In tegenstelling tot het effect op apo A-I had de behandeling van Hep G2 cellen met butyraat geen effect op het apo B100 mRNA niveau. De toename in de secretie van apo B100 bleek het gevolg te zijn van een remming van de intracellulaire degradatie van apo B100 (hoofdstuk 3). In het controle medium bleek slechts 18 ± 4% van het nieuw gesynthetiseerde apo B100 door de Hep G2 cellen te worden uitgescheiden. Wanneer de Hep G2 cellen werden geïncubeerd in aanwezigheid van butyraat, steeg dit percentage tot 32 ± 6%.

Het is bekend dat apo B100 niet door hepatocyten wordt gesecreteerd, wanneer het niet goed kan worden verpakt in een lipoproteïne deeltje. De intracellulaire hoeveelheid lipidemateriaal dat beschikbaar is voor de assemblage van een apo B100-bevattend lipoproteïne is dan ook zeer belangrijk voor de gesecreteerde hoeveelheid apo B100. Bij Hep G2 cellen die zijn geïncubeerd in aanwezigheid van 2 mM butyraat werd een toename gevonden in de hoeveelheid triglyceriden (+30%) in de cel. De hoeveelheid cholesterol en cholesterolesters veranderde niet. De toename in de secretie van apo B100 door butyraat ging gepaard met een toename in de secretie van triglyceriden (+90%) en cholesterolesters (+78%). De secretie van cholesterol veranderde niet. Deze veranderingen hadden geen gevolg voor de dichtheid van het apo B100-bevattende lipoproteïne deeltje dat werd uitgescheiden. Wanneer de hoeveelheden triglyceriden, cholesterolesters en cholesterol die in de cel aanwezig zijn en door Hep G2 cellen worden uitgescheiden worden samengevoegd, blijkt er alleen een toename voor de triglyceriden gevonden te worden. Dit wijst er op dat behandeling van Hep G2 cellen met butyraat leidt tot een verhoogde beschikbaarheid van triglyceriden voor de assemblage van apo B100-bevattende lipoproteïnen en dat hierdoor de intracellulaire afbraak van apo B100 wordt tegen gegaan, zodat er meer apo B100 kan worden gesecreteerd.

Met de humane hepatoma cellijn Hep G2 werd het effect van cyclosporine A (CsA) op de apo B100 secretie bestudeerd. CsA is een immunosuppressivum dat veelvuldig gebruikt wordt bij patienten na een orgaantransplantatie, maar dat ook steeds meer bij patiënten met auto-immuun ziekten. Behandeling met CsA leidt echter tot een verhoging van de LDL-cholesterol concentratie in het bloed. De studie beschreven in hoofdstuk 4 gaat in op de mogelijkheid dat de verhoogde concentratie van LDL in het bloed het gevolg is van een toegenomen synthese of secretie van apo B100 door de lever.

Incubatie van Hep G2 cellen met CsA gaf een dosis- en tijdsafhankelijke afname van de apo B100 secretie te zien. Maximale remming (-50%) werd gevonden met 5 μM CsA en werd al bereikt binnen 8 uur. Dit effect van CsA was specifiek voor apo B100. De secretie van apo A-I, albumine en [^{35}S]-methionine gelabelde eiwitten veranderde niet. De

afname in de ophoping van apo B100 in het cultuurmedium is in tegenspraak met de verminderd opname van LDL door Hep G2 cellen o.i.v. CsA. Dit houdt in dat de synthese of de secretie van nieuw gesynthetiseerd apo B100 geremd wordt. Dit is in overeenstemming met de afname van de secretie van gelabeld apo B100, zoals gevonden wordt bij metabole labeling met [^{35}S]-methionine.

Het apo B100 mRNA niveau van de Hep G2 cellen wordt echter niet beïnvloed door CsA. Dit betekent dat de afname in de secretie niet op transcriptie niveau gereguleerd wordt. Zoals hiervoor al vermeld, is de hoeveelheid lipidemateriaal dat in de cel beschikbaar is voor de assemblage van een apo B100-bevattend lipoproteïne belangrijk voor de secretie van apo B100. Bij de incubaties met CsA traden er echter geen veranderingen op in de intracellulaire concentraties van triglyceriden, cholesterolesters en cholesterol. Naast de afname in de apo B100 secretie werd bij Hep G2 cellen tevens een daling in de secretie van triglyceriden (-47%), cholesterol esters (-27%) en cholesterol (-18%) gevonden. De verschillen in de afname van de secretie van deze lipiden veroorzaakten geen verandering in de dichtheid van de in het cultuur medium aanwezige apo B100-bevattende lipoproteïnen. De totale hoeveelheid van de in de Hep G2 cellen aanwezige en de door de cellen gesecreteerde lipiden veranderde niet bij behandeling met CsA, zodat de afname in de apo B100 secretie niet het gevolg lijkt van een verminderde beschikbaarheid van lipiden voor de assemblage van apo B100 bevattende lipoproteïnen.

Uit "pulse-chase" studies bleek dat de intracellulaire hoeveelheid nieuw gesynthetiseerd apo B100 al met 50% is afgenomen na de 10 min "pulse" periode en dat er nadien relatief evenveel apo B100 intracellulair werd afgebroken. Dit houdt in dat de synthese van apo B100 of een proces direct gekoppeld aan de synthese van apo B100 door CsA geremd wordt. Een mogelijke verklaring voor dit laatste is dat CsA de vouwing van apo B100 verstoort. Er is nl. beschreven dat CsA met hoge affiniteit bindt aan cyclophiline, een peptidyl-prolyl cis-trans isomerase en zo de activiteit van dit enzym remt. Cyclophiline katalyseert de vouwing van met name grote eiwitten, voordat deze over het membraan van het endoplasmatisch reticulum getransporteerd worden. Een incorrecte vouwing van apo B100 tijdens de translatie zou zo tot de afbraak van apo B100 kunnen leiden en uiteindelijk tot een verminderde secretie van apo B100, waardoor ook een daling in de secretie van de lipiden zal optreden. Deze studie laat daarmee zien dat de apo B100 secretie geremd kan worden via een nog niet eerder beschreven mechanisme.

Deze resultaten geven aan dat de verhoogde LDL-cholesterol concentraties bij patienten, die met CsA behandeld worden, niet het gevolg lijkt te zijn van een toename van de secretie van apo B100-bevattende lipoproteïnen door de lever. De toename in LDL-cholesterol wordt mogelijk veroorzaakt door een remming van de binding en opname van LDL.

Naast de studies met de humane hepatoma cellijn Hep G2 hebben we experimenten uitgevoerd met primaire hepatocyten van de Java aap. Hepatoma cellijnen kunnen het nadeel hebben dat door dedifferentiatie specifieke levercelfuncties verloren. Een mogelijk voorbeeld hiervan is de stimulatie van de apo A-I synthese door retinoïden bij primaire hepatocyten van de Java aap (hoofdstuk 5). Deze stimulatie door retinoïden werd niet gevonden bij Hep G2 cellen. Mogelijk mist de Hep G2 cel factoren, b.v. één of meerdere van de retinoïd receptoren, die noodzakelijk zijn voor de inductie van de apo A-I synthese.

Bij de primaire hepatocyten van de Java aap wordt met retinol (vitamine A) en retinoïnezuur (vitamine A-zuur) een tijds- en dosis-afhankelijke toename van de apo A-I secretie gevonden. Maximale stimulatie (met een factor 2.7) werd gevonden bij apehepatocyten die gedurende 72 uur met 10 μM retinoïnezuur waren geïncubeerd. De secretie van apo B100 en van de [^{35}S]-methionine gelabelde eiwitten veranderde niet. Dit wijst er op dat het effect van retinoïnezuur specifiek is voor apo A-I.

De metabole labeling laat verder zien dat retinoïnezuur de synthese of de secretie van nieuw gesynthetiseerd apo A-I stimuleert. Verder onderzoek toonde aan dat retinoïnezuur het apo A-I mRNA niveau in de apehepatocyten verhoogt. Na 72 uur incuberen met 10 μM retinoïnezuur werd een drievoudige toename in het apo A-I mRNA niveau gevonden. De toename in het apo A-I mRNA niveau was gekoppeld aan een tweevoudige stimulatie van de transcriptie van het apo A-I gen. Deze resultaten geven aan dat de stimulatie van de apo A-I synthese door retinoïden bij primaire hepatocyten van de Java aap op transcriptie niveau gereguleerd wordt.

De in de natuur voorkomende retinoïden; all-trans-retinoïnezuur en de 9-cis en 13-cis isomeren van retinoïnezuur hadden een vergelijkbaar stimulerend effect op de apo A-I secretie. De stimulatie van de apo A-I secretie na 72 uur incuberen met 10 μM retinol was echter lager (1.6 x). Deze gegevens zouden kunnen wijzen op een mogelijke betrokkenheid van metabolieten van vitamine A bij de regulatie van het apo A-I gehalte *in vivo*. Naast de natuurlijke retinoïden zijn een vijftal synthetische retinoïden onderzocht op hun mogelijke effect op de apo A-I secretie. De resultaten met de verschillende natuurlijke en synthetische retinoïden suggereren dat een eindstandige carbonzuur-groep belangrijk is voor de stimulatie van de apo A-I synthese door de apehepatocyten.

Recent werd gerapporteerd dat de retinoïd receptoren RARα (Retinoic Acid Receptor-α) en RXRα (Retinoid X Receptor-α) betrokken zijn bij de activatie van de promotor van het apo A-I gen. De natuurlijke liganden voor RARα en RXRα zijn waarschijnlijk, respectievelijk, all-trans-retinoïnezuur en 9-cis-retinoïnezuur. Dat all-trans-retinoïnezuur en 9-cis-retinoïnezuur toch een zelfde stimulatie geven van de apo A-I secretie is waarschijnlijk het gevolg van de isomerisatie van deze retinoïden. All-trans-retinoïnezuur werd voor een deel spontaan omgezet (zonder dat hepatocyten aanwezig waren) in 9-cis- en 13-cis-retinoïnezuur (hoofdstuk 6). Vergelijkbaar hiermee werd 9-cis-retinoïnezuur gedeeltelijk

omgezet in all-trans- en 13-cis-retinoïnezuur. Hierdoor zijn bij incubatie met all-trans-retinoïnezuur of 9-cis-retinoïnezuur beide retinoïden naast elkaar in cultuurmedium aanwezig. Dit houdt tevens in dat de liganden voor de retinoïd receptoren RARα en RXRα beide aanwezig zijn. Wanneer apehepatocyten werden geïncubeerd met all-trans-retinoïnezuur werd eveneens zowel all-trans als 9-cis- en 13-cis-retinoïnezuur in cellen en medium gevonden. Echter het merendeel van het all-trans-retinoïnezuur werd door de apehepatocyten gemetaboliseerd tot niet nader geïdentificeerde produkten. Van de 10 μM all-trans-retinoïnezuur die bij het begin van de 24 uurs incubatie in het medium aanwezig is, was na afloop minder dan 2% terug te vinden in cellen plus medium. Wanneer 9-cis- en 13-cis-retinoïnezuur hierbij worden opgeteld werd nog altijd minder dan 5% van het aan het medium toegevoegde all-trans-retinoïnezuur terug gevonden. Zoals hiervoor al vermeld is, is een 24 uurs preïncubatie nodig voordat een effect van de retinoïden op de apo A-I secretie wordt waargenomen. Om deze tijdsafhankelijke stimulatie van de apo A-I secretie te kunnen verklaren, werd onderzocht hoe snel een effect van all-trans- of 9-cis-retinoïnezuur waarneembaar was op de transcriptie van het apo A-I gen en het apo A-I mRNA niveau. Al na een incubatie van 4 uur met 10 μM all-trans-retinoïnezuur en/of 10 μM 9-cis-retinoïnezuur werd een stimulatie van de transcriptie van het apo A-I gen gevonden. Ondanks dit snelle effect op de transcriptie werd geen toename van het apo A-I mRNA niveau gevonden na een 24 uurs incubatie met all-trans- en 9-cis-retinoïnezuur. Een stimulatie van het apo A-I mRNA niveau werd pas gemeten nadat de apehepatocyten twee maal 24 uur waren geïncubeerd met 10 μM all-trans-retinoïnezuur (+136%) of 10 μM 9-cis-retinoïnezuur (+126%). Een verdere stimulatie van het apo A-I mRNA niveau werd gevonden na de derde 24 uurs incubatie met all-trans-retinoïnezuur (+347%) of 9-cis-retinoïnezuur (+379%). Waarschijnlijk wordt een toename in het apo A-I mRNA niveau, als gevolg van de stimulatie van de expressie van het apo A-I gen, pas na langere incubaties waargenomen door een hoge stabiliteit van het apo A-I mRNA.

Naast apo A-I en apo B100 bleek dat primaire hepatocyten van de Java aap ook lipoprotein(a) (Lp(a)) ophopen in het cultuurmedium. De dichtheid van dit Lp(a) was vergelijkbaar met Lp(a) aanwezig van het serum van mens of aap. Naast Lp(a) was ook vrij apo(a) in het cultuurmedium van de apehepatocyten aanwezig. Apo(a) werd eveneens in de hepatocyten zelf gevonden. Dit in tegenstelling tot Lp(a) dat niet in de cellen kon worden aangetoond. Deze resultaten suggereren dat de apehepatocyten apo(a) synthetiseren en secreteren en dat mogelijk pas buiten de hepatocyt de associatie optreedt tussen apo(a) en een apo B100-bevattend lipoproteïne.

De ophoping van Lp(a) en apo(a) in het cultuur medium van apehepatocyten bleek beïnvloedbaar door cholesterol-rijke lipoproteïnen. β-VLDL en LDL (geïsoleerd uit het serum van een konijn) verminderde de ophoping van Lp(a) met respectievelijk 49% en

38%. Dit effect kon niet worden verklaard door een afname van de produktie van apo(a). De produktie van de totale hoeveelheid apo(a) (vrij apo(a) en apo(a) in Lp(a)) nam nl. juist toe o.i.v. β-VLDL (+26%) en LDL (+76%). Een mogelijke verklaring voor deze discrepantie tussen de effecten op Lp(a) en apo(a) zou kunnen zijn dat het gesecreteerde ape-apo(a) bindt aan β-VLDL of LDL van het konijn, dat aan het medium is toegevoegd en daardoor mogelijk competeert met het gesecreteerde ape-LDL. Apo(a) gekoppeld aan een apo B-bevattend lipoproteïne van konijnen kan niet gedetecteerd worden met antilichamen tegen humaan apo B. Wanneer dit inderdaad het geval is, is meting van het totale apo(a) zinvoller in regulatie studies. Onze gegevens suggereren dat aanbod van cholesterol (of cholesterolesters) de apo(a) synthese stimuleert. Hoewel niet kan worden uitgesloten dat een andere factor, aanwezig in β-VLDL en LDL van het konijn, de synthese of secretie van apo(a) door apehepatocyten stimuleert.

ABBREVIATIONS

ACAT	Acyl-coenzyme A: Cholesterol-acyltransferase
apo	apolipoprotein
CsA	Cyclosporin A
CETP	Cholesteryl Ester Transfer Protein
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
HDL	High Density Lipoprotein
HL	Hepatic Lipase
HMGCoA	3-Hydroxy-3-Methylglutaryl-Coenzyme A
IDL	Intermediate Density Lipoprotein
LCAT	Lecithin Cholesterol Acyltransferase
LDL	Low Density Lipoiprotein
Lp(a)	Lipoprotein(a)
LPDS	Lipoprotein Depleted Serum
LPL	Lipoprotein Lipase
PBS	Phosphate Buffered Saline
RAR	Retinoic Acid Receptor
RXR	Retinoid X Receptor
SD	Standard Deviation
VLDL	Very Low Density Lipoprotein

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