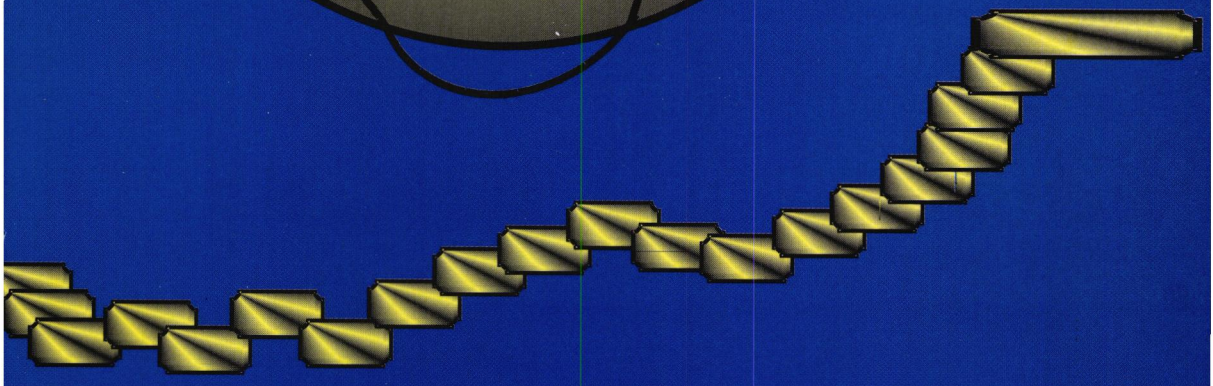
Regulation of apolipoprotein(a) and apolipoprotein A-I synthesis



Diana Neele

REGULATION OF APOLIPOPROTEIN(a) AND APOLIPOPROTEIN A-I SYNTHESIS

Proefschrift
ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. W.A. Wagenaar,
hoogleraar in de faculteit der Sociale Wetenschappen,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 22 oktober 1998
te klokke15.15 uur

door

Adriana Maria Neele
geboren te Zaanstad in 1968

Promotiecommissie:

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The studies presented in this thesis were performed at the Gaubius Laboratory of TNO Prevention and Health, Leiden. This work was financially suppported by the Netherlands Heart Foundation (project # 92.328)

ISBN 90-5412-049-5

Financial support by the Gaubius Laboratory of TNO Prevention and Health of this thesis is gratefully acknowledged.

The printing of this thesis was further financially supported by the Dr. Ir. J.H. van der Laar Stichting, J.E. Jurriaanse Stichting and Parke-Davis B.V.

aan mijn moeder,
ter nagedachtenis aan mijn vader,
voor Goffe

Stellingen behorende bij het proefschrift:
Regulation of apolipoprotein(a) and apolipoprotein A-I synthesis

1. Een hoge lp(a) concentratie in het bloed vergroot de kans op hart- en vaatziekten.
   Stein et al., Arch Intern Med. 1997;157:1170-1176

2. Retinoiden hebben een gunstige invloed op de apo A-I (verhoging) en apo(a) (verlaging) synthese *in vitro,* maar ze zijn in de praktijk niet bruikbaar.
   Dit proefschrift

3. De verhoogde plasma lp(a) concentratie in slecht ingestelde diabetes mellitus type I patienten kan verklaard worden door een direct effect van insuline op de apo(a) synthese.
   Dit proefschrift

4. De bevinding van Ramharack *et al.* dat fibraten de plasma lp(a) concentratie en het apo(a) mRNA in apen verlagen wordt niet bevestigd door experimenten beschreven in dit proefschrift.
   Dit proefschrift en Ramharack et al., J Lipid Res 1995; 36: 1294-1304

5. Het feit dat triazolodiazepines *in vitro* de apo(a) synthese drastisch verlagen biedt mogelijkheden voor de ontwikkeling van medicijnen ter verlaging van hoge lp(a) plasma niveau's.
   Dit proefschrift

6. Apolipoprotein(a) is, wat functie betreft, na 35 jaar onderzoek nog immer een mysterieus eiwit.
   Brown & Goldstein, Nature 1987;330:113-114

7. Hoewel er meerdere behandelingsmethoden zijn ter verlaging van een hoge plasma lp(a) concentratie is er niet één echt geschikt voor klinische toepassing.
   Angelin, Curr Opin Lipidol 1997; 8: 337-341 en Berglund, Curr Opin Lipidol 1995; 6: 48-56

8. De in epidemiologisch onderzoek gevonden inverse relatie tussen consumptie van groene en zwarte thee en plasma lipiden berust op confounders.
   Princen et al., Arterioscler Thromb Vasc Biol 1998; 18: 833-841

9. De meting van de resistentie van LDL tegen oxidatieve stress ex vivo heeft geen sterk voorspellende waarde voor het inschatten van het risico op coronaire hartziekten.
Van de Vijver et al., Arterioscler Thromb Vasc Biol 1998; 18: 193-199

10. Het is niet uit te sluiten dat kringle 5 van plasminogeen ook bijdraagt aan de remming van angiogenese beschreven door O'Reilly *et al.*, aangezien bij de bereiding van het angiostatine preparaat (kringle 1-4 van plasminogeen) elastase is gebruikt.
O'Reilly et al., Cell 1994; 79: 315-328 en Cao et al., J Biol Chem 1997; 272: 22924-22928

11. Het kijken naar een voetbalwedstrijd van het Nederlands elftal is voor een fanatieke voetbalfan bijna net zo uitputtend als er zelf één spelen.

12. Zonder stimulerende middelen behaalt geen wielrenner de top.

Leiden, 22 oktober 1998 Diana Neele

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Chapter 1

General introduction

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1.1 LIPOPROTEINS AND LIPID METABOLISM

1.1.1 Introduction

Cardiovascular disease is the leading cause of death in the Western industrialized countries. A major cause of cardiovascular disease is atherosclerosis in which the accumulation of fatty deposits in the arterial wall plays an important role. Important risk factors for the development of atherosclerosis besides high blood pressure, diabetes and smoking are elevated levels of cholesterol and/or triglycerides in the blood. Although extreme high levels of cholesterol and triglycerides can be life threatening they are physiologically important components of the human body. Cholesterol is used for the synthesis of plasma membranes, steroid hormones and bile acids whereas triglycerides are sources of energy for muscle and various other tissues and for energy storage in adipose tissue (1-4).

1.1.2 Lipoprotein metabolism

Cholesterol and triglycerides are hydrophobic molecules which are transported in the blood together with proteins in a complex called lipoproteins. Lipoproteins are spherical macromolecular particles in which the hydrophobic core containing triglycerides and cholesteryl esters is emulsified by a shell composed of phospholipids, unesterified cholesterol and one or more specific proteins called apolipoproteins. Four major lipoprotein classes can be distinguished according to their size and buoyant density, determined by ultracentrifugation: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL). In addition to these four major lipoproteins, another lipoprotein particle, called lipoprotein(a) can be found in humans. It is composed of LDL and a high molecular weight glycoprotein, designated apolipoprotein(a) (Table I).

The lipoprotein classes differ in size, electrophoretic mobility, lipid and apolipoprotein composition and these parameters determine the functions of the lipoproteins in the lipid metabolism. The lipoprotein metabolism can be divided into three different pathways, i) the endogenous pathway, ii) the exogenous pathway and iii) the reverse cholesterol pathway (5-9).

In the exogenous pathway dietary triglycerides and cholesterol are absorbed by the intestine and packaged into chylomicrons. These triglyceride-rich particles are secreted into the blood stream via the thoracic duct lymph. Upon entering the blood circulation, the chylomicrons are rapidly hydrolyzed by lipoprotein lipase (LPL) thereby delivering free fatty acids (FFA) to peripheral tissues. During this process apo A-I and excess surface phospholipids are split off from the chylomicrons as surface remnants to form HDL. The resulting chylomicron remnants are mainly taken up via apo E-specific recognition sites on

the parenchymal cells of the liver (10) (fig 1).

Table I. Composition of human plasma lipoprotein classes

	Chylomicron	VLDL	LDL	HDL	lp(a)
Diameter (nm)	75-1200	30-80	19-25	5-12	25
Density (g/ml)	< 0.96	0.96-1.006	1.019-1.063	1.063-1.210	1.060
Electrophoretic mobility	Origin	pre-ß	ß	α	pre-ß
Triglycerides (% weight)	80-95	45-65	13	15	3-11
Phospholipids (% weight)	8	20	28	45	15-22
Free cholesterol (% weight)	1-3	4-8	10	10	6-8
Cholesteryl ester (% weight)	3	15	48	30	32-50
Proteins (% weight)	1-2	6-10	18-22	45-55	20-26
Apolipoproteins	A-I, A-IV, B-48, C-I, C-II, C-III, E	B-100, C-I, C-II, C-III, E	B-100	A-I, A-II,E	B-100, (a)

In the endogenous pathway the liver secretes VLDL particles which contain cholesterol and triglycerides. The VLDL particles are also hydrolyzed by LPL, resulting in the formation of VLDL remnant lipoproteins or intermediate density lipoproteins (IDL). IDL particles are partly cleared from the circulation by the LDL-receptors and/or remnant receptors. The remaining particles are further hydrolyzed by another lipolytic enzyme hepatic lipase (HL). This results in the formation of LDL which is rich in cholesteryl esters and contains apo B-100 as the sole apolipoprotein. Apo B-100 serves as the ligand in the LDL receptor-mediated uptake. The LDL receptor is present on liver cells and also on extra-hepatic tissues such as muscle, adipose tissue and steroid hormone producing glands. When LDL is chemically or physically modified it is removed from the circulation by LDL receptor-independent pathways. High plasma levels of LDL results in an increased LDL concentration in the intima. In the arterial wall LDL is oxidized by the endothelial cells, smooth muscle cells or macrophages. This process leads to monocytes entering the intima, recruited by the chemotactic factor present in oxidized LDL. Subsequently the monocytes differentiate into tissue macrophages and take up oxidized LDL via various

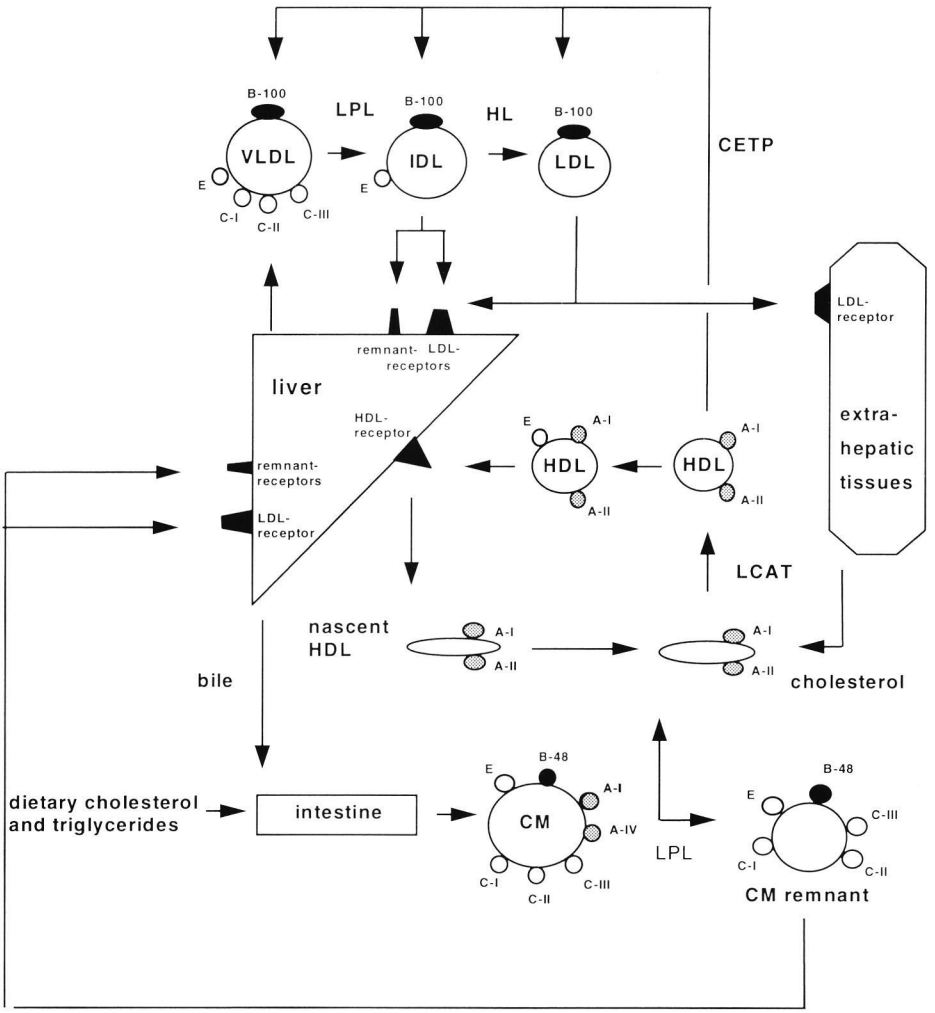

Figure 1.
Schematic representation of the pathways in lipoprotein metabolism.
CM = chylomicron, VLDL = very low density lipoprotein IDL = Intermediate density lipoprotein, LDL = low density lipoprotein, HDL=high density lipoprotein, LPL = lipoprotein lipase, HL = hepatic lipase, LCAT = lecithin:cholesterol acyl transferase, CETP = cholesterol ester transfer protein.

scavenger receptors which leads to the formation of foam cells. This process is assumed to be one of the initial steps in the formation of atherosclerotic lesions (11,12) (fig 1).

The liver is the main organ where excess of cholesterol can be removed from the body, by conversion into bile acids and secretion into the bile. Cholesterol present in peripheral tissues can be transported back to the liver by HDL particles. This is the so-called reverse cholesterol transport pathway. Nascent HDL is secreted by the liver as lamellar disks and contains only apo A-I, apo A-II and phospholipids. Transfer of chylomicron remnants which are released during lipolysis increase the size of nascent HDL which subsequently takes up cholesterol from extra-hepatic tissues. This cholesterol is esterified by the enzyme lecithin:cholesterol acyl transferase (LCAT) with apo A-I as a co-factor. The HDL cholesterol is then delivered to the liver via several pathways. Cholesteryl esters from HDL may be transferred to VLDL, IDL and LDL by cholesteryl ester transfer protein (CETP), and these particles are taken up by the liver as described above. HDL can also acquire apo E in the circulation and since apo E is recognized by different hepatic receptors, it can be taken up by the liver. Another way for HDL to deliver its cholesteryl esters to the liver is by the selective uptake of these particlesby the liver. This process is most likely regulated by the scavenger receptor SR-BI (13) (fig 1).

The metabolic pathway of apolipoprotein(a), lipoprotein(a) and the regulation of the synthesis of apo A-I will be discussed in more detail in the following part of this introduction.

1.2 REGULATION OF APOLIPOPROTEIN A-I LEVELS

It is well established that an inverse relationship exists between plasma levels of HDL-cholesterol and the risk of the development of CHD (14,15). This relationship is also valid for apo A-I, the major protein constituent of HDL. Apo A-I synthesis is reported to be associated with the HDL-cholesterol level in the circulation *in vivo* (16,17). Apo A-I is synthesized by the liver and intestine and secreted in a lipid-free or lipid-poor particle (18). Kinetic studies in humans have established that variation in plasma HDL cholesterol and apo A-I levels between individuals is primarily due to variation in the rate of apo A-I catabolism and not apo A-I production (19,20). Several genetic factors may play an important role in modulating the plasma levels of apo A-I and HDL through different mechanisms. Studies in transgenic mice overexpressing human apo A-I emphasize the significance of apo A-I production. These mice have high plasma HDL and apo A-I levels and are more resistant to atherogenic diets (21). Similar results were obtained in apo A-I transgenic rabbits, a better model for human atherosclerosis (22). These data indicate that upregulation of the apo A-I synthesis may be useful in increasing the HDL level, which may

subsequently decrease the risk of development of atherosclerosis.

Factors that influence HDL-lipid composition (and therefore also the size) appear to be the most important in modulating the apo A-I catabolism. Studies in humans and transgenic and knock-out animals have established that genetic variation in LCAT, CETP and hepatic lipase affect HDL and apo A-I levels by modifying the lipid content of the HDL particles (14).

The transgenic apo A-I mice and rabbits provide a good model for examining the effects of diets and drugs on apo A-I synthesis. The influence of the most commonly used drugs and hormones on the regulation of the apo A-I synthesis will be discussed below.

Fibrates, like gemfibrozil, bezafibrate, fenofibrate and ciprofibrate are drugs which are widely used in the treatment of hypertriglyceridemia and hypercholesterolemia. These drugs lower particularly triglyceride levels and increase HDL-cholesterol levels in hyperlipidemic patients (23-25). The rise in HDL-cholesterol, induced by fibrate treatment, may partially be secondary to the decrease in triglyceride-rich lipoproteins, i.e. less exchange of triglycerides and cholesteryl esters occurs between HDL and triglyceride rich lipoproteins, leading to an increased level of HDL (23). On the other hand, studies in both humans and animals suggest that fibrates also stimulates apo A-I synthesis and increases apo A-I mRNA levels (26-28). HMG-CoA reductase inhibitors such as lovastatin, pravastatin, fluvastatin and atorvastatin which are clinically used to lower LDL-cholesterol have a slight increasing or no effect on apo A-I synthesis (29,30). HDL cholesterol levels are modestly increased, generally between 5-15%, after statin treatment (29-32).

Different hormones are reported to influence the apo A-I synthesis and secretion. The effect of insulin on apo A-I synthesis is not completely clear at present. Stimulation of the apo A-I secretion and mRNA level by insulin has been reported in rat hepatocytes (33-34). However, these findings are disputed by others who report a decrease of apo A-I secretion in HepG2 cells and rat hepatocytes after insulin treatment (35,36). Another set of hormones which have been shown to influence apo A-I synthesis are corticosteroids. Dexamethasone, a synthetic corticosteroid, increased the apo A-I synthesis and mRNA levels in rat hepatocytes and HepG2 cells (36,37) and *in vivo* in rats (38). Saladin *et al.* (39) showed that dexamethasone induces the proximal apo A-I promoter, and showed furthermore that the glucocorticoid receptor and a labile cell-specific protein are required for the induction of the apo A-I gene transcription by dexamethasone.

It has been suggested that sex steroid hormones play an important role in the regulation of HDL plasma levels. Increased plasma apo A-I levels after estrogen treatment are reported in humans (40). Moreover, an increased apo A-I synthesis and mRNA level was found in HepG2 cells treated with 17-ß estradiol (41). A hormone that has been studied frequently for its effect on apolipoprotein synthesis is the thyroid hormone. This hormone increases the transcriptional activity of the apo A-I gene resulting in a increased apo A-I

synthesis (42-44).

Another group of compounds that affects the regulation of the synthesis of apo A-I are the retinoids. A previous report (45) from our group showed that retinoids, including retinol (vitamin A), all-*trans* retinoic acid and its naturally occurring isomers, stimulate apo A-I synthesis in primary hepatocyte cultures from cynomolgus monkey. Similar data were obtained by others in HepG2 cells and rat hepatocytes (46-48). However, retinoids decrease hepatic apo A-I gene expression *in vivo* (46) and decreased or unchanged HDL cholesterol and apo A-I levels were reported in humans who were treated with isotretinoin (13-*cis* retinoic acid) (49).

1.3 LIPOPROTEIN(a)

1.3.1 Structure of lp(a) and apo(a)

The existence of lipoprotein(a) (lp(a)) was first reported by Berg (50) in 1963 as an antigenic variant of LDL. In the early 1970 it was shown that although the lipid composition was similar to that of LDL, the lipoprotein particle lp(a) contained significantly more carbohydrate and had a pre-beta mobility (51). Later studies showed that lp(a) is a complex of LDL in which an additional protein component, apolipoprotein(a) (apo(a)) is linked to LDL via a disulfide bridge with apo B-100, the main protein component of LDL. A small part of the apo(a)/apoB-100 complex is found in VLDL and IDL like particles. Apo(a) is a highly glycosylated, hydrophilic protein that has very little affinity for lipids. It is remarkably polymorphic, with 34 size isoforms identified in human plasma so far, and a molecular weight of 300 to 800 kDa (52). Apo(a) is very homologous to plasminogen and the cDNA contains multiple copies of a sequence that encodes a protein motif which closely resembles the 4th kringle of plasminogen (75-85% homology) (53) (fig 2).
These segments are linked to a plasminogen-like kringle 5 and a protease domain in which the percentage of homology with the respective plasminogen domains is 91% and 94%, respectively (fig 2). A substitution at the cleavage site for tissue plasminogen activator prevents the proteolytic activation of apo(a). The apo(a) kringles have been classified into 10 kringle 4 subtypes which differ in amino acid substitution. The size of apo(a) is determined by the number of kringle 4 type 2 domains that are encoded in the apo(a) gene, which can vary from approximately 12 to 51 (52). The genes for apo(a) and plasminogen are physically linked in chromosome 6, 50 kb apart, and orientated in a head-to-head fashion. Two related apo(a) genes are located in the same gene cluster and one produces a mRNA transcript in the liver, but it is unknown whether its function is related to apo(a). The apo(a) gene spans 52.5 kb and consists of 19 exons separated by 18 introns (54). The apo(a) 5'-flanking region contains consensus sequences for the promoter

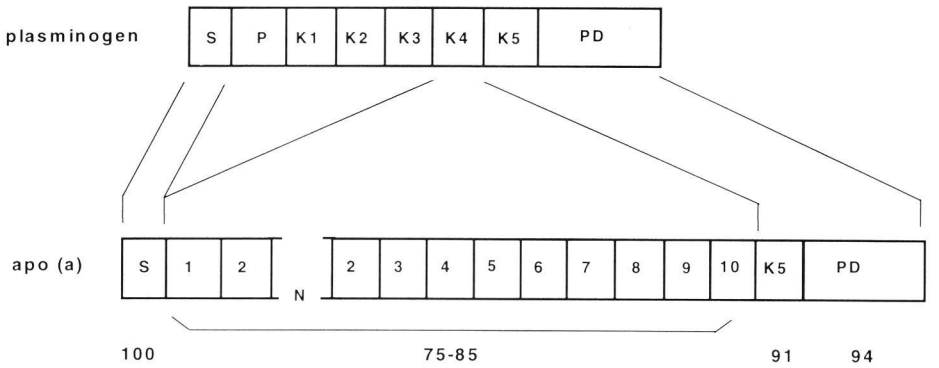

Figure 2. Comparison of the cDNAs for human plasminogen and apo(a). The numbers at the bottom indicate the percentage of homology for each domain. S = signal peptide, P = preactivation peptide, K1-K5 = kringle domains, PD = protease domain (This figure is modified from White *et al.* (75))

elements TATA and CAAT boxes and for liver-specific transcription factors such as HNF-1α and C/EBP, but also nuclear hormone receptor elements and IL-6 responsive element consensus sequences have been identified (55,56). Currently several research groups are focussing their attention on the gene cluster and promoter region of the apo(a) gene to find regulatory elements and to gain greater understanding of the factors that control the expression of the apo(a) gene.

1.3.2 Apo(a) gene

On the phylogenetic scale lp(a) is restricted to Old World monkeys (e.g. cynomolgus monkeys, baboons and chimpanzees) and humans (57,58), suggesting that the gene has evolved relatively recently. Exceptions are the hedgehog (59) and the marmoset, a species of the New World monkey (60) in which the presence of apo(a) has also been reported.

Plasma levels of lp(a) show enormous variation among individuals, ranging from about 0.1 to more than 200 mg/dl and are mainly regulated at apo(a) synthesis level and not at the level of lp(a) catabolism (61,62). In general, about 90% of the variation in lp(a) plasma levels can be attributed to the apo(a) gene locus (52,63). Furthermore, apo(a) size polymorphism accounts for 27-60% of this genetic variability, depending on the ethnic origin of the population studied (64,65). There exists a correlation between apo(a) size polymorphism and the plasma lp(a) levels. However, this is not a strict correlation and the same isoform can be associated with lp(a) levels which may vary over a 200-fold range

(66,67), thus the apo(a) locus may influence the lp(a) concentration through a mechanism that is independent of the apo(a) gene size. The influence may occur at the transcriptional or mRNA stability level because apo(a) mRNA concentration has been shown to correlate with lp(a) levels in cynomolgus monkeys (57,68). So far, a number of studies have revealed polymorphisms in the apo(a) promoter, however none of the polymorphisms were of a magnitude that might account for the apo(a) allele size-independent variation of lp(a) concentrations (69-71).

1.3.3 Synthesis, processing, secretion and assembly of lp(a)

Pulse-chase studies with primary cultures of monkey hepatocytes demonstrated that apo(a) is synthesized as a low molecular weight precursor that is converted to a mature form in the endoplasmic reticulum, which involves post-translational modification (i.e. glycosylation), after which it is secreted into the medium (72,73). Large apo(a) isoforms have a longer residence time in the endoplasmic reticulum than small isoforms and a higher proportion of the larger proteins is retained and degraded in the endoplasmatic reticulum. This may partly explain the observation that large isoforms are associated with low lp(a) levels and conversely, small isoforms are associated with high lp(a) levels (74,75). Nevertheless, a wide variation in the lp(a) plasma level exists between individuals with the same apo(a) isoforms. This variation may originate at the transcriptional level, since a correlation exists between hepatic apo(a) mRNA levels and plasma protein concentrations in monkeys and humans (57,76) (see section 1.2.1).

In vitro experiments showed that the apo(a)/apo B-100 complex was not detectable in the cell lysates; they were only detectable in the medium (73). This suggests that lp(a) assembly occurs extracellularly. Newly synthesized apo(a) binds with its kringle IV domain to the cell surface from where it can be captured by apo B-100 (on VLDL or LDL) and released from the cell as a lipoprotein particle (77). It is suggested to be a two-step process. Initially multiple noncovalent interactions serve to position apo(a) and apo B-100. Thereafter disulfide bonds are formed (78). *In vitro* mutagenesis studies showed that an unpaired cysteine residue in kringle 4 type 9 of apo(a) participates in the disulfide bond and formation with apo B-100 (78,79). The carboxyl terminal kringle 4 domains interact noncovalently with apo B-100 to contribute to the stability of lp(a) (77,80,81). Kringle 4 type 1 and 2 repeats, which are not involved in the binding to apo B-100 and extend from the surface of the particle (82), can possibly bind other substrates such as receptors or cells.

In contrast to the monkey hepatocytes, cultured human hepatocytes appear to contain and secrete unbound apo(a) as well as the apo(a)/apo B-100 complex. The amount of bound and unbound apo(a) differs between the various donor livers (83). It should be noted that this phenomenon is only reported in one paper and that Wilkinson *et al.* (84) showed that human liver homogenates do not contain apo(a)/apoB-100 complexes. On

the other hand, in human or monkey plasma apo(a) is always bound to apo B-100; unbound apo(a) is not detectable. Thus far the mechanism of the lp(a) assembly is not completely clear.

1.3.4 Metabolic pathway of lp(a)

Human metabolic studies have shown that a correlation exists between plasma lp(a) levels and lp(a) production rate, indicating that the lp(a) plasma concentration is mainly determined by lp(a) synthesis level and not by lp(a) catabolism (61,62). Not much is known about the catabolism and clearance of lp(a) from the circulation. LDL and lp(a) are structurally very similar, therefore the role of the LDL-receptor in the catabolism of lp(a) has been evaluated in several studies (75,85). Accumulating evidence suggests that lp(a) can bind to the LDL receptor but that the affinity is low as compared to that of LDL, suggesting that this is not a major determinant in lp(a) catabolism. Kostner *et al.* (86) first reported about the low affinity of lp(a) for the LDL receptor and suggested a "hitchhiking" process in which a lp(a)-LDL complex is taken up by the LDL receptor as a single ligand. Later Snyder *et al.* (87) showed by using primary human hepatocytes, macrophages and fibroblasts that the lp(a) binding and degradation by the LDL receptor was only 10%-30% of that of LDL. Moreover, the lp(a) catabolism in homozygous and heterozygous familial hypercholesterolemia (FH) patients who lack functional LDL receptors is not different when compared to the catabolism in control individuals (88). Similar data were found in hypercholesterolemic rhesus monkeys (89). Recently, Reblin *et al.* (90) showed by using mouse embryonic fibroblasts that lp(a) is a poor ligand for the LDL receptor and the LDL receptor-related protein. In contrast, in transgenic mice overexpressing the LDL receptor in the liver, lp(a) binds to the LDL receptor and is more rapidly cleared from the circulation suggesting that the LDL receptor is at least partly involved in the uptake of lp(a) (91) (fig 3).

In addition to the LDL receptor other pathways play a role in the catabolism of lp(a). A non-LDL-receptor pathway in fibroblast has been described (92) and Bottalico *et al.* (93) have shown that cholesterol loading of macrophages leads to enhancement of native lp(a) and apo(a) internalization and degradation by a non-LDL-receptor. In later studies the receptor for lp(a) on human macrophages was characterized. The foam cell lp(a)/apo(a) receptor consists of a cholesterol-regulatable binding activity and a short-lived component for internalization or lysosomal degradation (94). Recently, it was demonstrated that fibroblasts expressing the VLDL receptor can mediate endocytosis of lp(a), leading to its degradation within lysosomes (95). Kostner *et al.* (96) found that lp(a) interacts with kidney cells, which causes cleavage of the N-terminal part of apo(a) by a collagenase-type protease. Part of these apo(a) fragments are found in the urine, the core portion of lp(a)

in turn is cleared by receptors or specific binding systems in the liver (fig 3).

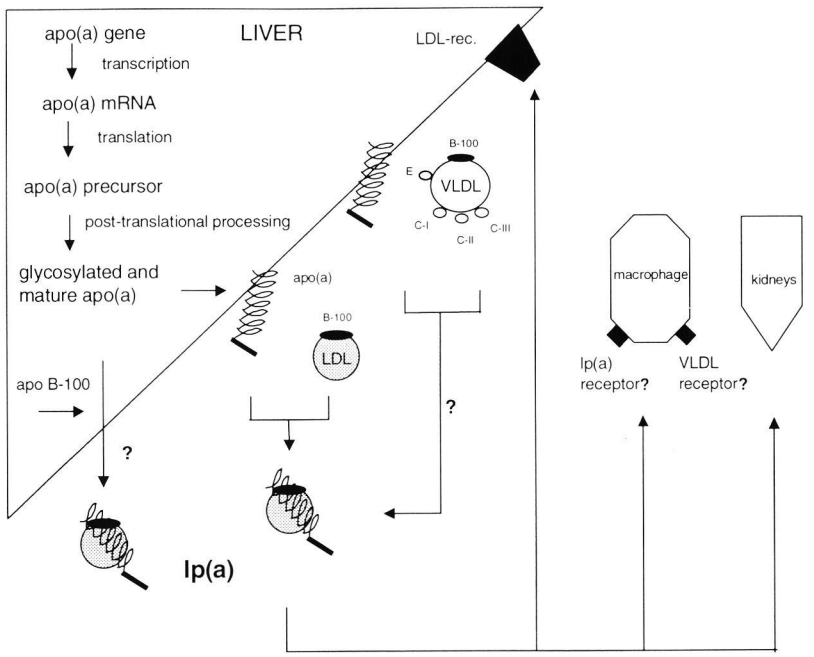

Figure 3. Schematic representation of the synthesis of apo(a) and assembly and metabolism of lp(a)

1.3.5 Prothrombotic effects of lp(a)

The structural similarity between apo(a) and plasminogen suggests that lp(a) may modulate thrombolysis. Although the apo(a) protease domain is incapable of active plasmin-type fibrinolytic activity, lp(a) can act as an inactive mimic of plasminogen. A number of *in vitro* studies have shown that lp(a) competes with plasminogen for binding to fibrin and cell-surfaces (97-100). As a consequence plasminogen escapes conversion to plasmin and is thus unable to function as a fibrinolytic agent, which results in impairment of thrombolysis. Ezratty *et al.* (101) found that lp(a) can also bind to platelets and that lp(a) attenuates the platelet-mediated binding and activation of plasminogen. However, not all studies show that lp(a) has an effect on fibrinolysis, but this may be due to the use of different lp(a) isoforms (102-104).

Data from *in vivo* studies concerning the effect of lp(a) on fibrinolysis are also not

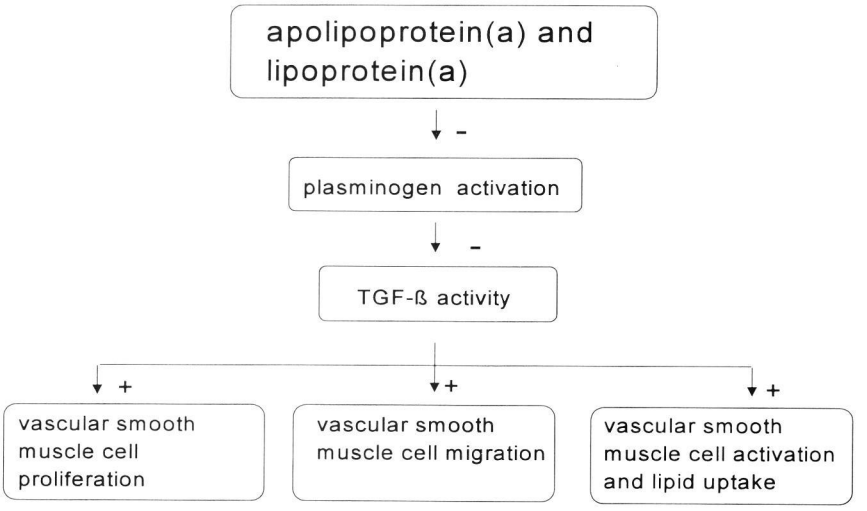

Figure 4. Actions of appo(a) and lp(a). TGF-ß = transforming growth factor-beta. This figure is modified from Grainger *et al.* (118)

consistent. Some studies failed to show an inverse relationship between lp(a) plasma levels and fibrinolysis (105-107), while others showed a clear negative effect of lp(a) on the fibrinolytic system (108-110). The different results are probably due to the wide variation in methodology and models used to assess thrombosis and fibrinolysis. Furthermore, most studies have not taken into account the effect that sequence polymorphisms and different lp(a) isoforms may have. Whether and how lp(a) influences fibrinolysis *in vivo*, remains to be clarified.

1.3.6 Proatherogenic effects of lp(a)

Lp(a) has been identified in human arterial atherosclerotic lesions and in saphenous vein grafts after coronary bypass surgery, and the degree of deposition was associated with the concentration of serum lp(a). When corrected for corresponding plasma levels, more apo(a) than apo B-100 is found in the lesions, suggesting that lp(a) is preferentially deposited in atherosclerotic plaques compared with LDL (111,112). Mice injected with 125I-labeled human lp(a) and human 125I-labeled LDL in equimolar amounts showed similar results: Lp(a) displayed a significantly higher accumulation compared to LDL in the arterial wall (113), suggesting that the apo(a) moiety is predominantly responsible for the localization of lp(a) in the lesions. The latter study is also in line with the observation that unbound apo(a) (not attached to LDL) in transgenic apo(a) mice is found in the vessel wall

(114,115). The mechanism by which lp(a) and apo(a) accumulate in the lesion is not completely clear, but it has been shown that lp(a) via apo(a) binds to matrix components which are, among others, found in atherosclerotic plaques such as fibrin, glycosaminoglycans and fibronectin. When lp(a) is localized near a vessel injury site it can be taken up by the macrophage and this may lead to foam cell formation (95). Furthermore, lp(a) and apo(a) can enhance vascular smooth muscle proliferation *in vitro* (98,116) and *in vivo* (117) in transgenic mice, by inhibiting the conversion of plasminogen to plasmin. This blocks the proteolytic activation of the autocrine inhibitor of smooth muscle cell proliferation, transforming growth factor-ß (TGF-ß) (114,116-119). Recently it was demonstrated that the amount of active TGF-ß in the circulation is inversely correlated with circulating lp(a) levels. The same studies showed that depressed TGF-ß activity was associated with advanced coronary artery atherosclerosis in patients with atherosclerotic lesions (119,120). At present it is not clear whether circulating active TGF-ß has a function or that it is just an indirect measurement for other processes involved in atherogenesis (fig 4).

1.3.7 Is lp(a) a risk factor for coronary heart disease?

Lp(a) continues to be a controversial player in lipidology but accumulating evidence shows that lp(a) plays a role in both atherogenesis and fibrinolysis. Several retrospective studies have found associations between high plasma levels of lp(a) and an increased risk of CHD (121-126). In particular, the concept that high levels of lp(a) in conjunction with increased concentrations of LDL-cholesterol are associated with an enhanced risk of cardiovascular disease is well established (127). However, the methodological limitations of retrospective studies, for instance inadequate sample storage temperature, inaccurate lp(a) measurements, differences in patient groups, or wide confidence intervals, makes it difficult to interpret the results (128).

The association between high lp(a) levels and CHD was partly clarified by several prospective studies. Thus far, 10 prospective studies have evaluated lp(a) as a predictor of CHD. The prognostic role of lp(a) in premature CHD was confirmed in 7 studies (129-135) and in 3 trials lp(a) levels were not found to be predictive of CHD events (136-138). Although these prospective studies have yielded inconclusive evidence for the role of lp(a) as a predictor of myocardial infarction or CHD death in the general population, they have consistently shown the importance of elevated lp(a) levels in predicting myocardial infarction and CHD death in men with hypercholesterolemia.

1.4 REGULATION AND MODULATION OF LIPOPROTEIN(a) LEVELS

1.4.1 Dietary factors

In humans and monkeys, no or relatively small changes in lp(a) levels have been found after dietary changes. The first dietary studies were performed by Albers *et al.* (139,140) who did not find changes in lp(a) levels when individuals were given a high-cholesterol diet, although apo B-100 levels were increased. Later studies reported similar results when normolipidemic men were fed a cholesterol-rich diet (141). In other studies in which subjects were given a low fat or low calorie diet also no changes in lp(a) levels were found (142,143) except in women with initial lp(a) levels above 30 mg/dl, in whom a mean decrease by 26.3% was observed with a 10 kg weight loss (143). Lichtenstein *et al.* (144) who treated patients with moderate hypercholesterolemia with different diets including a diet enriched in trans-fatty acids, did not find an effect on lp(a) levels. Although most diets do not change lp(a) levels it is reported that diets rich in trans-monounsaturated fatty acids or the trans-fatty acid elaidic acid slightly raise lp(a) levels (145,146). Increased lp(a) levels were also found in cynomolgus monkeys fed with a diet enriched in saturated fatty-acids compared with monkeys fed with diets enriched in mono- or polyunsatured fatty acids. This regulation took place at the (post)-transcriptional level since it was shown that apo(a) mRNA levels were lower in the livers of the monosaturated fatty acid-fed monkeys compared to the apo(a) mRNA levels of the saturated fatty acid-fed monkeys (147). A small but significant increase in lp(a) was also found by Rainwater *et al.* (148) after they fed baboons with diets enriched with cholesterol and saturated fat.

Fish oils have a beneficial effect on plasma lipoprotein level. They reduce the production of VLDL and may have a favorable effect on LDL receptor expression. The effect of fish oils on lp(a) levels is not convincing (149), some studies report a decrease (150), while others do not find any effect (151,152).

1.4.2 Hormones

1.4.2.1 Sex hormones and anabolic steroids

Several types of hormones influence lp(a) levels. The first hormone-like compound that was reported to lower lp(a) levels was stanozolol (153) and thereafter other androgens such as tibolone and danazole showed a similar decreasing effect on lp(a) plasma levels (26% and 79%, respectively) (154,155). Besides androgens, estrogens also decrease plasma lp(a) levels. In several studies in which postmenopausal woman received estrogen treatment lp(a) levels were reduced (156-158). Combination therapies of both estrogens and progestogens have a similar decreasing effect on lp(a) concentrations as therapy with estrogens alone (159).

1.4.2.2 Growth hormone and insulin-like growth factor-I

Over the past 5 years more than 20 studies examined the effect of growth hormone (GH) on plasma lp(a) levels. Almost all studies show that GH increases plasma lp(a) levels (110-200%) in healthy volunteers as well as in subjects with growth hormone deficiency (160-164). In contrast, LDL-cholesterol is decreased and hepatic LDL-receptors are induced by growth hormone (165). GH induces also the secretion of insulin-like growth factor-I (IGF-I). So it is hypothesized that IGF-I is responsible for the increase in lp(a) especially, since the IGF-I level is decreased during estrogen therapy (165). However, this is not the case; lp(a) is lowered after the administration of IGF-I to individuals (166-168). These results indicate that the lp(a) increasing effect of GH is not mediated by IGF-I. The mechanism by which GH and IGF-I influence lp(a) concentrations is not understood and warrants further study.

1.4.2.3 Insulin

It has been shown that lp(a) levels are elevated in patients with insulin-dependent diabetes mellitus (IDDM) (169-172). Several studies showed that insulin therapy associated with improved glycaemic control resulted in decreased lp(a) plasma levels (by about 30%) in IDDM patients (169,171). However, no effect on plasma lp(a) levels after improved glycaemic control was found in non-insulin-dependent diabetes mellitus (NIDDM) patients (173,174). The reports on a possible association between lp(a) and insulin levels in healthy subjects remain controversial. Inverse relationships have been described (175,176), but the absence of an association between insulin and lp(a) concentrations has also been reported (177). Furthermore, it is not clear whether insulin plays a role in the regulation of lp(a) plasma concentrations by influencing its synthesis or catabolism.

1.4.3 IL-6 and acute-phase reaction

Marked acute-phase responses of lp(a) have been reported after acute ischemic coronary syndromes and surgery (178,179). Elevations in lp(a) plasma levels of more than 200% have been observed after acute myocardial infarction and raised lp(a) levels associated with acute myeloblastic leukemia were shown to be reduced after chemotherapy that resulted in complete remission (180). It is also shown that lp(a) levels are closely related to the acute phase reaction, IL-6 levels and hypercoagulability in chronic hemodialysis patients (181). These observations are consistent with the presence of multiple IL-6 responsive element consensus sequences in the promoter region of the apo(a) gene (182). Moreover, IL-6 increased the transcriptional activity of the apo(a) promoter in promoter-reporter studies (183). However, some studies in patients suffering acute myocardial infarction do not support the view that lp(a) acts as an acute phase reactant (184,185). Frazer *et al.* (186) showed by using YAC transgenic apo(a) mice that

turpentine, an inflammatory stimulus known to increase plasma concentrations of IL-6, reduces apo(a) plasma concentrations and mRNA levels.

1.4.4 Drugs

1.4.4.1 Nicotinic acid and neomycin

Nicotinic acid reduces plasma VLDL and LDL levels and increases HDL levels (187). Clinical studies have indicated that nicotinic acid therapy alone or in combination with neomycin also reduces serum levels of lp(a) (188-191). Hypercholesterolemic patients with lp(a) levels above 30 mg/dl, who where treated for two months with nicotinic acid showed a decrease in lp(a) levels of 36%. The fractional catabolic rate of lp(a) was not changed, which suggests that the reduction in lp(a) levels was achieved through suppression of the synthetic rate (192). Because high doses of nicotinic acid (3-4 g/day) are needed to observe its effect on lp(a) levels, side effects became a problem. Nicotinic acid derivatives were developed to reduce these side effects. Although niceritrol, a nicotinic acid derivative, is able to lower lp(a) and is better tolerated than nicotinic acid, higher doses than initially thought were needed in order to achieve a significant lp(a) lowering (193-195). The exact mechanism behind the effect of these compounds is not clear at present.

1.4.4.2 HMG-CoA reductase inhibitors, fibrates and LDL-apheresis

HMG-CoA reductase inhibitors like lovastatin, simvastatin and pravastatin (statins), which are known to lower LDL-cholesterol are not able to lower lp(a) levels in hypercholesterolemic individuals (190,191,196-199). The most recent HMG-CoA reductase inhibitor, atorvastatin, does not seem to lower lp(a) either (200,201). In contrast, in some studies these compounds even increased the lp(a) levels (202,203). This group of drugs exerts its effect largely by the induction of the hepatic LDL receptor activity and the role of the LDL-receptor in the metabolism of lp(a) is still debated (see section:1.3.4).

Fibrates are a group of drugs which are widely used in treatment of hypertriglyceridemia and hypercholesterolemia. They are among the oldest lipid-lowering drugs, e.g. clofibrate was already used in the late 1950's (204). The drugs lower triglyceride levels and increase HDL-cholesterol levels in particular in hyperlipidemic patients (23-25). Data on the effects of fibrates on lp(a) levels are not conclusive; some *in vivo* studies report a decrease in lp(a) level (205-209), or an increase (206,210), and there are also studies which do not observe any effect on the lp(a) plasma levels (211-213).

Lipoprotein(a) plasma levels are significantly lowered by LDL apheresis (190,191,214-216). LDL-apheresis is the specific removal of apo B-containing lipoproteins from the circulation. Since the lp(a) particles contain apo B they are also removed during the apheresis treatment. This method is performed primarily in high risk patients with familial hypercholesterolemia because it is very costly and has to be repeated frequently.

Pokrovsky *et al.* (217) recently described a specific apheresis for lp(a) in which the LDL levels remains unchanged. This method may be used to study the effect of drastically lowering lp(a) levels without affecting LDL levels.

1.4.4.3 Miscellaneous

Other compounds that may influence the lp(a) plasma levels are N-acetylcysteine and CI-1011, a potent ACAT inhibitor. N-acetylcysteine, which cleaves the disulfide bridge between apo(a) and apo B-100 is reported to lower plasma lp(a) levels (218). However, this finding does not seem to be supported by two later studies (219,220).

CI-1011 seems to be a more promising tool for lowering lp(a) levels. Recently, Ramharack *et al.* (221) showed that CI-1011, a potent lipid regulator in rodents, is able to lower lp(a) plasma levels by 68% in cynomolgus monkeys. Furthermore, it was shown in the same paper that the compound decreased apo(a) synthesis in cynomolgus monkey hepatocyte cultures (221).

1.5 OUTLINE OF THIS THESIS

Since lipoprotein (a) is an atherogenic and prothrombotic plasma particle that is a risk factor for premature coronary heart disease, there is great interest in the regulation of the synthesis of this molecule. Despite the fact that plasma levels of lp(a) are almost entirely determined by inheritance, a limited number of drugs and hormones are reported to influence the levels of lp(a). At the time when the studies described in this thesis were initiated little was known about the regulation of the synthesis of lipoprotein(a) and only a few tools were available to lower lp(a) levels *in vivo* in man. Even now it is still not completely clear how compounds such as nicotinic acid and growth hormone influence apo(a) lp(a) plasma levels. Although some drugs are reported to lower lp(a) levels the use of these compounds is limited in clinical practice. The major problem with nicotinic acid and neomycin treatment is the high frequency of side effects. LDL apheresis is very costly and it is difficult to treat men with estrogens. So, developing drugs to lower lp(a) synthesis is of great interest. Lp(a) synthesis is difficult to study since it is only synthesized in humans and monkeys and only a limited number of cell-culture-systems exist in which regulation of apo(a) or lp(a) synthesis can be studied. Performing *in vivo* studies in humans or monkeys may cause problems: the studies are very costly and difficult to interpret because of the different isoforms, the wide variation in lp(a) levels and other factors such as hormones and acute-phase proteins that may influence the outcome. Moreover *in vivo* studies in humans do not permit the investigation of molecular factors that may affect the apo(a) gene transcription and synthesis. When this study was initiated the transgenic

apo(a) and lp(a) mice were not yet available. These mice might form a good model for validating our *in vitro* findings *in vivo*.

The first aim of the study was to achieve a better understanding of the regulation of the synthesis of apo(a) under the influence of different compounds which are known to modulate the lipid, lipoprotein or apolipoprotein metabolism. The second aim was to find potential new ways for lowering increased lp(a) levels. We had the opportunity of using primary cultures of cynomolgus monkey (Macaca fascicularis) and human hepatocytes in key experiments, which are two of the few cell models that synthesize apo(a) endogenously. Besides the effect of the compounds on apo(a) synthesis, their effect on apo A-I, apo B-100 and albumin synthesis was also determined.

In Chapter 2, we describe the effect of insulin on apo(a) synthesis since insulin dependent diabetes mellitus patients are reported to have higher lp(a) levels. It is not yet known whether insulin interferes with the lp(a) synthesis or catabolism. Chapter 3, 4 and 5 describe the effect of retinoids on the synthesis of apo(a) and lp(a) and apo A-I. It was known from previous work by our group that retinoids are potent inducers of apo A-I synthesis in cell culture systems, but that the induction is slow and time-dependent. In Chapter 3 the mechanism of induction of apo A-I by retinoids is described. In Chapter 4 the effect of retinoids on apo(a) synthesis in cynomolgus monkey hepatocytes and the effect of all-*trans* retinoic acid on lp(a) levels in rhesus monkeys *in vivo* is reported. To validate our *in vitro* findings with retinoids, we studied the short-term effect of isotretinoin (13-*cis* retinoic acid) on lp(a) levels *in vivo*, in 10 healthy men in a placebo-controlled study. This study, which was performed initially to study the effect on fibrinolysis, is described in Chapter 5. Since the effect of fibrates, widely used as lipid-lowering drugs, on lp(a) was not clear we tested the effect of commonly prescribed fibrates on apo(a) in our monkey and human culture systems, which is described in Chapter 6. Finally, as a potential novel tool for influencing lp(a) and HDL-levels, we tested the effect of a new class of compounds, the triazolodiazepines, for their effect on apo(a), apo A-I and apo B-100 synthesis in human and monkey hepatocytes (Chapter 7).

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Chapter 2

Insulin suppresses apolipoprotein(a) synthesis by primary cultures of cynomolgus monkey hepatocytes.

Diabetologia; in press

Insulin suppresses apolipoprotein(a) synthesis by primary cultures of cynomolgus monkey hepatocytes.

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Running title: Insulin suppresses apo(a) synthesis

ABSTRACT

Elevated plasma lipoprotein(a) (lp(a)) levels have been reported in patients with insulin-dependent diabetes mellitus (IDDM), which were lowered by insulin therapy. Data on lp(a) levels in non-insulin dependent diabetes mellitus patients (NIDDM) and on the effect of insulin on lp(a) levels in the latter patients are less consistent. To investigate the biochemical background of the changes observed, we studied the effect of insulin on apolipoprotein(a) (apo(a)) synthesis and mRNA levels in primary cultures of cynomolgus monkey hepatocytes.

Addition of low concentrations of insulin (10 nM) to the hepatocyte cultures had a mildly but significantly decreasing effect on apo(a) secretion (-16%). Maximal inhibition was 33% and obtained after incubation for 72 h with 1000 nM insulin. Apo B-100 secretion was 30%-36% decreased when using 10-1000 nM and no change was observed for the secretion of apo A-I and albumin which were measured as control proteins. Steady state apo(a) mRNA levels paralleled the decrease in apo(a) synthesis (-29% after incubating the cells for 48 h with 100 nM insulin), indicating that the decreased synthesis is regulated at the (post)-transcriptional level. Apo B-100 and apo A-I mRNA levels were not changed after incubation with insulin.

We conclude that relevant concentrations of insulin suppress apolipoprotein(a) synthesis in monkey hepatocytes which and that this effect occurs at the (post)-transcriptional level. These data may provide an explanation for the increased plasma levels of lp(a) as found in patients with insulin dependent diabetes mellitus.

INTRODUCTION

Lipoprotein(a) (lp(a)) is a lipoprotein particle in which apolipoprotein(a) is covalently linked to low density lipoprotein (LDL) via a disulfide bridge. Lp(a) plays an important role in both atherogenesis and fibrinolysis and high plasma levels of lp(a) are considered to be

a risk factor for vascular diseases (1-4). Plasma levels of lp(a) vary strongly between individuals and are almost entirely determined by inheritance (5-7). Only a limited number of drugs and hormone treatments are reported to influence the level of lp(a) in humans (8, 9). Down-regulation of apo(a) synthesis may be a way to decrease lp(a) levels, since apo(a) synthesis is the major level at which lp(a) concentrations *in vivo* are regulated (10, 11). In this way the risk of developing cardiovascular diseases may be reduced.

Diabetic patients are at high risk of developing cardiovascular disease, and in addition to other deviations in lipid metabolism, abnormal levels of lp(a) may contribute to the increased risk in these patients. It has been shown that lp(a) levels are elevated in patients with insulin-dependent diabetes mellitus (IDDM), especially in those with microalbuminuria or poor metabolic control (12-15). In contrast, in well-controlled IDDM patients no elevated lp(a) plasma levels were observed (16). Data from studies in patients with non-insulin-dependent diabetes mellitus (NIDDM) are less clear. Increased (17, 18), decreased (19) and unchanged (16, 20) lp(a) levels in NIDDM patients were found when compared with non-diabetic subjects.

Several studies showed that insulin therapy associated with improved glycaemic control resulted in decreased lp(a) plasma levels (by about 30%) in IDDM patients (12,15). However, no effect on plasma lp(a) levels after improved glycaemic control was found in NIDDM patients (21, 22). The reports on a possible association between lp(a) and insulin levels in healthy subjects remain controversial. Inverse relationships have been described (23, 24), but the absence of an association between insulin and lp(a) concentrations has also been reported (25). Furthermore, it is not clear whether insulin plays a role in the regulation of lp(a) plasma concentrations by influencing its synthesis or catabolism. Therefore, we have assessed the effect of insulin on apo(a) synthesis.

The availability of cynomolgus monkey hepatocytes, one of the few *in vitro* culture systems that synthesize apo(a) endogenously, enabled us to study the effect of insulin on apo(a) synthesis. These cells were shown to be a good model to study the regulation of apo A-I and apo B-100 synthesis (26-28) and the production of apo(a) (28, 29). Our data indicate that physiological concentrations of insulin inhibit apo(a) synthesis by suppression of apo(a) mRNA levels.

METHODS

Materials

Materials used for the isolation of the simian hepatocytes were obtained from sources described previously (26-28). Materials used for the culturing were obtained from Sigma or as indicated otherwise. Insulin (Actrapid Penfill 1.5, 100 IE/ml) was obtained from Novo

Nordisk Pharmaceutique S.A., France.

Simian hepatocyte isolation and culture

Simian hepatocytes were isolated from livers of both male and female monkeys. The monkeys were 1.5 to 3 years old and were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The monkeys were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitis vaccine at this institute. The monkeys were fed Primate Diet G.O. (Hope Farms B.V., Woerden, The Netherlands) *ad libitum* with one or two pieces of additional fruit per day and were fasted overnight before sacrifice. The isolation and culture procedure was performed as described previously (26-28). Viability, based on the ability of hepatocytes to exclude trypan blue dye (0.11%), was 66-96%. Total cell yields varied from 0.74 to 2.3 x 10^9 viable cells. The cells were seeded on culture dishes at a density of 1.5 x 10^5 viable cells per square cm and maintained at 37°C in a 5% CO_2/95% air atmosphere. After a 24 h attachment period, the incubations without or with insulin were started in the RPMI 1640 medium (ICN), containing 10 µg/ml transferrin, 0.1 µM Cu^{2+}, 0.3 nM Se^{4+}, 50 pM Zn^{2+}, 10 µg/ml glucagon, 50 ng/ml EGF, 10 µU/ml growth hormone, 100 mU/ml prolactin, 5 µg/ml linoleic acid, 20 µg/ml BSA and 50 nmol/L dexamethasone as described by Gatmaitan *et al.*(30), and supplemented with 10% heat inactivated (30 min at 56°C) fetal calf serum (FCS) (Boehringer Mannheim), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 100 µg/ml kanamycin. The medium was renewed every 24 h. At the end of each incubation period the medium was collected and centrifuged for 30 seconds in an Eppendorf centrifuge at maximum speed to remove debris and detached cells. The supernatant was frozen in dry ice and stored at -20°C until apolipoproteins were measured. After the last incubation, the cells were washed three times with cold phosphate-buffered saline (PBS) and cellular protein was determined.

Apo(a) and other protein measurements

Total apo(a) concentrations were determined using the TintElize Lp(a) (Biopool AB, Umeå, Sweden). This ELISA uses polyclonal antibodies to human apo(a) both as catching and as detecting antibodies and detects in this manner free apo(a) as well as lp(a). The antibodies of this kit showed strong immunological cross-reactivity with lp(a) from cynomolgus monkeys with standard curves parallel to human lp(a), indicating a high level of homology between human and cynomolgus lp(a) (28).

Apo A-I and apo B-100 concentrations in the medium were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) with polyclonal antibodies to human apo A-I or human apo B-100, respectively, both as catching and detecting antibodies as described previously (26, 31). The standard curves for apo A-I and apo B-100 in human and

cynomolgus monkey sera and in medium of cultured cynomolgus hepatocytes were parallel, indicating that similar epitopes are recognized on apo A-I and apo B-100 of the two species (26).

Accumulation of albumin in the medium was measured by rocket immuno-electrophoresis using rabbit anti-human albumin (Dakopatts, Glostrup, Denmark) (31).

RNA isolation and hybridization

Total RNA was isolated from cynomolgus hepatocytes as described previously (26-28, 31). After washing the pellets with 70% (v/v) ethanol, RNA samples were dissolved in formamide, and the RNA concentration in each sample was determined spectrophotometrically. Equal amounts of total RNA (10 µg) from different incubations were fractionated by electrophoresis in an 0.8% agarose gel containing 0.27 mol/L formaldehyde. Subsequently the RNA was transferred to Hybond-N^+ (Amersham), in accordance with the manufacturer's instructions, and UV cross-linked. Probes, labeling conditions and hybridization were performed as described previously (26-28).

Apo(a) mRNA(s) were detected using a kringle IV synthetic double-stranded probe of 75 nucleotides with the sense sequence: GGGAATTCGAACCTGCCAAGCTTGGTC ATCTATGACACCACACTCGCATAGTCGGACCCCAGAATAAAGCTTGGG, based on the sequence published by McLean *et al* (32). This probe was labeled by the random primer method according to Megaprime™ DNA labelling systems, Amersham Life Science. After hybridization the blots were washed twice with 2x saline-sodium citrate (SSC/1%SDS) and twice with 1xSSC/1% SDS for 30 min at 65°C (1xSSC = 0.15 mol/L NaCl/ 0.015 mol/L sodium citrate, pH 7.0). The blots were exposed to a Fuji imaging plate type BAS-MP for 1 to 24 h. The relative amounts of mRNA were quantified using a phosphor-imager (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.08c.

Statistical analysis

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

RESULTS

Insulin decreases apo(a) secretion

In fig 1 the dose-dependency is shown of the effect of insulin on apo(a) secretion by cynomolgus monkey hepatocytes. The effects of insulin on the secretion of apo B-100, apo A-I and albumin are presented for comparison. Addition of increasing amounts of

insulin to the medium for 72 h resulted in a dose-dependent decrease of apo(a) secretion (fig 1). The effect was significant at a concentration of 10 nM insulin (-16%), whereas a suppression of 33% was reached at 1000 nM insulin. Apo B-100 secretion was decreased by 30%-36% at different insulin concentrations (fig 1). In these experiments apo A-I secretion tended to be higher compared to the control, but this was not significant (fig 1).

Albumin secretion was determined as control protein, since it is the most abundantly synthesized protein in hepatocytes. After 72 h of incubation with 10 or 100 nM insulin, no effect on the albumin secretion by the cynomolgus monkey hepatocytes was observed. Synthesis rate was 32 ± 9 µg albumin/ 24 h per mg cell protein for the control incubations and 29 ± 7 µg albumin/ 24 h per mg cell protein (mean ± S.E.M, n=3) for both the incubations with 10 and 100 nM insulin (fig 1).

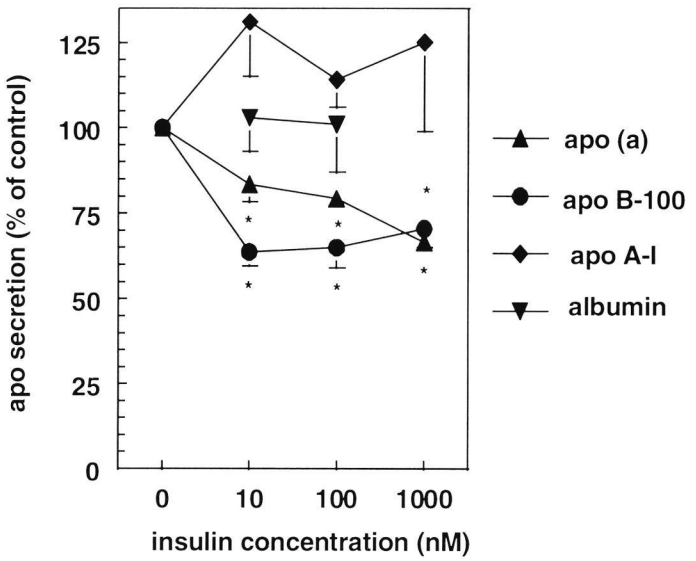

Fig 1. Dose-dependency of the effect of insulin on apolipoprotein synthesis
After a 24 h attachment and recovery period, hepatocytes were cultured for 72 h without or with different concentrations of insulin. Medium was renewed every 24 h. Apo(a), apo A-I and apo B-100 concentrations were determined as described in "Materials and Methods", and were normalized for cell protein in the dishes. Data are expressed as a percentage of control. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 3-4 independent hepatocyte isolations. Apo(a), apo B-100 and apo A-I production in control medium after 72 h of incubation for these four hepatocyte cultures was 381 ± 26, 515 ± 138 and 3501 ± 506 ng/24 h per mg cell protein.
* Indicates a significant difference ($P < 0.05$) between control and treated cells.

To assess the time-course of inhibition, cells were exposed to 10 and 100 nM insulin for different lengths of time between 24 and 96 h of culture. No significant change in apo(a) secretion was seen after the first 24 h incubation period. Maximal suppression of apo(a) secretion, when using 10 nM or 100 nM of insulin, was reached after 48 h of incubation (-21% and -26%, respectively) (fig 2a).

Apo B-100 secretion was only significantly decreased after 72 h of incubation with 10 nM insulin; during the first two incubation periods no significant effect was observed. Incubation with 100 nM insulin decreased apo B-100 secretion during all incubation periods (fig 2b). Apo A-I secretion did not change significantly during any of the incubation periods (fig 2c).

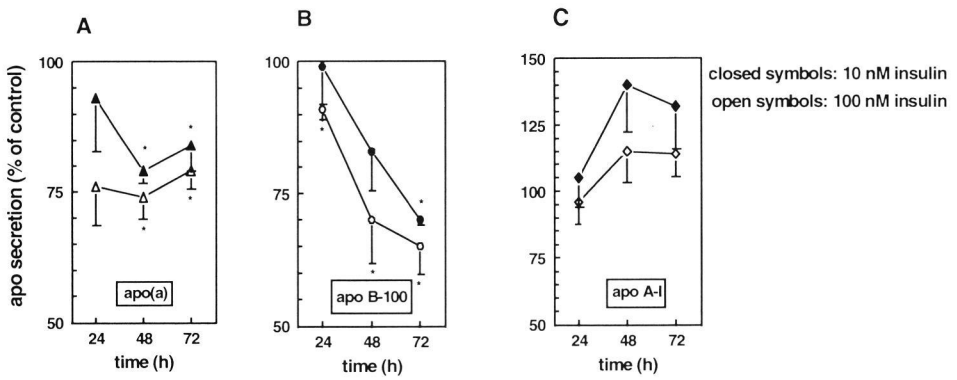

Fig 2. Time-dependency of the effect of insulin on apolipoprotein synthesis
Time effect of insulin on secretion of apo(a) (A), apo B-100 secretion (B), and apo A-I (C). After a 24 h attachment and recovery period, hepatocytes were cultured for 24, 48 or 72 h without or with 10 or 100 nM insulin. Medium was renewed every 24 h. Apo(a), apo A-I and apo B-100 concentrations were determined as described in "Materials and Methods", and were normalized for cell protein in the dishes. Data are expressed as a percentage of control. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 3-4 independent hepatocyte isolations.
Apo (a), apo B-100 and apo A-I production in control medium for these four hepatocyte cultures after 24 h of incubation was respectively 34.6 ± 11, 240 ± 54 and 1392 ± 372; after 48 h 129 ± 12, 368 ± 61 and 3098 ± 148; and after 72 h 381 ± 26, 515 ± 138 and 3501 ± 506 ng/24 h per mg cell protein.
* Indicates a significant difference ($P < 0.05$) between control and treated cells.

Insulin suppresses apo(a) mRNA levels

To assess at what level insulin inhibits apo(a) synthesis, apo(a) mRNA levels were determined (fig 3). Actin and GAPDH mRNA and 18S rRNA, which were not affected by insulin, were used as internal standards. Northern blot analysis showed that apo(a) mRNA was significantly decreased (- 29%) after incubation for 48 h with 100 nM insulin and when

corrected for GAPDH signal. The other control signals gave similar results. In the same culture experiments the apo B-100 and apo A-I mRNA levels did not change significantly when compared to the control and corrected for GAPDH, actin or 18S rRNA levels.

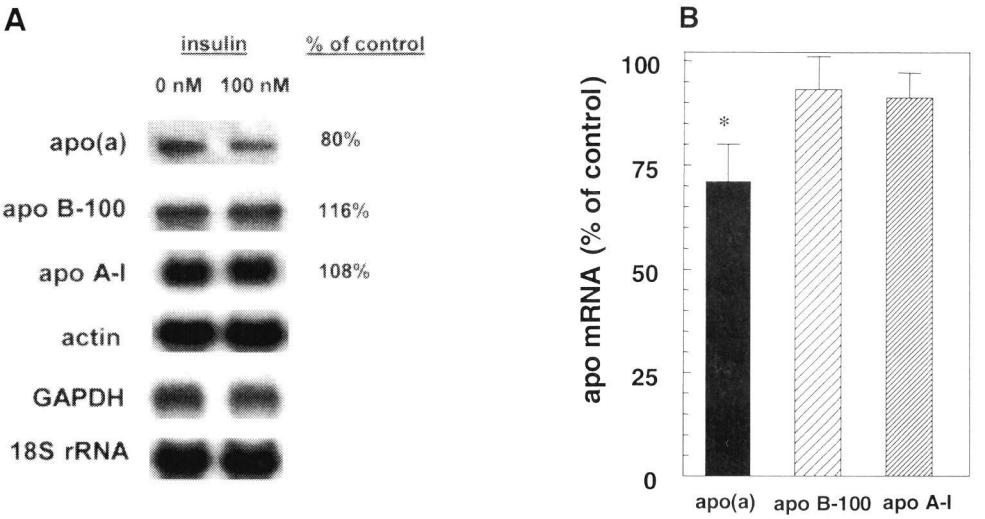

Fig 3. Effect of insulin on apo(a) mRNA levels

After incubation of the hepatocytes for 48 h with 100 nM insulin or control medium, total RNA was isolated and mRNA levels of apo(a), apo B-100, apo A-I, actin, GAPDH and 18S ribosomal RNA were assessed by Northern-blot hybridization. A) Phosphor Imager Scan of a representative Northern blot hybridized with ^{32}P-labeled probes for apo(a), apo B-100, apo A-I, actin, GAPDH, and 18S rRNA. All mRNA bands were quantified and the apo(a) signals were corrected for the actin, GAPDH and 18S signal, which were used as internal standards. B) Histogram of the effect of insulin on apo(a), apo B-100 and apo A-I mRNA .

Values are means ± S.E.M. of experiments with hepatocytes of 4 independent cultures.

DISCUSSION

In this study we showed that low concentrations of insulin already decreased apo(a) synthesis by a direct effect on cynomolgus monkey hepatocytes. Suppression of apo(a) synthesis was regulated at the (post)-transcriptional level, since apo(a) mRNA levels were decreased to the same extent as the apo(a) protein synthesis. Apo B-100 synthesis was also suppressed but its mRNA level was not affected by insulin. Apo A-I and albumin synthesis, measured as controls, did not change significantly under the influence of the hormone.

As far as we know this is the first study in which the influence of insulin on apo(a), apo A-I and apo B-100 synthesis is studied in an *in vitro* system which synthesizes all

apolipoproteins, including apo(a), endogenously. Our data on the effect of insulin on apo B-100 secretion (-35%) and mRNA compares well with several other *in vitro* studies using primary human and rat hepatocytes and HepG2 cells, and *in vivo* studies which show a similar down-regulation of apo B-100 by insulin (varying between 34% and 60%) (33-36). The decrease has been shown to be regulated post-transcriptionally (35-37) and is attributable, in part, to increased intracellular degradation of newly synthesized apo B-100 (37).

This study provides direct evidence that hepatic apo(a) synthesis is influenced by relevant (10 nM) insulin concentrations, considering the fact that hepatocytes degrade insulin extensively (38) and that the half-life of insulin, at least in primary rat hepatocyte cultures, is between 4 and 5 hours (36). Therefore, we also used relatively high initial insulin concentrations (100 nM, 1000 nM). Apo(a) secretion and mRNA were suppressed by about 20-35% when the primary hepatocytes were incubated with insulin. Although the effect was moderate, it was consistently seen in all independent cultures. Moreover, similar decreases in plasma lp(a) levels (about 30-35%) were found in *in vivo* studies, in which IDDM patients with a poor metabolic control were treated with insulin (12, 15), suggesting that the primary hepatocytes reflect the *in vivo* situation well. In contrast, no such effect was observed in NIDDM patients treated with insulin resulting in improved metabolic control (21, 22). The reason for this is not fully clear but differences in the metabolic state between IDDM and NIDDM may be responsible, since NIDDM patients already have high basal insulin levels. Further, the stage of the diabetic complications of the disease should also be kept in mind, because kidney diseases are known to elevate the lp(a) concentrations (39). Inherent to *in vivo* studies is the fact that different variables may influence the outcome of the investigations and thereby make it difficult to compare the studies. Further, by measuring plasma lp(a) levels no distinction can be made between an effect of insulin on the lp(a) synthesis and/or removal of lp(a) from the circulation.

Our results indicate that the regulation by insulin takes place at the apo(a) synthesis level, since apo(a) production by the hepatocytes was decreased. In addition, apo(a) mRNA levels were suppressed to a similar extent as the protein suggesting transcriptional regulation. In line with this contention, during the preparation of this manuscript, Suzuki *et al.* (40) reported in an abstract that apo(a) transcriptional activity was decreased by insulin, as shown by promoter-reporter studies with 1.4 kb of the 5' prime flanking region of the apo(a) gene in HepG2 cells. Thus, with respect to the level of regulation, suppression of apo(a) production clearly differs from apo B-100 secretion, which is inhibited at the co-translational level (35-37). In contrast to the decreasing effect of insulin on apo(a) and apo B-100 secretion, apo A-I secretion and mRNA remained unchanged in simian hepatocytes. Unchanged apo A-I and mRNA levels were also found in cultures of human jejunal explants treated with insulin (41). However, a decreased (42, 43) as well as an increased (44, 45) apo A-I synthesis was reported after treatment of rat hepatocytes and

HepG2 with insulin. These contradictory results may be due to the use of different species and culture models.

In conclusion, we found that relevant concentrations of insulin decrease apo(a) synthesis in primary cultures of cynomolgus monkey hepatocytes by suppression of mRNA levels. These data may provide an explanation for the increased levels of lp(a) as found in patients with poorly controlled insulin dependent diabetes mellitus and the normalisation thereof on insulin administration.

ACKNOWLEDGMENTS

This work was supported by The Netherlands Heart Foundation (grant no.92.328 to Diana M. Neele). We are grateful to Mr Jan Staal, Wim Vos and Joke van de Siepkamp (RIVM, Bilthoven) for their cooperation and zootechnical assistance and Natascha de Haas for technical assistance.

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Chapter 3

Mechanism of the slow induction of apolipoprotein A-I synthesis by retinoids in cynomolgus hepatocytes. Involvement of retinoic acid and retinoid X receptors.

J Lipid Res. 1997; 38: 2273-2280

Mechanism of the slow induction of apolipoprotein A-I synthesis by retinoids in cynomolgus hepatocytes. Involvement of retinoic acid and retinoid X receptors.

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Running title: Mechanism of the induction of apo A-I synthesis by retinoids.

ABSTRACT

We showed previously that retinoids stimulate apolipoprotein A-I (apoA-I) synthesis in cultured cynomolgus hepatocytes only after a 24-h lag phase. Here we report on the biochemical background of the slow response, the requirement for high retinoic acid concentrations and the involvement of different retinoid receptors.

The time course of the effect of 10 μM all-*trans* retinoic acid (at-RA) on apoA-I mRNA levels and protein secretion were comparable, i.e. minor increases were observed after a 24-h incubation and mRNA levels were increased 2.2- and 3.5-fold after 48 h and 72 h, respectively. In contrast, apoA-I gene transcription was already increased (2.6-fold) after a 4-h incubation with 10 μM at-RA.

At-RA disappeared rapidly from the cultures: after 2 h of incubation 40% of the added amount was left and after 24 h only 2%.

RARβ mRNA and gene expression were increased after incubation with 10 μM at-RA, whereas RARα and RXRα mRNA levels and expression remained unchanged. No transcriptional activity and mRNA for other retinoid receptors were detectable. Both RAR-selective (TTNPB) and RXR-selective (3-methyl-TTNEB) agonists induced apoA-I synthesis at 1 and 10 μM.

These results show that (i) the slow increase in apoA-I secretion is caused by a slow increase of its mRNA level; (ii) the apoA-I gene transcription in cynomolgus hepatocytes is induced rapidly by retinoids; (iii) the added at-RA disappeared rapidly from the cultures, explaining the necessity for high initial concentrations; (iv) RARα and/or RARβ and RXRα are involved in the activation of apoA-I expression by retinoids.

INTRODUCTION

Decreased plasma levels of high density lipoprotein (HDL) cholesterol are associated with an increased risk of the development of coronary heart disease (1,2). Apolipoprotein A-I (apo A-I), the major protein constituent of HDL, has been reported to be even more predictive than the HDL cholesterol (3,4). Apo A-I synthesis is reported to be associated with the HDL-cholesterol level in the circulation *in vivo* (5,6). Furthermore, transgenic mice overexpressing human apo A-I have high plasma HDL and apo A-I levels and are more resistant to atherogenic diets (7). These data indicate that upregulation of the apo A-I synthesis may be useful in increasing the HDL level, which may subsequently decrease the risk of development of atherosclerosis.

In a previous report (8) we showed that physiologically important modulators such as retinoids, including retinol (vitamin A), all-*trans* retinoic acid (at-RA) and its naturally occurring isomers, stimulate apo A-I synthesis in primary hepatocyte cultures from cynomolgus monkey. Similar data were obtained by others in HepG2 cells and rat hepatocytes (9-11). The stimulatory effect was observed only after prolonged incubation with retinoids and at relatively high concentrations (8). In the present study we further investigated the regulation of apo A-I secretion in cynomolgus monkey hepatocytes, and studied the biochemical background for the slow stimulation of apo A-I secretion and for the high initial retinoid concentrations needed.

It should be noted however, that the effects of retinoids in isolated cultured cells may differ from their effects in the more complex *in vivo* system in which modulation may be influenced by more factors. In contrast to the effect *in vitro*, retinoids showed a decreasing effect on apo A-I gene expression *in vivo* in rat (11), and decreased or unchanged HDL and apo A-I levels were reported in human studies with isotretinoin, the 13-cis isomer of at-RA (12,13).

It is well-known that retinoids can exert their biological action by binding to members of two families of nuclear retinoid receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), each family consisting of three receptor types; α, β and γ (for reviews:14-16). The effects of at-RA are mediated by the RARs, whereas the effects of 9-*cis* retinoic acid (9-*cis*-RA) are mediated by both RARs and RXRs (17,18). The retinoid receptors control the transcription of genes by interacting with retinoic acid receptor response elements (RAREs) in the promoter region of these particular genes. One of the genes with an upstream RARE is the gene encoding apo A-I (19). The RARE in the human apo A-I promoter has been shown to bind both RXR-RXR homodimers and RAR-RXR heterodimers, and to be essential for apo A-I promoter activity in transactivation assays, in which the respective receptors are overexpressed (19,20). However, which retinoid receptors are actively involved in the induction of apo A-I gene transcription in a

physiological context is unknown. As a first step to elucidate this, we studied the transcriptional activity and the mRNA levels of the genes coding for the different retinoid receptors. In addition, we applied the RAR-specific ligand TTNPB (21) and the RXR-specific ligand 3-methyl-TTNEB (22) to investigate the involvement of RARs and RXRs in the induction of apo A-I gene transcription.

We found that transcription of the apo A-I gene is rapidly activated by at-RA, but that this is followed by a slow, time-dependent increase of its mRNA level. Our data suggest that activation of apo A-I gene expression requires both RARα and/or RARβ and RXRα.

METHODS

Materials

All-*trans*-retinoic acid (at-RA) was purchased from Sigma Chemical Co. (St Louis, MO, USA). 9-*Cis*-retinoic acid (9-*cis*-RA) was a generous gift from Mr. P. Weber and Dr F. Schneider, Hoffmann-La Roche Ltd. (Basel, Switzerland). 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphtyl)-ethenyl] benzoic acid (3-methyl-TTNEB) and (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB) were kindly provided by Dr S. Karathanasis, American Cyanamid Company (Pearl River, NY, USA) and by Dr M. Issandou, Laboratoires Glaxo, Centre de Recherches (Les Ulis, France). Stock solutions of 10 mM were prepared in 100% dimethylsulphoxide (DMSO) (freshly prepared just before each experiment) and stored at -20 °C for no longer than 5 days. Immediately before use the retinoids were diluted in culture medium, in such a way that the final DMSO 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 (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 monkeys were fed Primate Diet G.O. (Hope Farms B.V., Woerden, The Netherlands) ad libitum with one or two pieces of additional fruit per day and were overnight fasted before sacrifice. The isolation procedure was exactly as described (8). For each independent culture experiment

hepatocytes from a single monkey were used. Total cell yields varied from 0.74 to 2.3 x 10^9 viable cells. Viability, based on the ability of hepatocytes to exclude trypan 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 (30 min at 56°C) fetal bovine serum (FBS), 2 mM L-glutamine, 20 mU/ml insulin (135 nM), 50 nM dexamethasone, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml kanamycin 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 retinoids were started with culture medium 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 DMSO, all incubations were performed with medium containing 0.1% (v/v) DMSO. The medium was renewed every 24 h.

Retinoid analysis

Retinoids were extracted from both cells and conditioned medium using the method described by Barua *et al.* (23). Retinoids were analyzed by reverse phase high performance liquid chromatography (HPLC) using a Spherisorb (Phase Separations) S5ODS2 C_{18} analytical column (25 x 0.46 cm), developed by a non-linear gradient modified from Eckhoff and Nau (24) 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 Absorbance Detector (Separations).

RNA hybridization

Total RNA was isolated from cynomolgus hepatocytes by the method of Chomczynski & Sacchi (25). 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 RNA 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). Analysis of the mRNA level of the different retinoid receptors was performed by Northern blot hybridization of total RNA, as described (8). 20 micrograms of total RNA were fractionated on a 0.8% (wt/vol) agarose gel containing 1 mol/L formaldehyde. The following fragments were used as probes in the hybridization experiments: 1.3 kb EcoRI-BglII fragment of a human RARα cDNA, a 1.4 kb BamH1-XbaI fragment of human RARβ cDNA, a 1.4 kb EcoRI-XbaI fragment of a mouse RARγ1 cDNA, in which a few bases have been changed in the A and F regions by site-directed

mutagenesis so that it encodes the human amino acid sequence; a 1.4 kb EcoRI-BglII fragment of a human RXRα cDNA, and a 1.4 kb BamH1-EcoRI fragment of human RXRγ. The RARα and RXRα were cloned in the laboratory of Dr J. Grippo, Hoffmann-La Roche, Nutley, U.S.A., and RARβ, RARγ, and RXRγ were cloned in the laboratory of Dr P. LeMotte, Hoffmann-La Roche, Basle, Switzerland. A 2.2 kb EcoRI fragment of the mouse RXRβ (H-2RIIBP) cDNA was kindly provided by Dr. K Ozato (26). Other probes were as described previously (8)

Nuclear run-off studies

Nuclear run-off studies were conducted as described (8,27). The [^{32}P]UTP-labeled RNAs from the nuclear run-off studies were hybridized with target DNA, being 5 μg of plasmid material containing DNA sequences of human apo A-I, different retinoid receptors, hamster actin or the empty PUC vector. After hybridization the filters were exposed to Hyperfilm MP (Amersham) for 2-5 days. Corrections for non-specific hybridization were made by subtracting the PUC signal. Quantitation of relative amounts of mRNA was conducted using the 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 mean ± SD.

RESULTS

Effect of retinoids on apo A-I mRNA levels and gene transcription

Previously we reported that retinoids increase the secretion of apo A-I time- and dose-dependently in primary cynomolgus hepatocytes. Incubation for 72 h with 1 and 10 μM at-RA resulted in a significant increase of apo A-I secretion (1.7- and 2.7- fold, respectively), whereas 0.1 μM at-RA had no effect. The effect of at-RA was observed only after a lag phase of 24 h (8). In the present paper, concomitantly with the secretion of apo A-I, the time course of the effect of at-RA and 9-*cis*-RA on the apo A-I mRNA level was investigated. Only minor changes were observed in the apo A-I mRNA level after 24 h of incubation with 10 μM at-RA or 10 μM 9-*cis*-RA (Fig. 1 and Table I). In the second 24 h incubation period the apo A-I mRNA level was 2.2-fold increased using at-RA and 2.3-fold with 9-*cis*-RA, followed by a further stimulation with at-RA (3.5-fold) and 9-*cis*-RA (3.8-fold) in the third 24 h period. As shown in Table IB the time course of the effect of these retinoids on the apo A-I secretion was comparable to that on the apo A-I mRNA level. at-RA and its 9-*cis*-isomer gave similar stimulatory effects.

This slow increase in the apo A-I mRNA level may result from a retarded transcriptional activation of the apo A-I gene. Therefore, we investigated the transcription

Table I. Time course of the effect of at-RA and 9-*cis*-RA on apo A-I mRNA levels in cynomolgus hepatocytes

A.

	apo A-I mRNA (% of control)		
incubation time:	24 h	48 h	72 h
standard medium	100	83 ± 12	126 ± 19
10 μM at-RA	146 ± 23	215 ± 18*	354 ± 33*
10 μM 9-*cis*-RA	142 ± 15*	226 ± 13*	379 ± 25*

B.

	apo A-I secretion (% of control)		
incubation period:	0-24 h	24-48 h	48-72 h
10 μM at-RA	123 ± 17	188 ± 27*	334 ± 33*
10 μM 9-*cis*-RA	128 ± 10*	219 ± 30*	304 ± 30*

After a 24 h attachment period cynomolgus hepatocytes were cultured for 24, 48 or 72 h on control medium with at-RA (10 μM), 9-*cis*-RA (10 μM) or without retinoids (control). **A) The effect of the retinoids on apo A-I mRNA levels**. Apo A-I and actin mRNA levels at the indicated time points were assessed by densitometric scanning of the intensity of the bands on the autoradiographs (as illustrated in a representative autoradiograph in Fig. 1). Actin mRNA level, which was not affected by retinoids, 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 in medium without retinoids, with analysis of 3 and 6 μg total RNA and are means ± SD of three independent hepatocyte isolations. **B) The effect of retinoids on the apo A-I secretion during the 24 h culture periods in the experiments described above**. Apo A-I secretion was measured by Elisa procedure (8), normalized for cell protein and is expressed as a percentage of control incubations without retinoids. Apo A-I production in control medium during the three consecutive 24 h culture periods was 780 ± 384, 698 ± 412 and 732 ± 368 ng/24 h per mg of cell protein, respectively. These values are means ± SD of duplicate incubations from three independent hepatocyte isolations.

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

rate of the apo A-I gene, as assessed by a run-off assay, in hepatocytes incubated for different time periods in the presence or absence of at-RA (10 μM). As shown in Fig. 2 the transcriptional activity of the apo A-I gene was already markedly increased (2.6-fold) after a 4 h incubation with at-RA. After 10 h of incubation a comparable enhancement of the gene expression was observed. However, the transcription rate decreased following

prolonged incubation: after 24 h of incubation stimulation was only 1.4-fold. To investigate whether the hepatocytes are equally able to respond to at-RA after prolonged culture, hepatocytes were incubated for 72 h in the presence of 10 μM at-RA, with renewal of the medium 8 h before isolation of the nuclei. This resulted in a similar extent of activation of apo A-I gene transcription (2.5-fold) as found after a 4 or 10 h incubation period (Fig. 2).

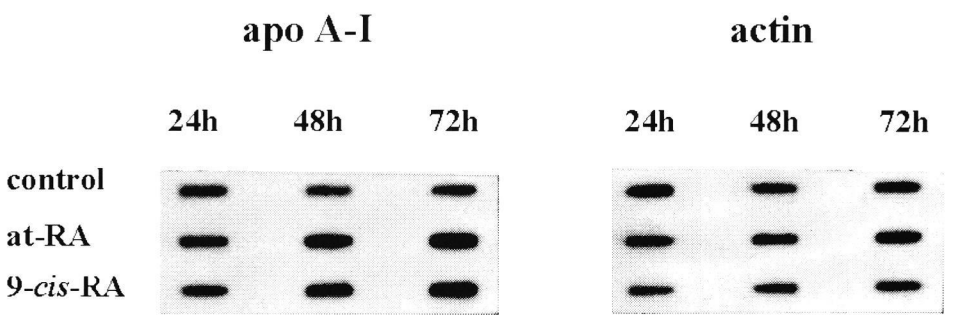

Fig 1. Time course of the effect of at-RA and 9-*cis*-RA on apo A-I mRNA level in cynomolgus hepatocytes

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, which had been incubated for 24, 48, or 72 h on standard medium without (control) or with 10 μM at-RA or 10 μM 9-*cis*-RA. Left and right autoradiograph show the results of the hybridization of apo A-I mRNA and actin mRNA, respectively. Actin mRNA was not affected by the retinoids. The experiment shown is representative of three independent experiments from which the data are shown in Table I.

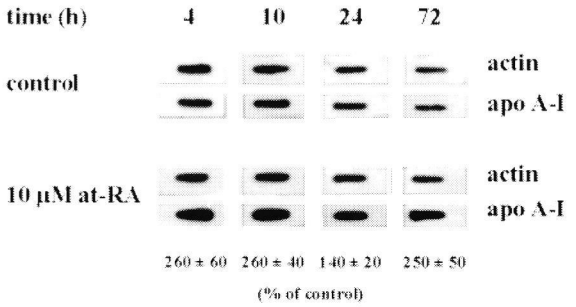

Fig 2. Transcriptional activation of the apo A-I gene in response to at-RA

After a 24 h attachment period cynomolgus hepatocytes were treated for 4, 10, 24 or 72 h without (control) or with 10 μM at-RA. For the 72 h incubation period the culture medium with or without at-RA was changed every 24 h and 8 h before isolation of the nuclei. At the bottom of the autoradiographs the transcriptional activity of the apo A-I gene in the presence of at-RA is listed, expressed as a percentage of the control incubation at the same time point. The density of the bands in the autoradiographs was assessed by densitometric scanning. The transcriptional activity of the apo A-I gene was normalized for the transcriptional activity of the actin gene, which was not affected by retinoids. Data shown are means ± SD for three independent hepatocyte isolations. All values differed significantly ($P < 0.05$) from the control incubation without retinoic acid.

Disappearance of added at-RA from the cultures

An explanation for the fall in apo A-I gene expression after a 24 h incubation with at-RA may be active metabolism by the hepatocytes of the ligand involved in the retinoid receptor-mediated increase in the apo A-I gene expression. Therefore, we measured the turnover of at-RA in the presence and absence of hepatocytes. As shown in Table IIA less than 2% of the added amount of at-RA could be recovered from cells and medium after a 24 h incubation. After a 2 h incubation nearly 60% of the added at-RA was already metabolized by the hepatocytes. When medium with at-RA, without hepatocytes, was incubated at 37°C we also observed the disappearance of at-RA (Table IIB), but at a much slower rate.

Table II. Disappearance of at-RA from the cultures.

		retinoid (nmol)			
		at-RA	9-*cis*-RA	13-*cis*-RA	total
A. Incubation with hepatocytes					
2 h	cells	0.69 ± 0.04	0.01 ± 0.00	0.11 ± 0.02	4.15 ± 0.30 (41.5 ± 3.0%)
	medium	2.74 ± 0.18	0.02 ± 0.00	0.58 ± 0.06	
4 h	cells	0.38 ± 0.20	0.01 ± 0.01	0.07 ± 0.01	3.31 ± 0.41 (33.1 ± 4.1%)
	medium	2.30 ± 0.15	0.04 ± 0.02	0.51 ± 0.06	
24 h	cells	0.06 ± 0.01	0.00 ± 0.00	0.02 ± 0.02	0.18 ± 0.09 (1.8 ± 0.9%)
	medium	0.07 ± 0.05	0.00 ± 0.00	0.03 ± 0.03	
B. Incubation without hepatocytes					
2 h	medium	9.01 ± 0.02	0.02 ± 0.00	0.91 ± 0.03	9.91 ± 0.05 (99.1 ± 0.5%)
4 h	medium	6.72 ± 0.06	0.06 ± 0.06	0.76 ± 0.05	7.54 ± 0.17 (75.4 ± 1.7%)
24 h	medium	4.23 ± 1.74	0.37 ± 0.19	1.20 ± 0.58	5.80 ± 2.21 (58.0 ± 22.1%)

Hepatocytes were allowed to attach for 24 h. 1 ml Medium containing 10 nmol at-RA was added to culture dishes (A) with hepatocytes (0.7 - 0.9 mg cell protein per dish) or (B) without hepatocytes. Media and cells were harvested after a 2, 4 or 24 h incubation at 37°C. The amount of at-RA together with 9-*cis*-RA and 13-*cis*-retinoic acid (13-*cis*-RA) was measured as described in the Methods section. The amount of the retinoids, expressed as a percentage of the starting amount of at-RA, is indicated in parentheses. For the incubation with hepatocytes the total amount of the retinoids is the total of cells and medium. Data shown are means ± SD for three independent isolations.

Effect of retinoids on mRNA levels and transcriptional activity of retinoid receptors

To investigate which retinoid receptors are involved in the transcriptional activation of the apo A-I gene, we studied the mRNA level and the transcriptional activity of the genes coding for the retinoid receptors in cynomolgus hepatocytes treated with at-RA or 9-*cis*-RA. Two RARα mRNAs (~3.6 and ~2.8 kb) were present in both control and treated hepatocytes (Fig. 3). Treatment with at-RA or 9-*cis*-RA did not affect the amount of the two RARα mRNA isoforms. In contrast, the RARβ mRNA (~ 3.4 kb) level, which was low in control hepatocytes, was induced about 6 to 8-fold by treatment with 10 μM at-RA and 4 to 5-fold by 10 μM 9-*cis*-RA relative to actin mRNA. No effect of retinoids on the RXRα mRNA (~ 5 kb) level was observed. In accordance with the mRNA levels, nuclear run-off assays showed clear gene expression of RARα and low expression of the RXRα gene; neither gene was induced by at-RA (Fig. 4). Gene expression of RARβ was very low in control cells, but was stimulated 1.8-fold by at-RA. No transcriptional activity and mRNA of the genes coding for RXRβ, RXRγ and RARγ were detectable in cynomolgus hepatocytes during culture.

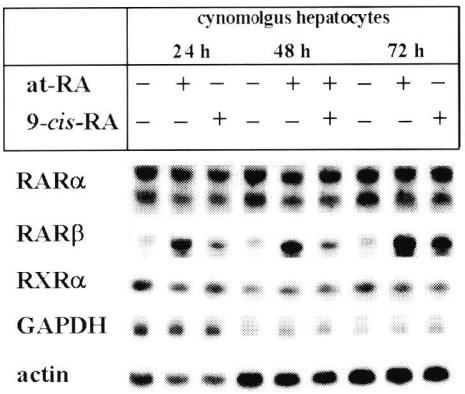

Fig 3. Effect of at-RA and 9-*cis*-RA on RARα, RARβ and RXRα mRNA levels in cynomolgus hepatocytes

Autoradiographs of a Northern blot hybridized with [^{32}P]-labeled probes for RARα, RARβ, RXRα, GAPDH and actin mRNA. Total RNA from cynomolgus monkey hepatocytes was isolated after incubation for 24, 48, or 72 h on standard medium without (-) or with (+) 10 μM at-RA or 10 μM 9-*cis*-RA, following a 24 h attachment period. 20 μg of RNA was applied on to the gel. Medium was renewed every 24 h. Incubation conditions are indicated at the top of the autoradiographs. The experiment shown is representative of three independent experiments. The density of the bands in the autoradiographs was assessed by densiometric scanning. After correcting for the actin mRNA level, used to assess differences in the amount of total RNA applied to the gels, RARα and RXRα mRNA levels did not change with respect to control upon incubation with either at-RA or 9-*cis*-RA. RARβ mRNA values as % of control at the same time point were 806 ± 181 and 416 ± 90 (24 h); 636 ± 135 and 398 ± 83 (48 h); 617 ± 149 and 460 ± 66 (72 h) after incubation with 10 μM at-RA and 10 μM 9-*cis*-RA, respectively. All data are means ± SD for three independent hepatocyte isolations. All data for RARβ mRNA differed significantly ($P>0.05$) from control incubations without retinoic acid.

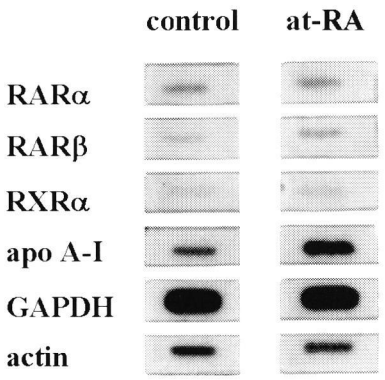

Fig 4. Effect of at-RA on transcriptional activity of RARα, RARβ and RXRα genes in cynomolgus hepatocytes

Autoradiographs of the run-off transcripts for RARα, RARβ, RXRα, apoA-I, actin and GAPDH of nuclei from simian hepatocytes treated without (control) or with 10 μM at-RA (RA) for 24 h. Four hours before harvesting the cells new medium with or without at-RA (10 μM) was added. The experiment shown is representative of three independent experiments. The density of the bands on the autoradiographs was assesssed by densiometric scanning. Transcriptional activity of the actin gene was used as an internal standard. RARα and RXRα gene expression did not change with respect to control upon incubation with at-RA. RARβ gene expression was increased being 1.80 ± 50% of the control value (n=3, P>0.05 with respect to control) upon incubation with 10 μM at-RA. All data are expresses as % of control and means ± SD for three independent hepatocyte isolations.

Effect of retinoid receptor-selective ligands on apo A-I synthesis

To assess which retinoid receptors are involved in the induction of apo A-I synthesis, hepatocytes were incubated with selective RAR- (TTNPB) and RXR- (3-methyl-TTNEB) agonists. Fig. 5 shows that both agonists induced apo A-I synthesis in a dose-dependent way and were equally active. Significant induction of apo A-I secretion was observed at 1 and 10 μM. In parallel experiments performed with the hepatocytes from the same isolations, at-RA showed a similar induction (166 ± 35% of control at 1 μM (n=6) and 232 ± 51% of the control at 10 μM (n=6)) as the agonists.

DISCUSSION

In this paper we have shown that unlike a rapid induction of apo A-I gene transcription by retinoids, apo A-I mRNA levels in cynomolgus hepatocytes are increased slowly and time-dependently, explaining the slow increase of apo A-I synthesis. at-RA disappeared very quickly from the cultures, explaining the necessity for high initial concentrations. Active transcription of RARα, RARβ and RXRα genes occurred and their mRNAs were present in cynomolgus hepatocytes, and RARβ mRNA levels and gene expression were induced by retinoids. Together with experiments using retinoid receptor-selective ligands these findings suggest the involvement of RARα and/or RARβ and RXRα in the stimulation of apo A-I synthesis.

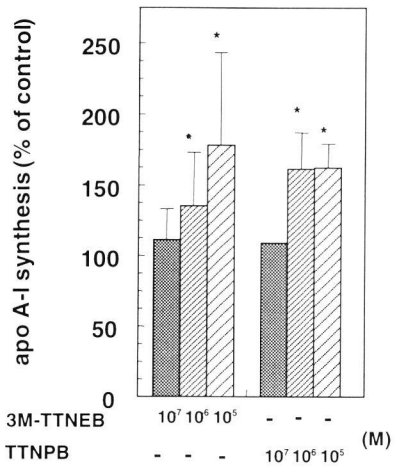

Fig 5. Effect of retinoid selective agonists 3-methyl-TTNEB and TTNPB on apo A-I synthesis in cynomolgus hepatocytes

After a 24-hour attachment period hepatocytes were incubated for three consecutive 24 h periods with increasing concentrations of 3-methyl-TTNEB (RXR-agonist) and TTNPB (RAR-agonist). Apolipoprotein A-I synthesis was determined in the last 24 h period as described in "Materials and Methods" and expressed as a percentage of control. The values are normalized for the amount of cell protein in the culture dishes, and are means ± SD of duplicate incubations from 3-6 independent hepatocyte isolations. * Indicates a significant difference ($P<0.05$) between control and treated cells.

The reason for the discrepancy between apo A-I gene transcription and mRNA level is not fully clear. The transcription rate of the apo A-I gene increased 2 to 3-fold shortly after addition of at-RA to cynomolgus hepatocytes, but decreased after prolonged incubation in the same medium. We have shown that in the presence of cynomolgus hepatocytes at-RA disappeared rapidly from the medium. This suggests that at longer incubation times without renewing of the medium, as was the case after a continuous 24 h-incubation period, retinoids may not be present at high enough levels for maximal induction of apo A-I gene expression. We suggest that apo A-I mRNA levels are increased in a pulsatory way and that the induction of RARβ may play a part in this process, possibly by extending the period of increased gene transcription. On the other hand, the possibility that the induction of RARβ is coincidental cannot be excluded. Nonetheless, the presence of a cellular pool of apo A-I mRNA, which is relatively stable and large with respect to the suggested small pulsatory increases, may explain the slow and time-dependent increase in apo A-I mRNA and secretion.

An increase in RARβ mRNA levels and transcriptional activity by at-RA and 9-*cis*-RA as found in cynomolgus monkey hepatocytes, has also been reported in various other cell-culture systems such as F9 teratocarcinoma cells and primary rat hepatocytes after treatment with retinoids (9, 28-30). This induction is consistent with the presence of a retinoic acid responsive element (RARE) in the promoter region of the RARβ gene (31).

Widom et al. (32) and Zhang et al. (20) reported that the retinoid receptor RXRα is important in the regulation of the apo A-I gene expression. This was concluded from experiments using cells transfected with RXRα. From our experiments with a RXRα-

selective agonist we can conclude that the RXRα receptor also appears to be involved in the regulation of the transcriptional activation of apo A-I by retinoids in primary cultures of cynomolgus monkey hepatocytes; no expression of RXRβ and RXRγ was detectable. However, the presence of the mRNAs coding for RARα and RARβ and the finding that a RAR-selective agonist induced the apo A-I secretion, indicates that these two receptors may also be involved in regulation. In agreement with the latter contention, it has been shown recently that both partners of the RAR/RXR heterodimer can activate transcription of RA-responsive genes (33-35). Whether retinoids act through activation of RXRα homodimers and/or via heterodimers of the RXRα receptor with the RARα or RARβ receptor remains to be elucidated.

In conclusion, we have shown that the slow increase in apo A-I secretion by retinoids in simian hepatocytes is not caused by a retarded transcriptional activation, but results from a slow time-dependent elevation of the apo A-I mRNA level. Transcriptional activation of the apo A-I gene by retinoids may occur via RXRα, possibly together with RARα or RARβ.

ACKNOWLEDGEMENTS

This work was supported by The Netherlands Heart Foundation (grant no. 87.035 to Allard Kaptein, 89.079 to Jaap Twisk and 92.328 to Diana M. Neele). Our grateful thanks are extended to Mr. Arie Timmerman (RIVM, Bilthoven) for his enthusiastic cooperation and excellent zootechnical assistance, and Mrs. Elly de Wit, Mrs. Karin Toet and Mr. Jos Grimbergen for skillful technical assistance.

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Chapter 4

Retinoids inhibit the synthesis of apolipoprotein(a) in cynomolgus monkey hepatocytes via involvement of the retinoic acid receptor (RAR). Absence of effect of oral retinoic acid *in vivo* in monkeys.

Submitted

Retinoids inhibit the synthesis of apolipoprotein(a) in cynomolgus monkey hepatocytes via involvement of the retinoic acid receptor (RAR). Absence of effect of oral retinoic acid *in vivo* in monkeys.

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Running title: Regulation of apolipoprotein(a) synthesis by retinoids

ABSTRACT

We have shown previously that retinoids induce apo A-I gene expression in cultured cynomolgus hepatocytes and do not have an effect on apo B-100 synthesis. In the present study the effect of retinoids on apo(a) synthesis in cultured hepatocytes and *in vivo* in rhesus monkeys was investigated.

Primary cynomolgus monkey hepatocytes synthesize and secrete apo(a). The addition of all-*trans* retinoic acid (at-RA) to the medium of the hepatocytes resulted in a dose- and time-dependent decrease of *de novo* apo(a) synthesis. Maximal inhibition was 54% after 72h of incubation with 10 µmol/L at-RA. Apo B-100 synthesis remained constant, while apo A-I synthesis was increased by 112% after treatment with 10 µmol/L at-RA for 72h, indicating that at-RA does not have a general effect on apolipoprotein synthesis in hepatocytes. 9-*Cis*-RA (-36%) and 13-*cis*-RA (-20%) also inhibited the apo(a) synthesis, whereas retinol was not active.

To investigate which retinoid receptors are involved in the inhibition of apo(a) synthesis, specific RXR- and RAR-ligands were used. 3-methyl-TTNEB, a specific RXR-agonist, did not have an effect on the apo(a) synthesis, whereas incubation with TTNPB, a specific RAR-agonist, resulted in a decrease of 34%. Steady state apo(a) mRNA levels were decreased by 42% after incubating the cells for 48 h with 10 µmol/L at-RA, indicating that the decreased synthesis is regulated at the (post)-transcriptional level. However, *in vivo*, plasma lp(a) levels did not change significantly after treatment of monkeys with 1 mg/kg at-RA per day for 4 weeks.

We conclude that retinoids down-regulate apo(a) synthesis and mRNA via involvement of RAR and not the RXR-homodimer in cynomolgus hepatocytes, but have no effect on lp(a) levels *in vivo*.

INTRODUCTION

Lipoprotein(a) (lp(a)) is a lipoprotein particle in which apolipoprotein(a) is attached to low density lipoprotein (LDL) via a disulfide bridge. Lp(a) plays an important role in both atherogenesis and fibrinolysis and high plasma levels of lp(a) are considered to be a risk factor for vascular diseases (for reviews:1-5). Although plasma levels of lp(a) are almost entirely determined by inheritance, a limited number of drugs are reported to influence the level of lp(a) in humans. Nicotinic acid (6) and (sex) steroids (7,8) are reported to decrease the plasma level of lp(a). Data on the effect of fibrates on lp(a) levels are still conflicting; no effect on lp(a) levels (9) as well as a decrease of lp(a) serum level (10, 11) and an increase of lp(a) serum level is reported (12). Down-regulation of apo(a) may be a way to decrease lp(a) levels, thereby reducing the risk of developing cardiovascular diseases.

There is a limited number of cell-culture-systems in which regulation of apo(a) or lp(a) synthesis can be studied. Primary cultures of monkey hepatocytes is one of the few cell models which synthesizes and secretes apo(a) (13). We have used primary hepatocytes cultures from cynomolgus monkey (Macaca fascicularis), because these cells were shown to be a good model for studying regulation apo A-I and apo B-100 synthesis (14,15) and the production of apo(a) (16). In addition, the cynomolgus monkey has been shown to be a suitable model for studying lipid and lipid metabolism (16-18) and the development of atherosclerosis (19).

We have previously shown that all-*trans* retinoic acid (at-RA), its natural isomers and retinol (vitamin A) increase apo A-I synthesis and do not affect apo B-100 synthesis in primary cultures of cynomolgus monkey hepatocytes (14,15). These results were confirmed by others showing an increase in apo A-I synthesis in HepG2 cells incubated with retinoids (20,21). Recently also an increase in apo A-II expression in HepG2 cells and human hepatocytes was reported (22). It should be noted however, that the effects of retinoids in isolated cultured cells may differ from their effects in the more complex *in vivo* system in which modulation may be influenced by more factors. In contrast to the stimulatory effect *in vitro*, retinoids showed a decreasing effect on apo A-I gene expression and no effect on apo A-II gene expression *in vivo* in rat (23), and decreased or unchanged HDL and apo A-I levels were reported in human studies with isotretinoin, the 13-cis isomer of at-RA (24,25). Furthermore, apo CIII mRNA is shown to be induced in the intestine of the rat but not in the liver after vitamin A treatment (26). Recently, increased levels of lp(a) were found in two acute promyelocytic leukemia patients treated with at-RA. In the same paper it was shown that at-RA could stimulate apo(a) promoter activity when a relatively short promoter-reporter construct was transfected in HepG2 cells (27). In view of the multiple and divergent effects of retinoids on apolipoprotein synthesis *in vivo* and *in vitro* we investigated the effect of retinoids on the synthesis of apo(a) in cultured cynomolgus hepatocytes and in rhesus monkey *in vivo*.

The effects of retinoids are mediated by the two families of nuclear retinoid receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The two receptor types, each of which consists of 3 subclasses (α,β and γ), are members of the thyroid/steroid receptor superfamily and function as ligand dependent transcription factors. The retinoid receptors bind as RAR/RXR heterodimers or RXR/RXR homodimers to specific retinoic acid response elements (RAREs) in target genes (for reviews: 28-30).

The present study shows that apo(a) synthesis and mRNA levels are significantly decreased when simian hepatocytes are incubated with retinoids. By using specific ligands for the RAR, TTNPB, and the RXR, 3-methyl-TTNEB, (31,32) we were able to demonstrate that RAR and not the RXR homodimer is involved in the suppression of apo(a) synthesis. In contrast, no significant changes in lp(a) levels were found *in vivo* in rhesus monkeys treated with at-RA.

METHODS

Materials

All-*trans* retinoic acid (at-RA), retinol and 13-*cis* retinoic acid (13-*cis*-RA) were purchased from Sigma Chemical Co (ST Louis, MO, USA). 9-*Cis* retinoic acid (9-*cis*-RA) was a generous gift from Mr P. Weber and Dr F. Schneider, Hoffmann-La Roche Ltd. (Basel, Switzerland). 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)-ethenyl] benzoic acid (3-methyl-TTNEB) and (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB) were kindly provided by Dr S. Karathanasis, American Cyanamid Company (Pearl River, NY, USA) and by Dr M. Issandou, Laboratoires Glaxo, Centre de Recherches (Les Ulis,France). Retinoids are light-sensitive, therefore the stock solutions, which were prepared in DMSO and preserved at -20°C in the dark, were diluted in culture medium just before it was used to renew the medium of the cells. All incubations, control and with retinoids at various concentrations, were performed with medium containing 0.1% (v/v) DMSO. The experiments were performed in subdued light. Materials used for the isolation and culturing of the simian hepatocytes were obtained from sources described previously (14).

In vivo study with rhesus monkeys

A small *in vivo* study with 6 rhesus monkeys (Macaca Mulatta) was performed within the Biomed II PRIMEFITT programme, granted by the European Union and coordinated by a colleague, Dr J.J. Emeis, at our institute. Although the study was designed for another purpose, we were allowed to measure total cholesterol, triglyceride, apo B-100 and lp(a) levels in the plasma of the monkeys. The rhesus monkeys weighing 4.4 to 5.9 kg were

between 3 and 6 years old and were fed a standard diet. Three monkeys were treated orally with 1 mg/kg at-RA per day (dissolved in sunflower oil and spread on a piece of bread) for 4 weeks and 3 with placebo (sunflower oil and bread only). Fasting blood samples were obtained once before treatment was initiated and at 1, 2 and 4 weeks thereafter, then one wash-out sample after 2 weeks was obtained. EDTA plasma was prepared and frozen in liquid nitrogen immediately after the samples were collected and stored at

-80°C. Levels of total plasma cholesterol and triglycerides were determined using commercially available kits (# 125512 and # 701904, respectively, Boehringer Mannheim GmbH). Apo B-100 and lipoprotein(a) were measured by rate immunonephelometry using an automated Beckman 'Array' analyser (Beckman Instruments) as described previously (33)

Cynomolgus monkey hepatocyte isolation and culture

Simian hepatocytes were isolated from livers of both male and female monkeys. The monkeys were 1.5 to 3 years old and were obtained from the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, The Netherlands. The monkeys were bred at the RIVM and served as donors for kidneys used in the production of poliomyelitis vaccine at this institute. The monkeys were fed Primate Diet G.O. (Hope Farms B.V., Woerden, The Netherlands) ad libitum with one or two pieces of additional fruit per day and were fasted overnight before sacrifice. The isolation procedure was performed as described previously (14). Viability, based on the ability of hepatocytes to exclude trypan blue dye (0.11%) was 66-96%. Total cell yields varied from 0.74 to 2.3 x 10^9 viable cells. The cells were seeded on culture dishes at a density of 1.5 x 10^6 viable cells per square cm and were maintained for the first 24 h in Williams E medium supplemented with 10% heat inactivated (30 min at 56°C) fetal calf serum (FCS) (Boehringer Mannheim), 2 mmol/L L-glutamine, 20 mU/mL insulin (135 nmol/L), 50 nmol/L dexamethasone, 100 U/mL penicillin, 100 µg/mL streptomycin and 100 µg/mL kanamycin at 37°C in a 5% CO_2/95% air atmosphere. After 14-16 h the non-adherent cells were washed from the plates, using the same culture medium as described above. Twenty-four hours after seeding, the incubations with the retinoids were started in the same culture medium, but with a lower insulin concentration, 10 nmol/L instead of 135 nmol/L. The medium was renewed every 24 h. At the end of each incubation period the medium was collected and centrifuged for 30 seconds in an Eppendorf centrifuge at maximum speed to remove debris and detached cells. The supernatant was frozen in dry ice and stored at -20°C until the apolipoproteins were measured. After the last incubation, the cells were washed three times with cold phosphate-buffered saline (PBS) and cellular protein was determined.

Apo(a), apo A-I and apo B-100 ELISA

Total apo(a) concentrations were determined using the TintElize Lp(a) (Biopool AB, Umeå, Sweden). This ELISA uses polyclonal antibodies to human apo(a) both as catching and as detecting antibodies and detects in this manner free apo(a) as well as lp(a). The antibodies of this kit showed strong immunological cross-reactivity with lp(a) from cynomolgus monkeys with standard-curves parallel to human lp(a), indicating a high level of homology between human and cynomolgus lp(a), in accordance with Makino *et al* (34) and Azrolan *et al* (16). The lp(a) Innotest ELISA was used to measure solely the lipoprotein lp(a). The latter ELISA only detects apo(a) in the lipoprotein lp(a) and no free apo(a), since monoclonal anti-apo(a) is used as catching antibody and polyclonal anti-apo B-100 as detecting antibody.

Apo A-1 and apo B-100 concentrations in the medium were measured using a sandwich enzyme-linked immunosorbent assay (ELISA) with polyclonal antibodies to human apo A-I or human apo B-100, respectively, both as catching and detecting antibodies as described previously (35). The standard curves for apo A-I and apo B-100 in human and cynomolgus monkey sera and in the medium of cultured cynomolgus hepatocytes were parallel, indicating that similar epitopes on apo A-I and apo B-100 of the two species are recognized.

Analysis of apo(a), apo A-I and, apo B-100 containing lipoproteins

Analysis of apo(a)-, apo A-I-, apo B-100-containing lipoproteins secreted by primary hepatocytes was performed as previously described by Kaptein *et al* (36). Briefly, culture medium was collected at the end of a 48 h incubation period with simian hepatocytes cultured in lipoprotein depleted medium. Lipoprotein depleted serum (LPDS) was prepared from heat inactivated FCS by density gradient ultracentrifugation as described (36). After the medium was harvested, protease inhibitors were added and density-gradient ultracentrifugation was performed (36). Subsequently, the gradient was fractionated and the density was measured. The fractions were dialyzed, and the lp(a) (Innotest ELISA), apo(a) (Tint Elize Lp(a), Biopool), apo A-I and apo B-100 concentrations in the fractions were determined by ELISA procedure.

Metabolic labeling and immunoprecipitation

The amount of newly synthesized proteins, in particular apo(a), was investigated by measuring the incorporation of 50 µCi/ml Tran [^{35}S] label (methionine and cysteine) (ICN, USA) into *de novo* synthesized proteins which were secreted in the culture medium. Total apo(a) (free and bound in lp(a)) was immunoprecipitated from 750 µl medium by incubating it overnight at 4°C with polyclonal anti-lp(a) directed specifically against the apo(a) part of lp(a) and not cross-reacting with apo B-100 (Behring Diagnostica, Marburg).

By using this polyclonal anti-lp(a), part of apo B-100 present in the lp(a) particle does coprecipitate. Protein G agarose (75µl of a 50% suspension) was added and samples were further incubated for 1 h at room temperature while being shaken. To reduce non-specific background protein G was pre-incubated for 30 min with unlabeled conditioned culture medium and washed 2 times with phosphate-buffered saline (PBS). The control medium containing all metabolically radiolabeled proteins and the immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions by the method of Laemmli, with a resolving gel containing a gradient of 3% to 10% (wt/vol) acrylamide and a stacking gel of 3.5% (wt/vol) acrylamide. Protein molecular-mass standards (Biorad, Richmond, CA, USA) were used for calibration of the gel. The gels were dried and exposed to a Fuji imaging plate type BAS-MP for 24 h. The relative amounts of newly synthesized protein were quantified using a phosphor-image (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.08c.

RNA isolation and hybridization

Total RNA was isolated from cynomolgus hepatocytes by the method of Chomczynski and Sacchi (37). After the pellets were washed with 70% (v/v) ethanol, RNA samples were dissolved in formamide, and the RNA concentration in each sample was determined spectrophotometrically, with the assumption that one A_{260} unit corresponds to 40 µg RNA/mL. Equal amounts of total RNA (10 µg) from different incubations were fractionated by electrophoresis in an 0.8% agarose gel containing 0.27 mol/L formaldehyde. Subsequently the RNA was transferred to Hybond-N^+ (Amersham), in accordance with the manufacturer's instructions, and UV cross-linked. Probes, labeling conditions and hybridization were performed as described previously (14).

Apo(a) mRNA(s) were detected using a kringle IV synthetic double-stranded probe of 75 nucleotides with the sense sequence: GGGAATTCGAACCTGCCAAGCTTGGTCAT CTATGACACCACACTCGCATAGTCGGACCCCAGAATAAAGCTTGGG, based on the sequence published by McLean *et al* (38). This probe was labeled by the random primer method according to Megaprime™ DNA labelling systems, Amersham Life Science. After hybridization the blots were washed twice with 2x saline-sodium citrate (SSC)/1%SDS and twice with 1xSSC/1% SDS for 30 min at 65°C (1xSSC = 0.15 mol/L NaCl/ 0.015 mol/L sodium citrate, pH 7.0). The blots were exposed to a Fuji imaging plate type BAS-MP for 1 to 24 h. The relative amounts of mRNA were quantified using a phosphor-imager (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.08c. The blots were re-used after they were stripped with 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 1% SDS pH 7.5.

Statistical analysis

For the *in vitro* data statistical significance of differences was calculated by Student's t-test for paired data with the level of significance being $P \leq 0.05$ or as indicated otherwise.

To evaluate the effect of retinoid treatment *in vivo,* values before and after placebo and retinoic acid treatment, changes in both groups, and the area under the curve were compared using the Mann-Whitney-test. Percentage changes were calculated as 100 x [(posttreatment value - pretreatment value) / pretreatment value]. Values are presented as mean ± SEM. Statistical analysis was performed using SPSS for Windows. Values of $P \leq 0.05$ were considered significantly different.

RESULTS

Characterization of lipoprotein production by primary cultures of hepatocytes from cynomolgus monkey

Primary cultures of hepatocytes from cynomolgus monkey accumulate apo(a), apo A-I and apo B-100 in the culture medium as detected by specific ELISAs for these proteins. To test in which form the apolipoproteins accumulate i.e. as free proteins or in lipoproteins, medium was separated by density gradient ultracentrifugation (Fig 1). Apo(a) was found in a lipoprotein particle with a buoyant density of serum lp(a) (1.04-1.10 g/mL) and at the bottom of the density gradient (>1.21 g/mL) as a free protein.

By using two different apo(a) ELISAs (see section Materials and Methods) the percentage of apo(a) bound to apo B-100 in a lipoprotein(a) particle was found to be 40% ± 4% in the first 48 h culture period and 56% ± 6% in the second 48 h culture period for 4 independent cultures. The total apo(a) production in control medium during the first incubation period was 53 ± 18 ng/24 h per milligram of cell protein (mean ± SD), 80 ± 55 during the second period and 124 ± 70 (range, 68-210 ng/24 h per milligram of cell protein) during the third incubation period in 4 independent hepatocyte cultures. The genotypes of the cynomolgus monkeys differed from S_2 to S_5 (data not shown).

For comparison, apo A-I was found in the density range of 1.12-1.25 g/mL, indicating that apo A-I is present in the culture medium in lipoprotein particles with the buoyant density of HDL but also in a lipid-poor or lipid-free form. Most apo A-I was found in the lipid-poor or free fraction. Most apo B-100 was present in a lipoprotein particle with the buoyant density of of LDL (1.02-1.06 g/mL) and a small amount of apo B-100 was found in a particle with the buoyant density of VLDL (<1.009 g/mL). Apo A-I and apo B-100 production in control medium during the third 24 h incubation period (from 72-96 h of culture age) for 7 independent hepatocyte cultures was 1542 ± 809 (range, 795-2888) and 555 ± 251 (range, 289-1015) ng/24 h per milligram of cell protein, respectively. The apo

A-I and B-100 production of primary cultures of cynomolgus monkeys remained nearly constant during the culture periods.

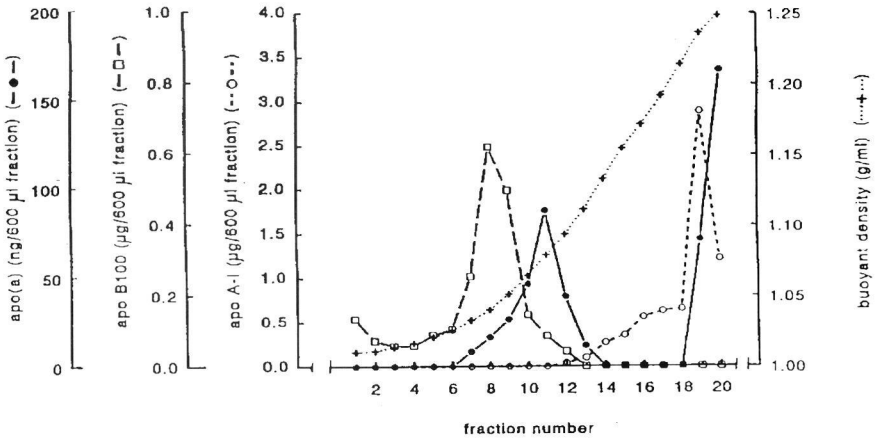

Fig 1. Density gradient pattern of apo(a)-, apo B-100- and apo A-I- containing lipoproteins in the culture medium of cynomolgus monkey hepatocytes
Cynomolgus monkey hepatocytes were cultured for 48 h in standard medium with lipoprotein deficient FCS, after a 48 h preincubation time in the same medium. 4 mL medium was used for density gradient ultracentrifugation as described in "Materials and Methods". Apolipoprotein concentrations in the fractions were determined by ELISA after dialysis against PBS containing 0.1% casein and 2.5 mmol/L EDTA.

Dose-dependency and time course of the effect of at-RA on the apo(a) synthesis

In Fig 2a the dose-dependency is shown of the effect of at-RA on apo(a) synthesis by cynomolgus monkey hepatocytes. The effects of at-RA on the synthesis of apo A-I and apo B-100 are presented for comparison. The addition of increasing amounts of at-RA to the medium resulted in a dose-dependent decrease of apo(a) synthesis. The effect was significant after the addition of 1 µmol/L at-RA for 72 h (-25%). Maximal suppression of 54% was observed after incubation with 10 µmol/L at-RA for 72 h. The amount of apo(a) contained in lp(a) in the medium was decreased to the same extent (-42%) as the total amount of apo(a). In these experiments apo A-I synthesis was increased by 66% with 1 µmol/L at-RA and by 125% with 10 µmol/L. Apo B-100 synthesis was not significantly affected at any of the concentrations.

Time dependency of the effect of at-RA is shown in Fig 2b. No significant change in apo(a) synthesis was seen after the first 24 h incubation period. After 48 h of incubation apo(a) synthesis was decreased by 38%, and the effect of at-RA was even more

pronounced after 72 h of incubation (-54%). Apo A-I synthesis was slightly, but significantly increased (+24%) after 24 h with 10 µmol/L at-RA, and by 79% and 125% after 48 h and 72 h of incubation, respectively. Apo B-100 synthesis did not change significantly during the incubation periods.

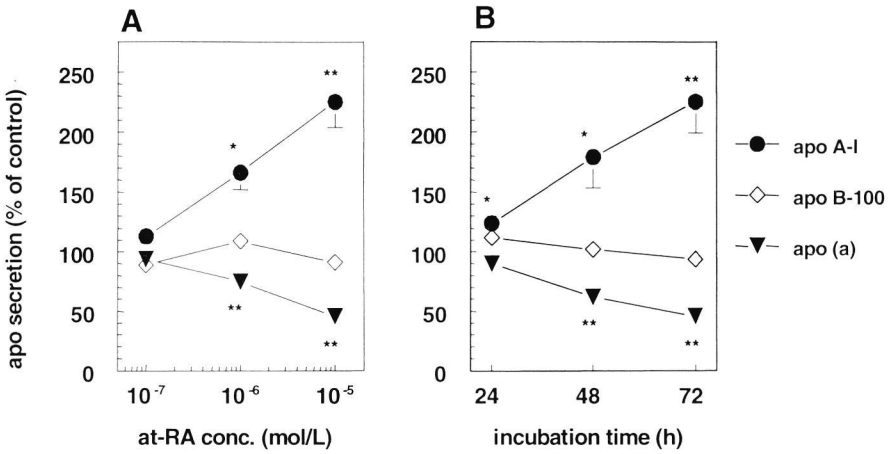

Fig 2. Dose- and time-dependency of the effect of at-RA on the apolipoprotein synthesis of cynomolgus monkey hepatocytes

After a 24 h attachment and recovery period, hepatocytes were cultured for 24, 48 or 72 h with or without different concentrations of at-RA. Medium was renewed every 24 h. Apo(a), apo A-I and apo B-100 concentrations were determined as described in "Materials and Methods", and were normalized for cell protein in the dishes. Data are expressed as a percentage of control.

A) Hepatocytes were incubated for 72 h with various concentrations of at-RA. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 4-7 independent hepatocyte isolations.

B) Hepatocytes were incubated for 24 h, 48 h and 72 h with 10 µmol/L at-RA. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 4-6 independent hepatocyte isolations.

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

** Indicates a significant difference ($P < 0.005$) between control and treated cells.

Effect of at-RA on *de novo* synthesis of apo(a)

The decrease in apo(a) secretion after at-RA incubation was investigated in more detail by measuring the incorporation of [^{35}S]-methionine and cysteine in newly synthesized and secreted apo(a). Metabolically labeled proteins in the medium and immunoprecipitated apo(a) were analyzed on SDS-PAGE (Fig 3). As is shown, apo(a) synthesis is decreased after retinoid incubation which is both visible in the lanes containing total medium and the immunoprecipitated apo(a). Apo A-I synthesis is increased, while apo B-100 remains similar after 72 h of incubation with 10 µM at-RA.

Effect of different natural retinoids on apo(a) synthesis

In order to investigate the effect of other naturally occurring retinoids on apo(a) synthesis, incubations with these retinoids were performed. In addition to apo(a), apo B-100 and apo A-I were determined in the same experiments for comparison. The results are shown in Table 1. 9-*Cis*-RA and 13-*cis*-RA, which are both natural isomers of at-RA, gave similar results to those obtained with at-RA. Apo(a) synthesis was suppressed by 36% and 20%, respectively and the apo A-I synthesis was elevated by 129% and 82%, respectively. Retinol (vitamin A), the precursor of at-RA, did not change the apo(a) synthesis, whereas apo A-I synthesis was induced by 66%. The retinoids did not change apo B-100 synthesis, except for 9-*cis*-RA, which increased apo B-100 slightly but significantly.

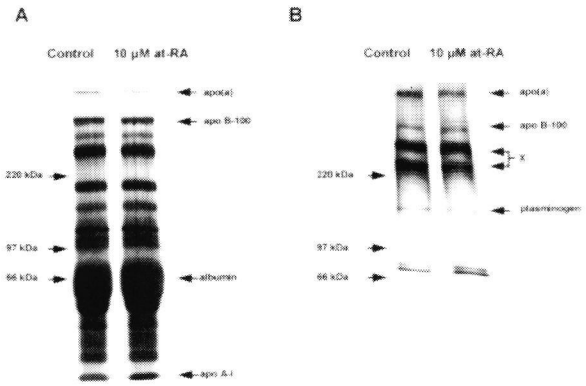

Fig 3. Effect of at-RA on *de novo* synthesis of apo(a)
A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of metabolically radiolabeled proteins in the culture medium of cynomolgus monkey hepatocytes. Hepatocytes were incubated for 24 h in standard medium with or without 10 µmol/L at-RA containing 50 µCi/ml Tran [^{35}S] label (methionine and cysteine) after two 24-hour preincubation periods in the same medium without labeled methionine and cysteine. B) Immunoprecipitation of apo(a) from metabolically radiolabeled culture media (as described above) after separation by SDS-PAGE. Molecular-mass standards are indicated on the left side and the positions of apo(a), apo B-100, albumin and apo A-I on the right side. X represents non-specifically precipitated protein.

Effect of retinoid receptor-specific ligands on apo(a) synthesis

To assess which retinoid receptors are involved in the inhibition of apo(a) synthesis, hepatocytes were incubated with retinoid receptor-specific ligands (31,32). Fig 4 shows the effects of the retinoid X receptor (RXR)-specific ligand 3-methyl-TTNEB, and of the retinoic acid receptor (RAR)-specific ligand TTNPB, on apo(a) synthesis. Apo(a) synthesis was inhibited by 34% when the hepatocytes were incubated for 72 h with 10 µmol/L of the

Table I. Effect of various retinoids on apo(a), A-I and B-100 synthesis

Compound added to the medium		synthesis (% of control) apo(a)	apo A-I	apo B-100
10 µmol/L at-RA	:	46 ± 10 **	212 ± 56 **	91 ± 4
10 µmol/L 9-cis-RA	:	64 ± 6 **	229 ± 29 *	141 ± 2 *
10 µmol/L 13-cis-RA	:	80 ± 1 **	182 ± 16 *	136 ± 39
10 µmol/L retinol	:	102 ± 14	166 ± 20 *	120 ± 15

Primary cynomolgus monkey hepatocytes were incubated for 72 h with different retinoids. Apo(a) synthesis was determined as described in the Materials and Methods section and expressed as a percentage of control. Apo A-I and apo B-100 were determined for comparison. The values are normalized for the amount of cell protein in the culture dishes. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 3-6 independent hepatocyte isolations.

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

** Indicates a significant difference ($P < 0.005$) between control and treated cells.

RAR-agonist. Interestingly, incubation with 10 µmol/L of the RXR-agonist did not inhibit the apo(a) synthesis in cynomolgus monkey hepatocytes. In the same incubations apo A-I synthesis was dose-dependently increased with 3-methyl-TTNEB, reaching a maximal induction of 78% at 10 µmol/L (data not shown). These data indicate that the RXR-agonist is active in simian hepatocytes and that the RXR-homodimer is not involved in the suppression of apo(a) synthesis. On the contrary, the RAR appears to be involved in the regulation of apo(a) synthesis by retinoids.

To investigate whether the inhibitory effect of TTNPB on apo(a) synthesis could be overcome or counteracted by incubation with the RXR-agonist, hepatocytes were incubated simultaneously with increasing amounts of TTNPB together with a high concentration of 3-methyl-TTNEB. This resulted in an inhibition of apo(a) synthesis which was similar to the decrease in cells incubated with TTNPB alone, indicating that binding of 3-methyl-TTNEB to the RXR receptor does not influence the effect of the RAR-agonist on apo(a) synthesis.

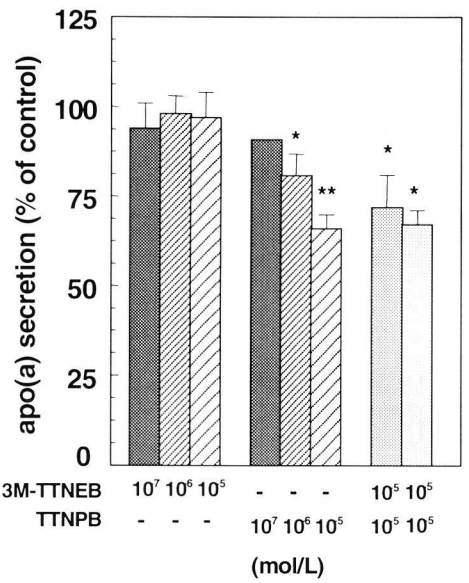

Fig 4. Effect of retinoid receptor-specific ligands on apo(a) synthesis

Primary cynomolgus monkey hepatocytes were incubated for three consecutive 24 h periods with increasing concentrations of TTNPB (RAR-agonist) and 3-methyl-TTNEB (RXR-agonist), and with a combination of both compounds. Apolipoprotein(a) synthesis was determined in the last 24 h period as described in" Materials and Methods" and expressed as a percentage of control. The values are normalized for the amount of cell protein in the culture dishes, and are means ± S.E.M. of duplicate incubations of hepatocytes from 3-6 independent hepatocyte isolations.

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

** Indicates a significant difference ($P < 0.005$) between control and treated cells.

Effect of at-RA on apo(a) mRNA levels

mRNA levels were determined to investigate at which level retinoids inhibit apo(a) synthesis. The mRNA levels of apo(a), GAPDH, actin as well as 18S ribosomal RNA were assessed by Northern blot hybridization (Fig 5a). Actin and GAPDH mRNA and 18S ribosomal RNA, which were not affected by retinoids, were used as internal standards. Northern blot hybridization of total RNA from cultured hepatocytes revealed the existence of one or two distinct messengers, which ranged in size from about 10 to 12 kb and represents transcripts of different apo(a) alleles (Fig 5a). Quantification of the different apo(a) mRNA bands demonstrated that all apo(a) mRNAs were susceptible to suppression to the same extent. The apo(a) mRNA(s) were decreased by 42% ± 8% (Mean ± SEM.) after incubation of the hepatocytes for 48 h with 10 µmol/L at-RA (Fig 5b). The apo B-100 mRNA level remained constant in these experiments (data not shown).

Effect of at-RA in rhesus monkeys *in vivo*

In vivo studies in monkeys were performed to investigate whether retinoids have a decreasing effect on plasma lp(a) levels.The monkeys received 1 mg/kg at-RA per day or placebo orally for 4 weeks. The interindividual variation of the lp(a) concentration between the monkeys (minimum 15.3 mg/dL, maximum 82.2 mg/dL) was high in both the control and the at-RA group. After supplementation of the monkeys with at-RA no significant change in plasma lp(a) level at any time point was observed when compared to the control group (table II).

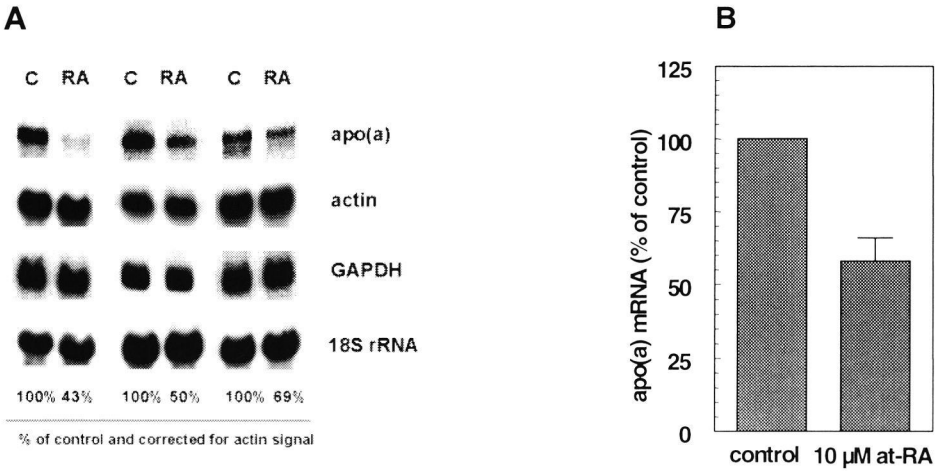

Fig 5. Effect of at-RA on apo(a) mRNA levels in cynomolgus monkey hepatocytes
Total RNA was isolated from the hepatocytes which were incubated for 48 h with 10 µmol/L at-RA or control medium. A) Phosphor Imager Scan of three representative Northern blots hybridized with 32P-labeled probes for apo(a), actin, GAPDH, and 18S rRNA. All mRNA bands were quantified and the apo(a) signals were corrected for the actin, GAPDH and 18S signal, which were used as internal standards. B) Histogram of the effect of at-RA on apo(a) mRNA. Mean ± S.E.M., n=4.

The changes in plasma triglyceride levels differed significantly between the retinoid and placebo group after 1 week of treatment (44% versus -5%; p=0.05). The triglyceride levels of the monkeys fluctuated during the experiment both in the control group and in the retinoid-treated group. We calculated the area under the curve to determine the effect of at-RA in time. The triglyceride levels in the treated group were significantly increased compared to the control group, on the average by 14% (p = 0.05), indicating that the monkeys responded to the retinoid. After the wash-out period the increased triglyceride levels of the retinoid treated animals returned to baseline levels again. Total cholesterol and apo B-100 were not changed during retinoid treatment when compared to the control group (data not shown).

DISCUSSION

In this study we showed that retinoids play a physiological role in regulating the expression of the apo(a) gene. However, in contrast to a response *in vitro* no effect was found *in vivo*. Retinoids inhibited the synthesis of apo(a) in a dose- and time-dependent way in cynomolgus monkey hepatocytes and regulation took place at the (post)-transcriptional level. By using receptor-specific retinoids we found that the RXR-homodimer is not involved in the regulation, whereas the RAR appears to be responsible for the suppression of apo(a) synthesis.

Table II. Effect of at-RA on plasma lp(a) and triglyceride levels *in vivo* in monkeys

	baseline	week 1	week 2	week 4
control group (n=3)				
lp(a) (mg/dL)	57.9 ± 21.4	62.1 ± 19.0	56.9 ± 19.1	64.9 ± 18.3
		(121% ± 18%)	(105% ± 9%)	(132% ± 27%)
change		4.2 ± 3.2	-1.0 ± 2.2	7.0 ± 3.1
		(21% ± 18%)	(5% ± 9%)	(32% ± 27%)
plasma triglycerides (mmol/l)	0.57 ± 0.17	0.51 ± 0.10	0.49 ± 0.10	0.68 ± 0.18
		(95% ± 8%)	(99% ± 22%)	(123% ± 8%)
change		- 0.06 ± 0.07	- 0.08 ± 0.17	0.12 ± 0.03
		(- 5% ± 9%)	(0.2% ± 23%)	(23% ± 8%)
retinoic acid group (n=3)				
lp(a) (mg/dL)	60.7 ± 8.8	63.1 ± 14.5	62.7 ± 13.9	60.8 ± 13.4
		(101% ± 10%)	(101% ± 12%)	(98% ± 9%)
change		2.3 ± 6.0	1.9 ± 6.3	0.1 ± 5.0
		(2% ± 10%)	(5% ± 9%)	(-2% ± 9%)
plasma triglycerides (mmol/l)	0.51 ± 0.10	0.77 ± 0.25	0.64 ± 0.08	0.71 ± 0.17
		(144% ±19%)*	(130% ± 13%)	(139% ± 12%)
change		0.26 ± 0.15*	0.13 ± 0.03	0.20 ± 0.08
		(44% ± 19%)*	(30% ± 13%)	(39% ± 13%)

Rhesus monkeys were treated daily for 4 weeks with placebo or 1 mg/kg all-trans retinoic acid orally. Fasting blood samples were obtained before treatment (baseline) and at 1, 2 and 4 weeks thereafter. Values of triglyceride and lp(a) levels and the change are mean ± S.E.M. The values between parentheses are percentages of baseline levels, or percentage changes. * Significantly different with respect to the control group.

The buoyant density of the lp(a) particle, which accumulated in the medium, was similar to that of lp(a) found in human or simian serum (39,40). After density gradient ultracentrifugation, about 50% of the total amount of apo(a) was found as a free protein, unlike the fact that apo B-100-containing lipoproteins, mainly LDL, was present in excess. Apo B-100 was synthesized in a 4 to 5 times higher amount than apo(a) in the third culture period (72-96 h). This indicates that not all of the synthesized apo(a) does associate with LDL to form a lp(a) particle in hepatocyte cultures. Our finding is in agreement with other cell-culture and *in vivo* studies, showing that lp(a) assembly occurs extracellularly (13,41,42), and that approximately half of apo(a) secreted by monkey hepatocytes is present as free apo(a) (13). More recently, it was demonstrated that newly-synthesized apo(a) binds to the hepatocyte surface, and that from this location apo(a) comes into

contact with apo B-100 and is released from the cell surface as a lipoprotein particle (43). Apo B-100 was primarily found in a particle with the buoyant density of LDL. Only a small part was present in a VLDL particle, suggesting that during the 24 h incubation period most of the VLDL was converted into LDL. On the other hand, it is also possible that apo B-100 is secreted mainly as an LDL-like particle, as in HepG2 cells (44). Apo A-I was found in the lipid-poor or lipid-free fraction as well as in a lipoprotein particle with the buoyant density of HDL. These findings indicate that primary cultures of cynomolgus monkey hepatocytes are a good model for studying (apo)lipoprotein synthesis and secretion.

We found that retinoids decrease apo(a) synthesis in a physiological culture system. When primary cynomolgus monkey hepatocytes were incubated with at-RA, a dose- and time-dependent decrease of apo(a) secretion was observed. Metabolic labeling studies showed that the *de novo* synthesis of apo(a) was decreased. Lp(a) concentration in the medium was lowered to the same extent as the apo(a) concentration. All independent hepatocyte cultures showed a similar decreasing effect on the different apo(a) mRNAs after treatment with retinoids, independent of differences in genotype. This shows that the decrease of apo(a) protein is not due to degradation of apo(a) but is the result of a decreased mRNA level. Our finding that apo(a) synthesis is decreased after a lag phase of 24 h, suggests that the half-life of apo(a) mRNA is relatively long (15). In the same culture experiments apo A-I and apo B-100 were determined for comparison. Apo A-I synthesis was induced, while apo B-100 synthesis remained unaffected, indicating that the decrease of apo(a) synthesis by retinoids is not part of a general effect of retinoids in simian hepatocytes. Relatively high concentrations of at-RA were needed to obtain effects. The reason for this is that retinoids are rapidly metabolized by cynomolgus hepatocytes. After 2 h of incubation approximately 60% of the added at-RA has disappeared from the medium, whereas after 24 h only 2% of at-RA is left (15). Thus, the high concentrations of retinoids applied in this study are necessary for maintaining a sufficiently high concentration during the 24 h incubation period.

At-RA is known to be the active metabolite of retinol and can in turn be converted into physiologically active compounds such as its 9-*cis* and 13-*cis* isomers (45-47). We have compared the effect of these natural retinoids. Retinol was not active in the suppression of apo(a) synthesis, although in the same experiments apo A-I synthesis was induced, but less potently than with at-RA. The reason for the discrepancy between apo A-I induction and the lack of apo(a) suppression is unclear. It is known that retinol is not active in binding to retinoid receptors and in transactivation of gene-transcription and that the compound first has to be metabolized to at-RA to become active (48). 9-*Cis*-RA and 13-*cis*-RA also had suppressing effects on apo(a) synthesis. A possible explanation for the similar potency of at-RA and 9-*cis*-RA may be the intracellular interconversion of these retinoids to each other in these metabolically active hepatocytes. Moreover, 9-*cis*-RA, which has been shown to be the natural ligand for the retinoid X receptor (RXR), is also able to activate

the retinoic acid receptor (RAR), as does at-RA only with the latter receptor (46,47,49). To establish which retinoid receptors are involved in the suppression of apo(a) synthesis, retinoid receptor-specific ligands were applied (31,32). As with at-RA, high concentrations of these ligands were necessary to observe effects, suggesting that cynomolgus hepatocytes also metabolize these compounds rapidly. When using the RXR-specific ligand, 3-methyl-TTNEB, no effects on apo(a) synthesis was observed, whereas apo A-I synthesis was enhanced. In contrast, the RAR-specific ligand, TTNPB, decreased apo(a) synthesis dose-dependently. These results indicate that the RXR-homodimer is not involved in the decrease of apo(a) synthesis, and that down-regulation proceeds via involvement of the RAR as partner of the RAR/RXR heterodimer. How this would happen is not fully understood, but there is increasing evidence that the RAR alone does not bind to DNA and cannot function effectively when it is not bound to the RXR (29,30,50-52). Our data suggest that RXR can act as a transcriptionally silent partner (30) and are in line with the finding that RXR-ligand binding is not necessary for the RXR/RAR-dimer to be active (30,53). We conclude that only RAR is actively involved in the decreased apo(a) synthesis.

The apo(a) mRNA level was found to be decreased after retinoid treatment. This finding together with the fact that RARs act as nuclear transcription factors, suggests that regulation takes place at transcription level. This contention is supported by the presence of several half-sites of the RARE sequence, AG$^{G}/_{T}$TCA (for review see; 28), in the 1.4 kb 5' flanking region of the human apo(a) gene and the baboon apo(a) gene as published by Wade *et al.* (54) and Hixson *et al.* (55), respectively. However, no direct repeat with 1-5 nucleotides spacing could be identified in these proximal sequences. It is possible that the full regulatory element cannot be located because of sequence-degeneration, or that the responsive element is located more upstream in the apo(a) promoter. This is conceivable, since a fully functional RARE was found far upstream in the t-PA promoter at position -7.3 kb (56), and a functional DR-5 RARE is observed 5.6 kb upstream from the transcriptional start site in the CRABII-gene (57). This contention is also supported by the recent finding of the presence of functional direct repeats (DR1) of the nuclear hormone receptor half sites AGGTCA in two enhancer regions located far upstream in the human apo(a) promoter (58).

While preparing this manuscript Ramharack *et al.* published data obtained with two culture experiments demonstrating a similar suppression of apo(a) synthesis by retinoids using cynomolgus hepatocytes (59). In contrast to the *in vitro* situation, treatment of a small number of rhesus monkeys with at-RA did not change plasma lp(a) levels. Unfortunately, we did not have the opportunity to measure apo(a) mRNA levels in the livers of these monkeys. In the latter experiment monkeys were treated with at-RA since this compound is used clinically in the treatment of leukemia. We chose a dose relevant to the human situation and used *in vivo* experiments addressing the pharmacokinetics and toxicology of RA *in vivo* (60,61). Similarly, in an intervention study with 10 healthy

volunteers receiving 80 mg per day of isotretinoin (13-*cis* retinoic acid) we found no changes in plasma lp(a) levels during treatment with the latter drug (25). Triglyceride levels which can serve as a positive control for the effect of retinoids (62) were increased in both experiments.

In contrast to the data of the hepatocyte cultures (this study and 59) and our data *in vivo* in monkeys and in healthy volunteers (this study and 25), Azuma *et al.* (27) reported recently an increased apo(a) promoter activity when using a relatively short human apo(a) promoter-reporter construct (-444 to -3 bp) which was transiently transfected in HepG2 cells and incubated with at-RA. Furthermore, they showed enhanced lp(a) levels in two patients with acute promyelocytic leukemia (APL) treated with at-RA. The reason for the discrepancies between our data and theirs is unclear but may be due to several factors. The cell-systems, transfected HepG2 cells are not comparable with primary hepatocytes. Differences in the expression of retinoid receptors may influence the results; retinoic acid receptors are essential for the decreasing effect on apo(a) by retinoids. Only 442 bp of the apo(a) promoter were used for the transfection, although it is suggested that fragments longer than 1.5 kb from the 5' flanking region of the apo(a) gene must be used in order to analyze the transcriptional regulation of the apo(a) gene (58,63). As stated above the RARE may be located further upstream. In addition, no control experiments were performed using mutation (deletion or substitution) constructs (27). Azuma *et al.* (27) also treated only two APL patients with 80 mg at-RA per day, whereas we treated ten volunteers with an equal amount of isotretinoin in a placebo-controlled design (25). In their study, the lp(a) levels were enhanced initially, but after about 2 weeks of treatment lp(a) levels started to decrease gradually to the original levels. It is well-known that APL patients are metabolically unstable which among other things is noticeable in the fluctuation of lp(a) values (27), and elevated serum lp(a) levels have been reported in leukemia (64). It can, therefore, not be excluded that the disease did influence the effects of retinoids on lipoproteins and that the increase in lp(a) is secondary to other changes.

An opposite effect of retinoids *in vivo* and *in vitro* is also described for apo A-I gene expression (23). Changes in the state of differentiation of cultured cells may underlie the discrepancy between data obtained in *in vitro* and *in vivo* experiments with retinoids. It is well known that retinoids are morphogens whose functions are most pronounced during development and cellular differentiation (29). Additionally, retinoids have been shown to have secondary effects on plasma lipid levels as exemplified by hypertriglyceridemia induced by retinoids (62). Lp(a) has been shown to be partially contained in triglyceride-rich particles (65,66), which could influence its metabolism.

In conclusion, we have shown that retinoids decrease apo(a) synthesis in primary cultures of cynomolgus monkey hepatocytes and that activation of the RAR is a prerequisite for this. However, no effect of retinoids was found on plasma lp(a) levels *in*

vivo in rhesus monkey. The results, therefore, show additional regulatory effects of retinoids on lp(a) levels *in vivo* and raise questions about the usefulness of retinoids in the treatment of atherosclerosis.

ACKNOWLEDGEMENTS

This work was supported by The Netherlands Heart Foundation (grant no.92.328 to Diana M. Neele) We would like to thank Mr Jan Staal, Wim Vos and Joke van de Siepkamp (RIVM, Bilthoven) for their cooperation and zootechnical assistance and Mr Allard Kaptein for the characterization of the lipoprotein production by the simian hepatocytes.

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Chapter 5

No short-term effect of oral isotretinoin (13-cis retinoic acid) on lipoprotein(a) and HDL subclasses lpA-I and lpA-I:A-II in healthy volunteers.

submitted

No short-term effect of oral isotretinoin (13-cis retinoic acid) on lipoprotein(a) and HDL subclasses lpA-I and lpA-I:A-II in healthy volunteers.

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Short title: No effect of isotretinoin on lp(a)

ABSTRACT

Objectives. We have previously shown that retinoids suppress apo(a) synthesis in cultured simian hepatocytes and may have divergent effects on apo A-I and apo A-II synthesis in human and monkey hepatocytes *in vitro* and in rat *in vivo*. Therefore, we evaluated the effect of isotretinoin on lipoprotein(a) (lp(a)), apo A-I, apo A-II and the HDL subclasses lpA-I and lpA-I:A-II.

Design. Randomized, double blind, placebo-controlled, cross-over study.

Subjects. 10 healthy male volunteers.

Intervention. Placebo and isotretinoin was administered two times a day (40 mg in the morning and 40 mg in the evening) for 5 days.

Results. As compared to treatment with placebo, isotretinoin had a significant increasing effect on plasma levels of total cholesterol (+10%), and apo B-100 (+9%), whereas triglycerides were borderline significantly increased (+35%). On the other hand, isotretinoin had no effect on plasma levels of lp(a), LDL-cholesterol, HDL-cholesterol and on lpA-I, lpA-I:A-II, apo A-I and apo A-II. No association was found between baseline lp(a) levels and the change after isotretinoin treatment.

Conclusions. Administration of isotretinoin to healthy volunteers enhances plasma levels of apo B-100 - containing lipoproteins without having short-term effects on lp(a) levels and on HDL-subclasses lpA-I and lpA-I:A-II.

INTRODUCTION

Lipoprotein(a) (lp(a)) is considered to be an independent risk factor for coronary heart disease and cerebrovascular disease [for review see: 1], although data from

epidemiological studies are not consistent [2]. The lipoprotein consists of an LDL-like particle in which apo B-100 is covalently linked to apolipoprotein(a) (apo(a)) via a disulphide bridge. Lp(a) levels vary strongly between individuals and are highly genetically controlled [3,4]. Although plasma levels of lp(a) are almost entirely determined by inheritance a limited number of drugs are described as influencing the levels of lp(a) in human [for review see: 5,6]. We have shown previously that retinoids, including all-trans retinoic acid (at-RA) and its 13-cis isomer (isotretinoin), decrease apo(a) synthesis and lp(a) levels in primary cultures of cynomolgus monkey hepatocytes [7]. Recently, decreased levels of lp(a) were found in 30 patients with severe acne treated with isotretinoin [8]. In contrast, increased levels of lp(a) were found in two acute promyelocytic leukemia patients treated with at-RA [9]. In the present study, we have evaluated the short-term influence of isotretinoin on lp(a) levels in 10 healthy men in a placebo-controlled study, performed initially to study the effect on fibrinolysis [10].

LpA-I is an HDL particle which only contains apo A-I and plays an important role in the protection against atherosclerosis. LpA-I:A-II, an HDL particle containing both apo A-I and apo A-II, is less effective than lpA-I in stimulating cholesterol removal from cells [11,12]. With respect to the regulation of apo A-I and apo A-II synthesis by retinoids, divergent effects have been reported. Apo A-I gene transcription was shown to be increased in primary cynomolgus monkey hepatocytes after treatment with different retinoids such as at-RA, 9-*cis*-RA and 13-*cis*-RA [13], and similar data were obtained in Caco-2 cells, HepG2 cells and rat hepatocytes [14-16], but opposite effects were found *in vivo* in rat [14]. Further, retinoids had an increasing effect on apo A-II synthesis in human hepatocytes and HepG2 cells [17], but no change in liver apo A-II mRNA levels was found in rat [14].

In man vitamin A derivative, isotretinoin (13-*cis*-RA) is shown to be effective in the treatment of dermatological diseases [18]. A side effect of treatment with retinoids is hyperlipidemia, including increases in triglycerides and cholesterol. In general, very low density lipoprotein (VLDL) triglyceride levels and cholesterol levels, predominantly contained in low density lipoproteins (LDL), as well as apo B-100 levels, are increased after long-term isotretinoin treatment [19-22]. Little or no change in HDL-cholesterol, apo A-I and apoA-II is found in these patients [19-22]. The effect of retinoids on the HDL subclasses lpA-I and lpA-I:A-II in healthy people is still unknown.

In this study we have investigated the effect of short-term treatment with isotretinoin on the plasma levels of lp(a), and on the distribution of apo A-I, apo A-II over HDL subclasses lpA-I and lpA-I:A-II, in 10 healthy male volunteers in a placebo-controlled intervention study.

MATERIALS AND METHODS

Experimental protocol

This study was conducted at the Centre for Human Drug Research, University Hospital Leiden, The Netherlands, initially to study the effect of isotretinoin on fibrinolysis [10]. The study was designed as a randomized, double-blind, placebo-controlled cross-over study with 10 healthy, male volunteers. The protocol was approved by the Ethics Committee of the University Hospital Leiden. Subjects had given written acknowledgment of informed consent to participate. All subjects were male, 21-26 years of age, non-smokers and were judged to be in good health on the basis of physical examination, ECG and routine blood and urine tests. During the study no drugs other than the one under investigation were used. The volunteers abstained for 48 h from alcohol and for 24 h from strenuous physical activities before the start of the study and during the study. Beverages containing caffeine were not allowed during the study. Each volunteer received 2 capsules containing 20 mg isotretinoin or placebo with a glass of water during a meal in the morning and in the evening (total dose of 80 mg/day), for 5 days. After a wash-out period of 2 weeks subjects were given the alternative treatment. Fasting blood samples were collected in ice-cold CTAD tubes (containing citrate, theophylline, adenosine and dipyridamol), and immediately placed on ice. Plasma was stored at -80 °C for all lipid and (apo)lipoprotein assays.

Analytical measurements

Lipids and apo(lipoproteins) were determined in plasma collected in the morning before the start of the intervention and at the end of the 5-day intervention period in the morning of the sixth day. Total cholesterol and triglyceride levels were determined using enzymatic methods (Boehringer CHOD-PAP-kit, No. 236691). HDL-cholesterol was measured after precipitation of particles containing apo B-100 using the precipitation method with sodium-phosphotungstate Mg^{2+}, as described [23]. LDL-cholesterol was calculated according to Friedewald and by a modification of the Friedewald formula to account for the fact that lp(a) contains 30% cholesterol by weight [24]. Apo A-I, apo B-100 and lipoprotein(a) were measured in all subjects by rate immunonephelometry using an automated Beckman 'Array' analyser (Beckman Instruments) as described previously [23]. Apo A-II and lpA-I concentrations were determined as previously described by ELISA [25] and immunoelectrophoresis [26], respectively. LpA-I:A-II was calculated by subtracting the lpA-I concentration from the apo A-I concentration.

Statistical analysis

To evaluate the effect of treatment on the variables, values before and after placebo and isotretinoin treatment, and changes in both groups were compared using the Wilcoxon

test for paired values. Percentage changes were calculated as 100 x [(posttreatment value - pretreatment value) / pretreatment value]. Values are presented as mean ± SD. Associations between parameters were quantified by means of the Spearman correlation coefficient. Statistical analysis was performed using SPSS for Windows. Values were considered significantly different at $P < 0.05$.

RESULTS

The well-known effects of isotretinoin on skin and mucous membranes (e.g. dry skin, cheilitis) were seen in all subjects. One volunteer had to be replaced during the first treatment period in which he received isotretinoin because of uneasiness and anxiety; all other volunteers completed the study. The mean plasma concentration of endogenous retinol did not change during the two treatment periods (between 398 and 549 ng/ml). On the last day of the isotretinoin treatment, plasma levels of isotretinoin varied between 102 and 296 ng/ml and peak levels, which were measured 2 to 6 h after drug intake, varied from 242 to 457 ng/ml. Two to 9% of the absorbed isotretinoin was isomerized to at-RA. at-RA peak levels were measured 2 to 6 h after drug intake and varied between 7 and 38 ng/ml, and the at-RA plasma levels on treatment day 5 varied between 2 to 29 ng/ml [10].

Lp(a) and HDL-subclasses lpA-I and lpA-I:A-II and HDL apolipoproteins were determined four times: before isotretinoin or placebo intake, after 5 days of treatment with placebo or isotretinoin, after the wash-out period of 14 days and after the second treatment period of 5 days. Total cholesterol, total triglycerides, LDL-cholesterol and apo B-100 were determined as control for the effect. Except for a slight effect on lpA-I, placebo had no effect on the measured parameters during the intervention study (table I). The total plasma cholesterol level within the subjects treated with isotretinoin increased significantly (10%, $P = 0.05$), when comparing the two treatment periods (isotretinoin and placebo) with each other (table I). Similar data were obtained for the triglyceride levels, but the increase just failed to be significant (35%, $P = 0.08$). LDL-cholesterol did not change, whereas apo B-100 increased significantly in the intervention group when compared to placebo (9%, $P = 0.04$) . Correcting the LDL-cholesterol value for cholesterol contained in the lp(a) particle (see Materials and Methods section) did not change the results (data not shown). When taking all lipoproteins containing apo B-100 together (total cholesterol - HDL cholesterol), thus including LDL as well as lp(a) and VLDL, a borderline significant increase (13%, $P = 0.07$) is observed after isotretinoin treatment as compared to placebo treatment. Apo B-100 levels were strongly associated with plasma total cholesterol ($R = 0.81$, $P = 0.005$) after isotretinoin treatment. The association between apo B-100 and triglycerides increased by treatment (before $R = 0.26$, $P = 0.47$; after $R = 0.59$, $P = 0.07$)

Table I. Effect of treatment with placebo or isotretinoin on concentrations of plasma lipids and lipoproteins

	Placebo			Isotretinoin			
	baseline (n=10)	absolute and percentage changes after treatment (n=10)	Pplacebo	baseline (n=10)	absolute and percentage changes after treatment (n=10)	Pisotretinoin	Pchange
plasma cholesterol (mmol/L)	3.38 ± 0.52 (2.48/3.94)	-0.05± 0.26 (-1% ± 8%)	0.44	3.43 ± 0.40 (2.60/3.91)	0.33 ± 0.49 (9% ± 14%)	0.03	0.05 (0.05)
plasma triglycerides (mmol/L)	0.77 ± 0.35 (0.35/1.37)	-0.10 ± 0.30 (-5% ± 29%)	0.39	0.75 ± 0.19 (0.40/1.08)	0.10 ± 0.21 (30% ± 28%)	0.01	0.06 (0.08)
LDL-cholesterol * (mmol/L)	2.10 ± 0.45 (1.45/2.74)	0.07 ± 0.30 (4% ± 15%)	1.00	2.23 ± 0.37 (1.59/2.79)	0.29 ± 0.44 (12% ± 18%)	0.04	0.14 (0.20)
apo B-100 (g/L)	0.74 ± 0.15 (0.52/0.94)	-0.01 ± 0.06 (-1% ± 8%)	0.65	0.71 ± 0.13 (0.55/0.93)	0.06 ± 0.08 (8% ± 11%)	0.04	0.03 (0.04)
lp(a) (g/L)	0.07 ± 0.11 (0.01/0.37) (median 0.03)	0.00 ± 0.02 (8% ± 32%)	1.00	0.06 ± 0.10 (0.01/0.39) (median 0.03)	0.01 ± 0.03 (21% ± 63%)	0.37	0.45 (0.39)
HDL-cholesterol (mmol/L)	0.94 ± 0.26 (0.58/1.31)	-0.08 ± 0.15 (-5% ± 14%)	0.17	0.85 ± 0.15 (0.61/1.10)	-0.07 ± 0.12 (-8% ± 13%)	0.09	0.88 (0.96)
lpA-I (g/L)	0.45 ± 0.08 (0.32/0.55)	-0.04 ± 0.04 (-8% ± 8%)	0.02	0.52 ± 0.07 (0.43/0.66)	-0.04 ± 0.07 (-5% ± 14%)	0.13	0.76 (0.92)
lpA-I/A-II (g/L)	0.81 ± 0.18 (0.48/1.06)	-0.01 ± 0.08 (1% ±12%)	0.68	0.78 ± 0.23 (0.41/1.08)	0.10 ± 0.31 (26% ± 56%)	0.44	0.33 (0.28)
apo A-I (g/L)	1.23 ± 0.22 (0.79/1.59)	-0.02 ± 0.10 (-5% ± 15%)	0.58	1.30 ± 0.24 (0.85/1.58)	0.05 ± 0.33 (8% ± 31%)	0.84	0.65 (0.51)
apo A-II (g/L)	0.52 ± 0.07 (0.40/0.63)	-0.03 ± 0.04 (-5% ± 9%)	0.08	0.59 ± 0.10 (0.40/0.73)	-0.01 ± 0.13 (3% ± 21%)	0.88	0.33 (0.29)

* Value of LDL-cholesterol obtained by the Friedewald formula. Baseline values are mean ± S.D. (range). Values of plasma lipids and lipoproteins of subjects after treatment with placebo or isotretinoin are mean ± S.D. absolute change (percentage change). Pplacebo and Pisotretinoin indicate the significance of placebo or isotretinoin treatment. Pchange indicates the significance of the absolute and percentage changes (parentheses) between placebo and isotretinoin treatment. Probability values were obtained by using Wilcoxon paired test.

Effect of isotretinoin on plasma lp(a), HDL subclasses and apolipoprotein A-I and A-II levels

The interindividual variation of the lp(a) concentration between the subjects (minimum 0.01 g/L, maximum 0.39 g/L) was high. After supplementation of the volunteers with isotretinoin some men showed an increase, and others a decrease in lp(a) concentration, which altogether resulted in an unchanged plasma lp(a) level (fig 1, table I). In addition, no association was found between baseline lp(a) levels of the subjects and the change in lp(a) after isotretinoin treatment ($\triangle$ $lp(a)_{after-before}$), indicating that high initial lp(a) levels were also not affected by the drug. Lp(a) levels were not associated with any of the other measured parameters at baseline or after intervention with isotretinoin.

HDL-cholesterol as well as the plasma levels of the HDL subclasses lpA-I and lpA-I:A-II and the apo A-I and apo A-II concentrations did not alter significantly in this placebo-controlled study (table I), but the composition of the HDL lipoproteins appeared to be changed. Before drug intake HDL-cholesterol was not significantly associated with apo A-I and lpA-I:A-II (R = 0.15, P = 0.67 and R = 0.21, P = 0.54, respectively), whereas after treatment the associations were strong and significant (R = 0.77, P = 0.01 and R = 0.74, P = 0.01, respectively). A significant negative correlation was observed for HDL subclass lpA-I and triglycerides (R = -0.68, P = 0.03) before treatment, which disappeared after isotretinoin treatment (R = -0.16, P = 0.66).

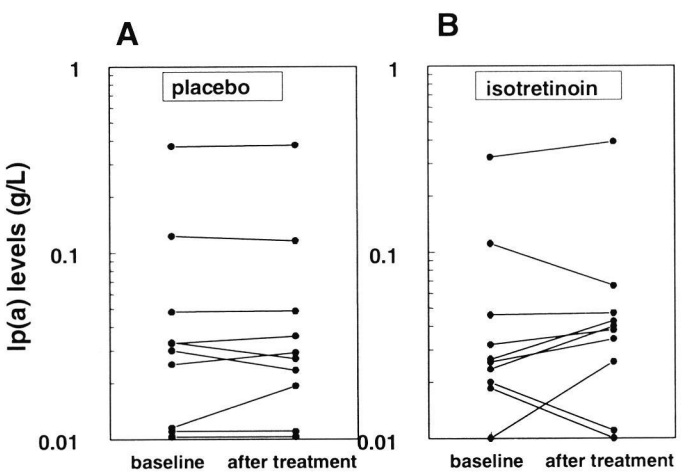

Fig 1. Individual lp(a) levels at baseline and after 5 days treatment with placebo (A) or isotretinion (B).

DISCUSSION

In this placebo-controlled study we showed that administration of 80 mg per day of isotretinoin for 5 days to healthy volunteers had a significant increasing effect on plasma total cholesterol and apo B-100 plasma concentrations, whereas plasma triglyceride levels were borderline significantly enhanced. Short-term isotretinoin treatment had no effect on lp(a), LDL-cholesterol, HDL-cholesterol, and on the HDL-subclasses lpA-I, lpA-I:A-II, or on plasma concentrations of apo A-I and apo A-II.

The data indicate that the increase of cholesterol after isotretinoin treatment takes place in the VLDL(-remnant) fraction, since LDL- and HDL-cholesterol levels were not affected. The rise most likely results from an increased particle number, since we and others [20,22] have shown that the plasma apo B-100 concentration was also enhanced after isotretinoin treatment. Maximum triglyceride levels were generally obtained after longer intervention periods with isotretinoin explaining the absence of a significant effect in our study. The precise biochemical background of the hyperlipidemic effect of retinoids is not known. Rats treated with much higher dosages of retinoids as used in humans showed a reduced VLDL uptake by the liver as result of an impaired lipolysis by a decreased lipoprotein lipase level [27,28]. In man also delayed clearance of plasma triglycerides has been demonstrated [29,30], but no changes in post-heparin plasma LPL activity were found [21,31-33]. Recently, some of us found that retinoids increase plasma apo C-III concentrations which may explain the delayed catabolism of triglyceride-rich remnant particles by interfering with apo E-mediated receptor clearance of these particles from plasma [Vu-Dac et al, unpublished data].

We have previously shown that apo(a) synthesis is decreased by about 50% in primary cultures of cynomolgus monkey hepatocytes incubated with at-RA. In the same experiments isotretinoin decreased apo(a) synthesis less potently but significantly by 20% [7]. To validate these findings we investigated the effect of isotretinoin *in vivo* in man in samples obtained initially for other purposes [10]. We found no changes in lp(a) levels during treatment with this drug. In contrast to our data, Azuma *et al.* [9] reported enhanced lp(a) levels in two patients with acute promyelocytic leukemia (APL) treated with at-RA. However, in patients with severe acne treated with 0.5 mg/kg/day isotretinoin decreased lp(a) levels were reported [8]. The reason for the discrepancies between our *in vivo* data and the data described by Azuma *et al.* [9] is unclear. In the latter study only two APL patients were treated with 80 mg per day at-RA, whereas we treated ten volunteers with an equal amount of isotretinoin in a placebo-controlled design. In their study, the lp(a) levels were enhanced initially, but after about 2 weeks of treatment lp(a) levels started to decrease gradually to the original levels. It is well-known that APL patients are metabolically unstable which among other things is noticeable in the fluctuation of lp(a) values [9], and elevated serum lp(a) levels have been reported in leukemia [34]. It can,

therefore, not be excluded that the disease did influence the effects of retinoids on lipoproteins and that the increase in lp(a) is secondary to other changes. The discrepancy between our data and the lp(a) decrease reported by Georgala *et al.* [8] may be related to the relatively high baseline levels of lp(a) in the latter study or the relatively short treatment period in our study. The half-life of plasma lp(a) has been reported to be between two and three days [35,36]. On the other hand, clear and significant changes in total cholesterol and apo B-100 were observed in the same period in our study. Furthermore, differential effects of retinoids have been reported *in vitro* and *in vivo* with respect to regulation of apo A-I and apo A-II [14,17] and the same phenomenon may occur with regulation of apo(a) synthesis.

In contrast to the increasing effect of retinoids on gene expression of apo A-I and apo A-II *in vitro* [13-17], retinoids showed a decreasing effect on apo A-I gene expression and had no effect on apo A-II mRNA *in vivo* in rat [14]. Decreased or unchanged HDL and apo A-I levels were reported in most patient studies with retinoids [8,20-22], but a partial normalisation of low plasma HDL was observed in patients with cystic acne treated with isotretinoin [33]. Therefore, the distribution of apo A-I and apo A-II over the lipoprotein particles lpAI and lpA-I:A-II was determined. We found no change in this distribution and in apo A-I, apo A-II and HDL-cholesterol level, although the HDL composition appeared to be altered. These results do not necessarily contradict with former studies in which an increase in apo A-I and apo A-II gene-expression by retinoids was found in cultured hepatocytes [13-17]. It is still conceivable that retinoids enhance apo A-I and apo A-II synthesis *in vivo*, which is not reflected in a raised apo A-I, apo A-II and HDL plasma level. No change or a slight decrease of the HDL level may partially be secondary to the reported increase in triglyceride-rich particles upon treatment with retinoids [22], which ultimately results in smaller HDL particles. Moreover, it has been shown recently that isotretinoin enhances the cholesteryl ester transfer protein (CETP) mediated cholesteryl ester transfer reaction [37]. An increased CETP activity also results in smaller HDL-particles which are known to be more rapidly cleared than larger HDL-particles [38]. Adverse effects on catabolism of triglyceride-rich particles elicited by retinoids *in vivo* may, therefore, over-shadow a potentially beneficial effect of the compounds on HDL levels. Studies showing no change or an increase of HDL-cholesterol unlike a rise in plasma triglycerides [22,33] may support this contention.

In conclusion this study shows that short-term isotretinoin treatment of healthy males leads to increased cholesterol and apo B-100 levels but has no effect on plasma levels of lp(a), apo A-I, apo A-II, lpA-I, lpA-I:A-II and HDL-cholesterol

ACKNOWLEDGEMENTS

This work was supported by The Netherlands Heart Foundation (92.328 to Diana M. Neele). Our grateful thanks are extended to Dr J.M.T. van Griensven (Centre for Human Drug Research, Leiden) for his valuable contribution to the initial study.

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Chapter 6

No effect of fibrates on synthesis of apolipoprotein(a) in primary cultures of cynomolgus monkey and human hepatocytes. Apolipoprotein A-I synthesis increased.

Biochem Biophys Res Commun 1998; 244: 374-378

No effect of fibrates on synthesis of apolipoprotein(a) in primary cultures of cynomolgus monkey and human hepatocytes. Apolipoprotein A-I synthesis increased.

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running title: No effect of fibrates on apo(a) synthesis

ABSTRACT

Fibrates have been shown to decrease plasma levels of triglyceride-rich lipoproteins and LDL, and to increase HDL. Data on the effect of fibrates on lipoprotein(a) levels in man are not consistent. Because lp(a) levels *in vivo* are mainly regulated at synthesis level, we studied the effect of fibrates on the synthesis of apolipoprotein(a) (apo(a)) in primary cultures of cynomolgus monkey and human hepatocytes. Furthermore, we assessed the effect of fibrates on apolipoprotein A-I (apo A-I) synthesis and investigated whether different fibrates have different effects on the apo(a) and apo A-I synthesis.

The addition of gemfibrozil to cultures of monkey and human hepatocytes had no effect on apo(a) synthesis, but resulted in a dose- and time-dependent increase of apo A-I synthesis and mRNA. In simian hepatocytes maximal stimulation was 2.5-fold after incubation for 72 h with 1.0 mM gemfibrozil, whereas apo A-I synthesis was induced 1.8- and 2.0-fold by using 0.1 mM and 0.3 mM, respectively. Similar results were obtained by using human hepatocytes; apo(a) synthesis remained unchanged, while apo A-I secretion was 2.0-fold increased at 1 mM gemfibozil. Other fibrates like bezafibrate, clofibrate and clofibric acid did not change apo(a) synthesis either. In contrast, they enhanced the synthesis of apo A-I (1.5-, 1.8- and 1.8-fold, respectively), although less potently than gemfibrozil.

We conclude that fibrates have no effect on apolipoprotein(a) synthesis in monkey and human hepatocytes and that these drugs induce apo A-I synthesis.

INTRODUCTION

Fibrates are a group of drugs which are widely used in treatment of hypertriglyceridemia and hypercholesterolemia. They are among the oldest lipid-lowering drugs, e.g. clofibrate was used as early as the late 1950's. Gemfibrozil is a so-called "second generation"

fibrate, whereas bezafibrate is a relatively new fibrate (1). The drugs lower triglyceride levels in particular and increase HDL-cholesterol levels in hyperlipidemic patients (2-4).

The hypotriglyceridemic action of fibrates is most probably mainly caused by the increase in LPL activity, which results in an increasing VLDL catabolism (5,6). Moreover, fibrates decrease apo C-III secretion (7), which also results in an increased triglyceride clearance, since apo C-III inhibits the lipolysis of triglycerides. With regard to the effects of fibrates on VLDL production, there is evidence from *in vivo* studies that some of these drugs reduce VLDL synthesis and secretion (8-11). The rise in HDL-cholesterol, induced by fibrate treatment, may in part be secondary to the decrease in triglyceride-rich lipoproteins, i.e. less exchange occurs between triglycerides and cholesteryl esters, leading to an increased level of HDL (2). On the other hand, studies in both humans and animals suggest that gemfibrozil also stimulates apo A-I synthesis and increases apo A-I mRNA levels (12-14).

The reports on the effect of fibrates on the synthesis of lipoprotein(a) (lp(a)) are conflicting. Lp(a) is an atherogenic and prothrombotic plasma particle that is considered to be a risk factor for premature coronary heart disease (CHD) (15). The lipoprotein consists of an LDL-like particle, in which apo B-100 is covalently linked to apolipoprotein(a) (apo(a)), via a disulfide linkage. Lp(a) levels vary strongly between individuals and are highly genetically controled (16-18). Although plasma levels of lp(a) are almost entirely determined by inheritance, a small number of drugs such as sex steroids, nicotinic acid and neomycin in combination with niacin are reported to lower the plasma levels of lp(a) in humans (19). As stated above data on the effects of fibrates on lp(a) levels are not conclusive; some *in vivo* studies report a decrease in lp(a) level (20-24), or an increase (10,24,25), or no effect at all (26-28). The divergent results of this group of drugs challenged us to study the effect of fibrates on apo(a) synthesis, since apo(a) synthesis is the level at which plasma lp(a) concentrations *in vivo* are mainly regulated (29,30). The availability of human and monkey hepatocytes, which are the only *in vitro* culture systems that synthesize apo(a) endogenously, enabled us to study the effect of gemfibrozil, bezafibrate clofibrate and clofibric acid on apo(a) synthesis.

MATERIALS & METHODS

Materials

Gemfibrozil was kindly provided by Warner-Lambert (Hoofddorp, The Netherlands), and bezafibrate by Boehringer Mannheim (Almere, The Netherlands). Clofibrate and clofibric acid were from Sigma Chemicals. Stock solutions of 1 M were prepared in DMSO and preserved at -20 °C. Just before each culture experiment, the fibrates were diluted in

culture medium, so that the DMSO concentration did not exceed 0.1% (v/v). Since the fibrates are not easy to dissolve, the solutions were incubated at 37°C for 1 h and vortexed repeatedly. Materials used for the isolation and culturing of the human and simian hepatocytes were obtained from sources previously stated (31-33).

Simian and human 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 isolation procedure was exactly as described (31,32). Human hepatocytes were isolated following a procedure similar to that described (33). The hepatocytes were isolated from donor livers obtained from the University Hospital, Leiden, The Netherlands and which could not be used for transplantation due to technical problems. For each independent culture experiment, freshly isolated hepatocytes from a single monkey or human liver were used. 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 (30 min at 56°C) fetal bovine serum (FBS), 2 mM L-glutamine, 20 mU/ml insulin (135 nM), 50 nM dexamethasone, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml kanamycin 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. 24 hours after seeding, the incubations with the fibrates were started using culture medium with a lower insulin concentration, 10 nM instead of 135 nM. The medium was renewed every 24 h and control incubations contained 0.1% (v/v) DMSO.

Apo(a), lp(a), apo A-I and apo B-100 ELISA.

Total apo(a) concentrations were determined using the TintElize Lp(a) (Biopool AB, Umeå, Sweden). This ELISA uses polyclonal antibodies to human apo(a) both as catching and as detecting antibodies and detects in this manner free apo(a) as well as lp(a). The antibody of this kit 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 Makino *et al.* (34) and Azrolan *et al.* (35) The lp(a) concentration was determined with the Innotest lp(a) (Innogenetics, Antwerp, Belgium) ELISA measuring only apo(a) bound in the lp(a) particle. In the latter assay both human apo(a) and apo B-100 antibodies are used. Apo A-I and apo B-100 concentrations in the collected culture medium were measured in triplicate using a sandwich enzyme-linked immunosorbent assay (ELISA) procedure with polyclonal antibodies to human apo A-I or human apo B-100, respectively, both as catching and detecting antibodies as described

(31,36). Cell-protein in the dishes was determined according to Lowry.

mRNA measurements

Quantification of mRNA levels was performed using Northern-blotting techniques as described (31,32).

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

The addition of increasing amounts of gemfibrozil to the culture medium of the simian hepatocytes did not affect the apo(a) synthesis (fig 1a). Similarly the percentage of apo(a) bound to apo B-100 in a lp(a) particle in the medium (56%) and the amount of apo(a) associated with the cells (31%) were not changed (data not shown). In contrast, apo A-I synthesis was dose-dependently increased in the same experiments (fig 1a).

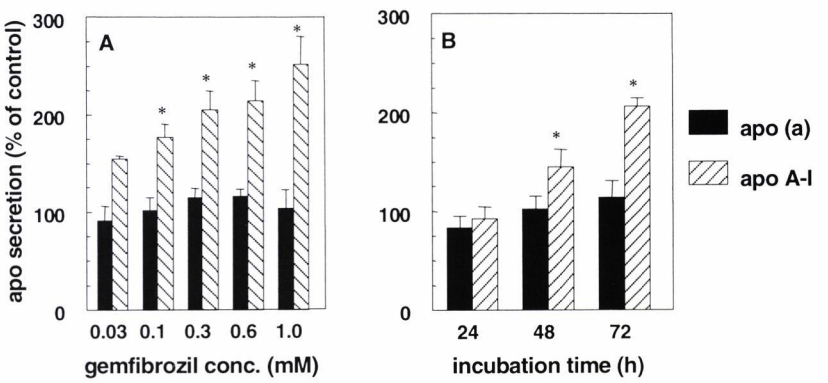

Fig 1. Dose- and time-dependency of the effect of gemfibrozil on the apolipoprotein synthesis in cynomolgus monkey hepatocytes

After a 24 h attachment and recovery period, hepatocytes were cultured for 24, 48 or 72 h with or without different concentrations of gemfibrozil. The medium was renewed every 24h. Apo(a) (black bars) and apo A-I (hatched bars) concentrations were determined as described in "Materials and Methods", and were normalized for cell protein in the dishes. Data are expressed as a percentage of control. A) Hepatocytes were incubated for 72h with various concentrations of gemfibrozil. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 3-9 independent hepatocyte isolations. B) Hepatocytes were incubated for 24h, 48 h and 72h with 1 mM gemfibrozil. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 4-5 independent hepatocyte isolations. * Indicates a significant difference ($P < 0.05$) between control and treated cells.

Incubation with 0.03 mM gemfibrozil for 72 h had no significant effect on apo A-I synthesis, but when using 0.1, 0.3, 0.6 and 1.0 mM gemfibrozil, apo A-I synthesis was increased 1.8, 2.0, 2.1 and 2.5-fold, respectively, as compared to the control. Albumin synthesis was not affected by the different concentrations of gemfibrozil, indicating that the concentrations used were not toxic to the hepatocytes and that gemfibrozil did not have a general effect on the protein synthesis of the hepatocytes.

Apo(a) synthesis did not change significantly during any of the incubation periods used (fig 1b). Apo A-I synthesis increased gradually with the increasing time of incubation (fig 1b), consistent with the slow and time-dependent increase observed with incubation of retinoids (31,32). Gemfibrozil (1.0 mM) increased levels apo A-I mRNA levels significantly 1.4 ± 0.3-fold after 48 h and 1.6 ± 0.3-fold after 72 h of incubation (n = 4 independent hepatocyte cultures).

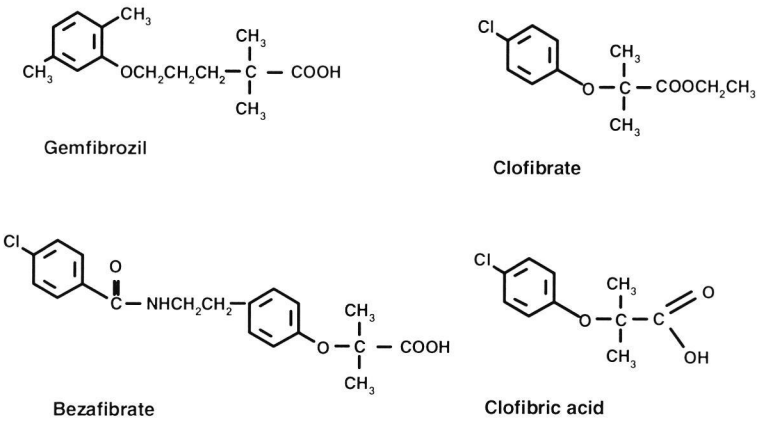

Fig 2. Chemical structures of the fibrates used

To investigate whether different fibrates have different effects on apo(a) and apo A-I synthesis, various fibrates in two different concentrations were tested. Gemfibrozil, bezafibrate and clofibric acid are free carboxylic acids and clofibrate is the ethyl-ester of clofibric acid (fig 2). Like gemfibrozil, the newer fibrate bezafibrate and the first generation fibrates clofibrate and clofibric acid had no effect on apo(a) synthesis (table I). All fibrates induced apo A-I synthesis, although less potently than gemfibrozil. The apo A-I induction was 1.5-fold for bezafibrate, and 1.8-fold for clofibrate and clofibric acid at 1.0 mM. To explore whether gemfibrozil has similar effects on the apo(a) and A-I synthesis in primary human hepatocytes, key experiments were performed using two independent cultures of

scarce available human hepatocytes. Due to low synthesis apo(a) was unfortunately only detectable in one culture, in which synthesis remained unchanged during the different incubation periods with increasing amounts of gemfibrozil. As in simian hepatocytes apo A-I synthesis was dose- and time-dependently increased (fig 3).

Table I. Structural specificity of fibrates

	Secretion (% of control)			
Fibrate added to the medium	apo (a)	(n)	apo A-I	(n)
0.3 mM Gemfibrozil	115 ± 9	(5)	205 ± 20 *	(6)
1.0 mM Gemfibrozil	103 ± 19	(7)	251 ± 29 *	(8)
0.3 mM Bezafibrate	120 ± 3	(2)	107 ± 9 †	(3)
1.0 mM Bezafibrate	114 ± 14	(4)	149 ± 14 * †	(5)
0.3 mM Clofibrate	106 ± 1	(2)	135 ± 16 †	(3)
1.0 mM Clofibrate	123 ± 11	(4)	175 ± 25 * †	(5)
0.3 mM Clofibric acid	118 ± 10	(2)	146 ± 14 †	(3)
1.0 mM Clofibric acid	108 ± 8	(4)	177 ± 10 * †	(7)

Primary hepatocytes from cynomolgus monkey were incubated with different fibrates (0.3 mM and 1.0 mM) for 24 h after two 24-hour preincubation periods with the same fibrate at the same concentration. Results are expressed as mean ± S.E.M. for the indicated number of independent culture experiments.

* indicates a significant difference ($P<0.05$) between control and treated cells

† indicates a significant difference ($P<0.05$) between the indicated fibrates and gemfibrozil at the same concentration.

DISCUSSION

We report in this paper that apo(a) synthesis is not influenced by gemfibrozil in primary cynomolgus monkey and human hepatocytes. Other commonly used fibrates like bezafibrate and clofibrate did not change the apo(a) synthesis either. Apo A-I protein and mRNA levels were increased after incubation with fibrates.

As far as we know this is the second paper concerning the effect of fibrates on apo(a) synthesis. In contrast to our findings, Ramharack *et al.* (21) found a decrease in lp(a) synthesis in cultured simian hepatocytes and decreased plasma lp(a) levels in cynomolgus monkeys after treatment with gemfibrozil. The reason for the discrepancy between their

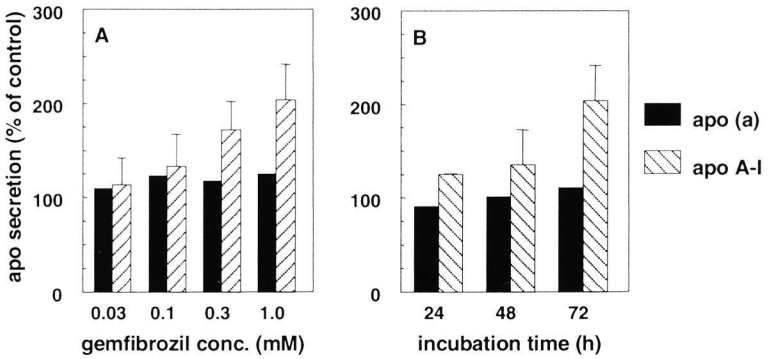

Fig 3. Dose- and time-dependency of the effect of gemfibrozil on the apolipoprotein synthesis in human hepatocytes

After a 24 h attachment and recovery period, hepatocytes were cultured for 24, 48 or 72 h with or without different concentrations of gemfibrozil. The medium was renewed every 24 h. Apo(a) (black bars) and apo A-I (hatched bars) concentrations were determined as described in "Materials and Methods", and were normalized for cell protein in the dishes. Data are expressed as a percentage of control. A) Hepatocytes were incubated for 72 h with various concentrations of gemfibrozil. B) Hepatocytes were incubated for 24 h, 48 h and 72 h with 1 mM gemfibrozil. Values are means (± range) of duplicate incubations of hepatocytes from two independent hepatocyte isolations for apo A-I and one for apo(a).

culture experiments and ours is unclear, but may be due to the fact that they used frozen hepatocytes derived from only one monkey while we used freshly isolated hepatocytes for each culture experiment using livers from 9 different monkeys. We also had the opportunity to use primary human hepatocytes, showing similar results to those obtained with the simian hepatocytes. Another discrepancy is that Ramharack *et al.* do not report an increase in apo A-I synthesis (they use apo A-I mRNA as one of the control signals), which is odd, since we found consistently that all fibrates increase apo A-I synthesis in human and monkey hepatocytes. Moreover, an induction of apo A-I synthesis by fibrates is also reported in several other papers (12-14, 37-39).

Decreased (20-24), increased (10,24,25) and unchanged (26-28) lp(a) serum levels are reported when hyperlipidemic subjects are treated with different fibrates. It is suggested that the divergent results are due to (i) differences in the patient groups, in which the lp(a) concentrations are highly skewed and often no correction is made for the phenotype, (ii) the duration of the intervention, (iii) the dose of fibrate administered, and (iv) the type of fibrate used. The latter two suggestions are however not likely when our *in vitro* data are taken into account, since the type of fibrate and the concentrations we used are comparable to the *in vivo* situation. When patients are treated with 600 mg gemfibrozil

twice a day the plasma concentration of gemfibrozil is about 0.1 mM (20 mg/L) (40). Anyhow, we did not observe a change in apo(a) synthesis and lp(a) levels in primary cultures of monkey and human hepatocytes with the fibrates used.

All fibrates used in our experiments increased the synthesis of apo A-I and slight but significant differences were observed between the different fibrates. Gemfibrozil, the most commonly used fibrate, was the most potent inducer of apo A-I synthesis. The older fibrate, clofibrate and its acid form, clofibric acid were equally potent inducers and the "newer" bezafibrate was less active in the induction of apo A-I synthesis. The induction of apo A-I synthesis and mRNA was significant after 48 h and 72 h of incubation with gemfibrozil. A similar slow stimulation of apo A-I synthesis and mRNA levels is seen when primary hepatocytes are incubated with retinoids (31,32). The increase in apo A-I mRNA levels has been shown to result from an enhancement of apo A-I mRNA stability (14), and from an induced transcription of the apo A-I gene (13). The latter was brought about by a subtle interplay between the PPAR binding A-site and the apo A-I basal promoter (13,37). In addition to the latter forms of regulation the rise in apo A-I levels (41,42) in patients treated with fibrates can also be caused in part by the rise in HDL levels, secondary to the decrease in triglyceride rich lipoproteins (2), which is caused by an increased LPL activity (5,6).

In conclusion, in this study we show that fibrates do not have an effect on apo(a) synthesis in primary cultures of human- and cynomolgus monkey hepatocytes. In comparison with bezafibrate, clofibrate and clofibric acid, gemfibrozil was the most potent inducer of apo A-I synthesis.

ACKNOWLEDGEMENTS

This work was supported by The Netherlands Heart Foundation (grant no. 92.328 to Diana M. Neele and Holger Huisman and grant no. 87.035 to Allard Kaptein. We would like to thank Mr Jan Staal, Wim Vos and Joke van de Siepkamp (RIVM, Bilthoven) for their cooperation and zootechnical assistance.

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Chapter 7

Triazolodiazepines induce apo A-I synthesis and suppress apo(a) synthesis in primary cultures of cynomolgus monkey and human hepatocytes. No effect on apo B-100 synthesis.

Submitted

Triazolodiazepines induce apo A-I synthesis and suppress apo(a) synthesis in primary cultures of cynomolgus monkey and human Hepatocytes. No effect on apo b-100 synthesis.

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ABSTRACT

In this study we investigated the effect of three triazolodiazepines, Ro 11-1464, U-34599 and U-51477 on the synthesis of apolipoprotein A-I (apo A-I), apo(a) and apo B-100 in cultured cynomolgus monkey and human hepatocytes. Ro 11-1464, a thienotriazolodiazepine with no activity on behaviour showed a dose- and time-dependent increase of apo A-I synthesis in simian hepatocytes. Induction of 3.7-fold was found after 72 h of incubation with 100 µmol/L Ro 11-1464, and addition of 10 and 30 µmol/L resulted in 1.8 and 2.3-fold increases, respectively. Concomitantly apo(a) synthesis was dose- and time-dependently suppressed, showing a 66% decrease after 72 h of incubation with 100 µmol/L Ro 11-1464. Apo B-100 and albumin synthesis remained constant, indicating that the compound does not have a general effect on protein synthesis in simian hepatocytes. The structurally related benzotriazolodiazepines, U-34599 and U-51477, were equally active in stimulation of apo A-I synthesis and in inhibition of apo(a) synthesis. The changes in apo A-I and apo(a) synthesis were regulated at the (post)-transcriptional level: Steady-state apo A-I mRNA levels were increased (2.3-fold) after incubating the cells for 48h with 100 µmol/L Ro 11-1464, whereas the apo(a) mRNA level was decreased (-50%). Similar data as obtained in simian hepatocytes were found using human hepatocytes. We conclude that triazolodiazepines induce apo A-I synthesis and mRNA and down-regulate apo(a) synthesis and mRNA in simian and human hepatocytes and have no effect on apo B-100 synthesis.

INTRODUCTION

Plasma levels of high density lipoprotein (HDL) cholesterol are negatively associated with the risk of the development of coronary heart disease (CHD)(1,2). Apolipoprotein A-I (apo A-I), the major protein constituent of HDL, has been reported to be even more predictive than the HDL cholesterol level (3,4) and apo A-I synthesis is associated with the HDL-cholesterol level in the circulation *in vivo* (5,6). Furthermore, transgenic mice overexpressing human apo A-I have high plasma HDL and apo A-I levels and are more resistant to atherogenic diets (7). These data indicate that upregulation of the apo A-I synthesis may be useful in increasing the HDL level, which may subsequently decrease the risk of development of atherosclerosis. Apo B-100 is the sole apolipoprotein on low density lipoprotein (LDL), the level of which is positively associated with the risk of developing CHD. When apo B-100 in the LDL -particle is linked via a disulfide bridge to apo(a) a lipoprotein particle designated lipoprotein(a) (lp(a)) arises. Lp(a) plays an important role in both atherogenesis and fibrinolysis and high plasma levels of lp(a) are considered to be a risk factor for vascular diseases (8-10). Lp(a) levels vary strongly between individuals and are highly genetically controled (11-13). A small number of drugs such as sex steroids, nicotinic acid and neomycin in combination with niacin are described to lower the plasma levels of lp(a) in human (14, 15). Down-regulation of apo(a) synthesis may be a way to decrease lp(a) levels, since lp(a) plasma concentrations are mainly determined by the apo(a) synthesis level (16, 17).

Benzodiazepines are the most commonly prescribed tranquilizers having anxiolytic, anticonvulsant and sedative effects (18). Some studies suggest that benzodiazepines may influence serum lipid levels, but the data are not consistent. Increased HDL-cholesterol levels in insulin-dependent diabetes mellitus patients treated with a benzodiazepine have been reported (19). In contrast, in the LRC Study lower plasma HDL levels and higher total triglyceride and VLDL-cholesterol levels were observed in benzodiazepine users (20). In the present study we have investigated the effect of triazolodiazepine, Ro 11-1464, and of two structurally related benzotriazolodiazepines on the apo A-I, apo B-100 and apo(a) synthesis in cynomolgus monkey and human hepatocytes.

The availability of primary monkey and human hepatocytes, which are the only *in vitro* culture systems that synthesize apo A-I, apo B-100 and apo(a) endogenously, enabled us to study the regulation of synthesis of these apolipoproteins at the same time. These cells were shown to be a good model to study regulation apo A-I and apo B-100 synthesis (21, 22) and production of apo(a) (23, 24).

The present study shows that triazolodiazepines induce apo A-I synthesis and mRNA levels and suppress apo(a) synthesis and mRNA levels in cultured hepatocytes.

MATERIALS & METHODS

Materials

Ro 11-1464 was kindly provided by Dr H.J. Kempen (Hoffmann-La Roche, Basel, Switzerland). Chemical structures of the compounds are shown in Fig 1. Stock solutions of 100 mmol/L were prepared in DMSO and preserved at -20°C. Just before each culture experiment, the triazolodiazepines were diluted in culture medium, in such a way that the DMSO concentration did not exceed 0.1% (v/v). Materials used for the isolation and culturing of the human and simian hepatocytes were obtained from sources previously stated (21, 22, 25).

Triazolodiazepines

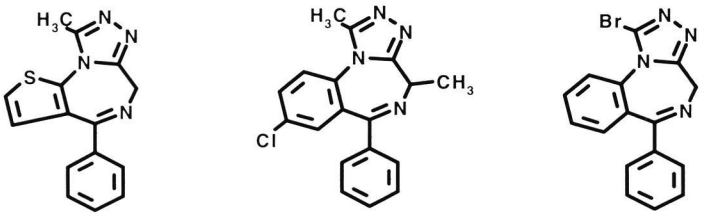

Fig 1. Chemical structures of the triazolobenzodiazepines

Simian and human 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 isolation procedure was exactly as described (21, 22, 24). Human hepatocytes were isolated following a procedure similar to that described previously (25). The hepatocytes were isolated from donor livers obtained from the University Hospital, Leiden, The Netherlands and which could not be used for transplantation due to technical problems. For each independent culture experiment, freshly isolated hepatocytes from a single monkey or human liver were used. The cells were seeded on culture dishes at a density of 2×10^5 viable cells per cm^2 and were maintained in Williams E medium supplemented with 10% heat-inactivated (30 min at 56°C) fetal calf serum (FCS), 2 mmol/L L-glutamine, 20 mU/ml insulin (135 nmol/L), 50 nmol/L

dexamethasone, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 100 µg/ml kanamycin 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. Twenty-four hours after seeding, the incubations with the triazolodiazepines in 0.1% DMSO were started using culture medium with a lower insulin concentration, 10 nmol/L instead of 135 nmol/L. The medium was renewed every 24 h and control incubations contained 0.1% (v/v) DMSO.

Measurement of apo A-I, apo B-100, apo(a) and albumin.

Total apo(a) concentrations were determined using the TintElize Lp(a) (Biopool AB, Umeå, Sweden). This ELISA uses polyclonal antibodies to human apo(a) both as catching and as detecting antibodies and detects in this manner free apo(a) as well as lp(a). The antibody of this kit showed strong immunological cross-reactivity with lp(a) from cynomolgus monkeys, indicating a high level of homology between human and cynomolgus lp(a) (23, 24). Apo A-I and apo B-100 concentrations in the collected culture medium were measured in triplicate using a sandwich enzyme-linked immunosorbent assay (ELISA) procedure with polyclonal antibodies to human apo A-I or human apo B-100, respectively, both as catching and detecting antibodies as described (21, 26). Cell-protein in the dishes was determined according to Lowry. Accumulation of albumin in the medium was measured by rocket immunoelectrophoresis according to the method described by Laurell (26, 27) using rabbit anti-human albumin (Dakopatts, Glostrup, Denmark).

RNA isolation and hybridization

Total RNA was isolated from cynomolgus or human hepatocytes by the method of Chomczynski and Sacchi (28) and the RNA samples were dissolved in formamide. Equal amounts of total RNA (10 µg) from different incubations were fractionated by electrophoresis in an 0.8% agarose gel containing 0.27 mol/L formaldehyde. Subsequently, the RNA was transferred to Hybond-N^+ (Amersham), in accordance with the manufacturer's instructions, and UV cross-linked. Probes, labeling conditions and hybridization were performed as described previously (21). Actin and GAPDH mRNA and 18S rRNA were used as internal standard since their levels were not affected by the triazolodiazepines used.

Apo(a) mRNA(s) were detected using a kringle IV synthetic double-stranded probe of 75 nucleotides with the sense sequence: GGGAATTCGAACCTGCCAAGC TTGGTCATCTATGACACCACACTCGCATAGTCGGACCCCAGAATAAAGCTTGGG, based on the sequence published by McLean *et al* (29). This probe was labeled by the random primer method according to Megaprime™ DNA labeling systems, Amersham

Life Science. After hybridization the blots were washed twice with 2x saline-sodium citrate (SSC)/1%SDS and twice with 1xSSC/1% SDS for 30 min at 65°C (1xSSC = 0.15 mol/L NaCl/ 0.015 mol/L sodium citrate, pH 7.0). The blots were exposed to a Fuji imaging plate type BAS-MP for 1 to 24 h. The relative amounts of mRNA were quantified using a phosphor-imager (Fuji Fujix BAS 1000) and the computer programs BAS-reader version 2.8 and TINA version 2.08c. The blots were re-used after they were stripped with 10 mmol/L Tris-HCl, 1 mmol/L EDTA, 1% SDS pH 7.5.

Statistical analysis

Statistical significance of differences was calculated by Student's t-test for paired data. Values of $P \leq 0.05$ were considered significantly different.

RESULTS

Ro 11-1464 increases apo A-I and decreases apo(a) synthesis in cultured simian hepatocytes

In Fig 2 the dose-dependency is shown of the effect of thienotriazolodiazepine Ro 11-1464 (see fig 1 for chemical structure) on the apolipoprotein synthesis by cynomolgus monkey hepatocytes. Ro 11-1464 increased the apo A-I synthesis dose - dependently, 1.8-fold when using 10 µmol/L Ro 11-1464 and 2.3 and 3.6-fold with 30 and 100 µmol/L, respectively (Fig 2a). Apo(a) synthesis, on the other hand, was dose-dependently decreased. Suppression of 24%, 52% and 66% was observed after 72 h of incubation with respectively 10, 30 and 100 µmol/L Ro 11-1464 (Fig 2b). To assess the time-course of the increased apo A-I synthesis and the decreased apo(a) synthesis, cells were exposed to 100 µM Ro 11-1464 for different lengths of time between 24 and 96 h of culture (Fig 3). Apo A-I synthesis was not changed after 24 h of incubation with the compound and thereafter increased gradually with increasing time of incubation (2.4 and 4.6-fold increase was observed after 48 h and 72 h of incubation, respectively) (Fig 3a), consistent with the slow and time-dependent increase observed after incubation of retinoids and fibrates (21, 22, 25). No significant change in apo(a) synthesis was seen after the first 24 h incubation period. After 48 h and 72 h of incubation apo(a) synthesis was significantly decreased by 22% and 42%, respectively (Fig 3b).

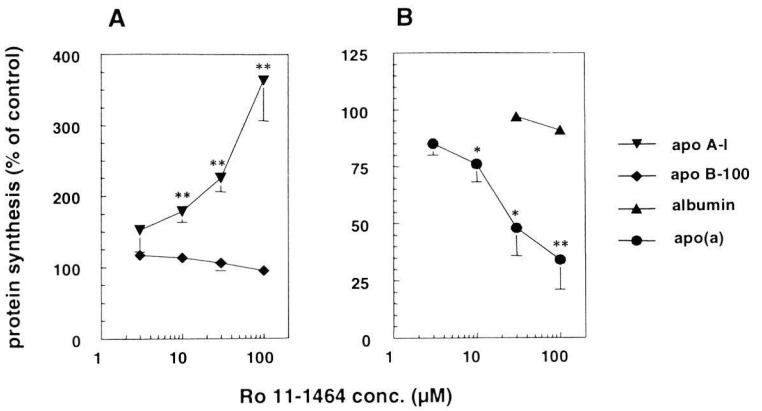

Fig 2. Dose-dependency of the effect of Ro 11-1464 on the apolipoprotein synthesis in cynomolgus monkey hepatocytes

After a 24 h attachment and recovery period, hepatocytes were cultured for 72 h with or without different concentrations of Ro 11-1464. Medium was renewed every 24 h. Apo A-I, apo(a) and apo B-100 and albumin concentrations were determined as described in "Materials and Methods", and were normalized for cell protein in the dishes. Data are expressed as a percentage of control. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 6 independent hepatocyte isolations. A) Apo A-I (▼) and apo B-100 (◆) synthesis. B) Apo(a) (●) and albumin (▲) synthesis.

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

** Indicates a significant difference ($P < 0.005$) between control and treated cells.

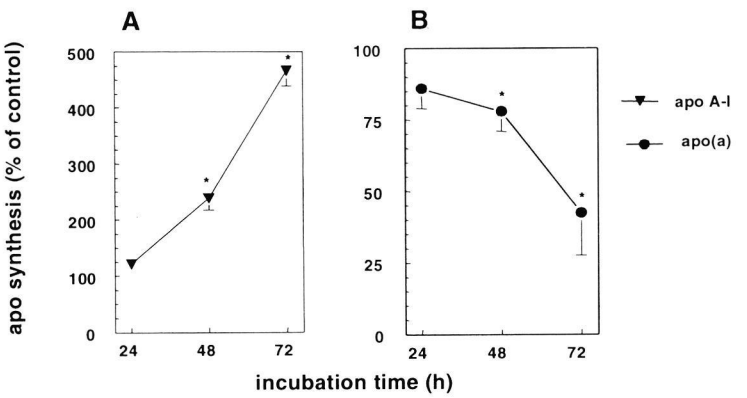

Fig 3. Time-dependency of the effect of Ro 11-1464 on the apolipoprotein synthesis in cynomolgus monkey hepatocytes.

After a 24 h attachment and recovery period, hepatocytes were cultured for 24, 48 or 72 h with or without 100 µmol/L Ro 11-1464. Medium was renewed every 24 h. Apo A-I and apo(a) concentrations were determined as described in "Materials and Methods", and were normalized for cell protein in the dishes. Data are expressed as a percentage of control. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 3 independent hepatocyte isolations. A) Apo A-I synthesis. B) Apo(a) synthesis.

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

Synthesis of apo B-100 was not significantly affected at any of the concentrations used (fig 2 and 3). Albumin secretion, determined as additional control protein, since it is the most abundantly synthesized protein in hepatocytes also did not change. Synthesis was 12 ± 1 and 11 ± 1 µg / 24 h per mg cell protein (mean ± range, n = 2) for the control and 100 µM Ro 11-1464 incubations, respectively. This indicates that the concentrations used were not toxic for the hepatocytes and that Ro 11-1464 does not have a general effect on hepatic protein synthesis.

Ro 11-1464 increases apo A-I and decreases apo(a) synthesis in primary human hepatocytes

To investigate whether Ro 11-1464 has similar effects on the apo A-I, apo(a) and apo B-100 synthesis in primary human hepatocytes, key experiments were performed using two independent cultures of scarcely available human hepatocytes.

Apo A-I synthesis was dose-dependently increased (Fig 4a). Although we used only two cultures, Ro 11-1464 seemed to be even more potent in human hepatocytes: apo A-I synthesis was 2.8, 6.2, and 7.2-fold increased after incubation for 72 h with respectively 10, 30 and 100 µmol/L Ro 11-1464. Due to low synthesis apo(a) was unfortunately only detectable in one culture, in which the synthesis decreased dramatically with increasing amounts of Ro 11-1464. Incubation with 10, 30 and 100 µmol/L Ro 11-1464 resulted in a decrease of 52%, 76% and 86%, respectively (Fig 4b). Apo B-100 and albumin synthesis in the human hepatocytes remained unchanged after incubation with Ro 11-1464 as in simian hepatocytes. Thus, the results obtained in primary cultures of human hepatocytes were similar to the results observed in monkey hepatocytes.

Structurally related benzotriazolodiazepines increase apo A-I and decrease apo(a) synthesis

To investigate the effect of structurally related benzotriazolodiazepines on apo A-I, apo(a) and apo B-100 synthesis in simian hepatocytes, various triazolodiazepines were tested. The benzotriazolodiazepines U-34599 and U-51477 (Fig 1), gave similar results to those obtained with the thienotriazolodiazepine Ro 11-1464. Apo A-I synthesis was elevated 3.6 and 3.9-fold, respectively with 100 µM of the compounds and apo(a) was suppressed by 58% and 38%, respectively. The compounds did not change the apo B-100 synthesis (Table I).

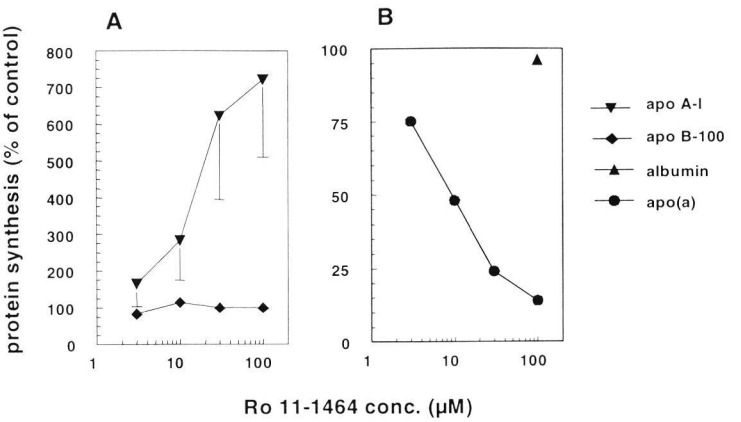

Fig 4. Dose-dependency of the effect of Ro 11-1464 on the apolipoprotein synthesis in human hepatocytes

After a 24 h attachment and recovery period, human hepatocytes were cultured for 72 h with or without different concentrations of Ro 11-1464. Medium was renewed every 24 h. Apo A-I, apo(a) and apo B-100 and albumin concentrations were determined as described in "Materials and Methods", and were normalized for cell protein in the dishes. Data are expressed as a percentage of control. Values are means (± range) of duplicate incubations of hepatocytes from 1 or 2 independent hepatocyte isolations. A) Apo A-I (▼) (n=2) and apo B-100 (◆) (n=2) synthesis. B) Apo(a) (●) (n=1) and albumin (▲) (n=2)

Table I. Effect of triazolobenzodiazepines on apo A-I, (a) and B-100 synthesis

	synthesis (% of control)		
triazolobenzodiazepines	**apo A-I**	**apo(a)**	**apo B-100**
100 µmol/L Ro 11-1464	363 ± 57%*	34 ± 13%*	95 ± 9%
100 µmol/L U-34599	359 ± 42%*	42 ± 11%*	102 ± 32%
100 µmol/L U-51477	390 ± 40%*	62 ± 22%*	95 ± 23%

Primary cynomolgus monkey hepatocytes were incubated for 72 h with various triazolodiazepines (for structures see fig 1). Apo A-I, apo(a) and apo B-100 synthesis were determined as described in the Materials and Methods section and expressed as a percentage of control. The values are normalized for the amount of cell protein in the culture dishes. Values are means ± S.E.M. of duplicate incubations of hepatocytes from 3-6 independent hepatocyte isolations.

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

Ro 11-1464 increases apo A-I and decreases apo (a) mRNA levels

mRNA levels were determined to investigate at which level Ro 11-1464 increases apo A-I and decreases apo(a) synthesis. Levels of actin and GAPDH mRNA and 18S ribosomal RNA, used as internal standards, were not affected by 100 µmol/L Ro 11-1464. Apo A-I mRNA increased in both monkey and human hepatocytes, 2.3- and 3.1-fold, respectively (Fig 5a,b). The apo(a) mRNA(s) (one or two distinct messengers, which ranged in size from about 10 to 12 kb and representing transcripts of different apo(a) alleles were observed) were decreased by about 50% in simian hepatocytes (Fig 5a,c). Similar results were found in a key experiment with human hepatocytes; apo(a) mRNA was decreased by 33%. The apo B-100 and albumin mRNA levels remained constant in both human and monkey hepatocytes (data not shown).

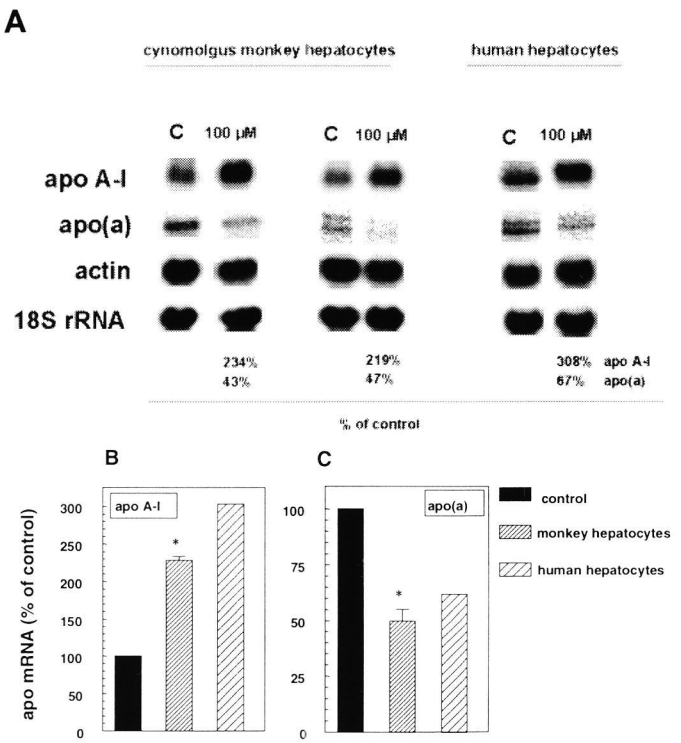

Fig 5. Effect of Ro 11-1464 on apo(a) mRNA levels in cynomolgus monkey hepatocytes
Total RNA was isolated from the hepatocytes which were incubated for 48 h with 100 µmol/L Ro 11-1464 or control medium. A) Phosphor Imager Scan of three representative Northern blots, two of monkey hepatocyte RNA and one of human hepatocyte RNA. The blots were hybridized with ^{32}P-labeled probes for apo(a), apo A-I, actin, and 18S rRNA. All mRNA bands were quantified and the apo(a) and apo A-I signals were corrected for 18S rRNA, which was used as internal standard. B) Histogram of the effect of Ro 11-1464 on apo A-I mRNA in monkey (mean ± S.E.M., n=3) and human hepatocytes (n=1) C) Histogram of the effect of Ro 11-1464 on apo(a) mRNA in monkey (mean ± S.E.M., n=3) and human hepatocytes (n=1)

DISCUSSION

In this paper we report that triazolodiazepines induce apo A-I and suppress apo(a) synthesis dose- and time-dependently in primary cynomolgus monkey and human hepatocytes. The regulation for both processes took place at the (post)-transcriptional level, since mRNA levels were changed. The two benzotriazolodiazepines U- 34599 and U-51477 displayed a similar effect as the thienotriazolodiazepine Ro 11-1464 on apo A-I and apo(a) synthesis. Apo B-100 and albumin synthesis were not affected by the compounds tested.

As far as we know this is the first paper concerning the effect of triazolodiazepines on apolipoprotein synthesis in a physiological culture system. Only a few *in vivo* studies appeared describing the effect of psychotropic benzodiazepines on serum lipoprotein levels, however, the results are not consistent. Because it has been reported that stress may affect serum lipid levels (30, 31), the effect of reducing stress on serum lipid levels was examined in patients with non-insulin dependent diabetes mellitus. The study showed that HDL-cholesterol levels were increased after administration of the anxiolytic fludiazepam, while total cholesterol levels remained unchanged (19). In contrast however, in the LRC Study in which the plasma lipid and lipoprotein levels of benzodiazepine users were compared to non-users, lower plasma HDL-cholesterol and higher total triglyceride and VLDL-cholesterol levels were observed in the group of benzodiazepine users (20). Lp(a) levels were not determined in any of these human studies. The reason for the discrepancies between the latter two studies is not known, but may be due to differences in patients groups and the kind of benzodiazepine used.

This study provides the first evidence that apo A-I and apo(a) synthesis are modulated by a direct effect of the triazolodiazepines on the hepatocyte. Apo A-I synthesis was dose- and time-dependently induced and apo(a) synthesis was dose- and time-dependently suppressed when primary monkey and human hepatocytes were incubated with Ro 11-1464, U-34599 and U-51477. The triazolodiazepine concentrations used are comparable to peak plasma levels found *in vivo,* being between 3 and 10 μM for triazolam, the most abundantly prescribed triazolobenzodiazepine in the United States (32). The effect of Ro 11-1464 in our study was significant for both apo A-I and apo(a) synthesis at 10 μM. We also used relatively high doses, comparable to the plasma concentrations of gemfibrozil (100 μM) (33) which also increases apo A-I synthesis (24, 34-36), but has no effect on apo(a) synthesis (24). Even higher concentrations are used to increase HDL-cholesterol and to decrease lp(a) levels with nicotinic acid, one of the few drugs which lowers lp(a) concentrations (peak plasma levels of 1.0-1.2 mM) (37).

The mechanism by which triazolobenzodiazepines influence the synthesis of apo A-I and apo(a) is not understood at present. We have shown that the regulation of both

processes is at the (post)-transcriptional level since the mRNA levels were changed, suggesting regulation of gene expression. However, no nuclear transcription receptors binding thieno- and benzotriazolodiazepines are described as far as we know. On the other hand the compounds may also affect stability of the mRNAs for apo A-I and apo(a). Further studies should elucidate the mechanism by which triazolodiazepines regulate the apo A-I and apo(a) synthesis, and *in vivo* studies may establish whether this class of compounds can be used in the treatment of atherosclerosis.

We conclude that triazolodiazepines induce apo A-I and suppress apo(a) synthesis and mRNA levels in simian and human hepatocytes, while apo B-100 synthesis is not affected. The induction of apo A-I synthesis and the down-regulation of the apo(a) synthesis together with unchanged apo B-100 synthesis may lead to a more favourable lipoprotein profile.

ACKNOWLEDGEMENTS

This work was supported by The Netherlands Heart Foundation (grant no. 92.328 to Diana M. Neele. We would like to thank Mr Jan Staal, Wim Vos and Joke van de Siepkamp (RIVM, Bilthoven) for their cooperation and zootechnical assistance.

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Chapter 8

Summary, Discussion and Future Perspectives.

SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

Summary and Discussion

Elevated plasma levels of LDL-cholesterol are positively associated with an increased risk of coronary heart disease (CHD), whereas an increased HDL-cholesterol level is negatively associated with CHD. Lp(a) which is composed of LDL and a high molecular weight glycoprotein, designated apo(a), plays an important role in both atherogenesis and fibrinolysis, and high plasma levels of lp(a) are considered to be a risk factor for vascular diseases. One way to reduce the risk of developing atherosclerosis is by manipulating the production of lipoproteins and in particular the synthesis of the apolipoproteins. Increasing the synthesis of apo A-I and decreasing apo B-100 and apo(a) synthesis may lead to enhanced HDL and decreased LDL and lp(a) levels and thus to a more favorable lipoprotein profile.

The lp(a) plasma concentration is under strong genetic control. However, this does not mean that there is no way to influence the lp(a) levels. A limited number of drugs have been reported to influence the lp(a) levels. Down-regulation of apo(a) synthesis may be a way of decreasing lp(a) levels, since apo(a) synthesis is the major level at which lp(a) concentrations *in vivo* are regulated.

In the studies described in this thesis we investigated the regulation of the synthesis of apo A-I and apo(a) by physiological modulators and we searched for ways to influence the regulation of the synthesis of these apolipoproteins in cultured hepatocytes. The availability of primary monkey and human hepatocytes, which are the only *in vitro* culture systems that synthesize apo(a) endogenously, enabled us to study the regulation of apo(a), apo A-I and apo B-100 simultaneously. Apo A-I is synthesized in equal amounts in the liver and intestine, whereas apo(a) and apo B-100 are synthesized only in the liver. At the time these studies were initiated little was known about the regulation of apo(a) and hardly any drugs were available to lower plasma lp(a) levels. The studies were started by evaluating whether cultured cynomolgus monkey hepatocytes are a good model for studying the regulation of apo A-I and apo B-100 synthesis and the production of apo(a) (chapter 4). The buoyant density of the lp(a) particle, which accumulated in the medium, was similar to that of lp(a) found in human or simian serum. About 50% of the total amount of apo(a) was found as a free protein, despite the fact that apo B-100-containing lipoproteins, mainly LDL, was present in excess. This indicates that not all of the synthesized apo(a) associates with LDL to form a lp(a) particle in hepatocyte cultures. Our finding is in agreement with other cell-culture and *in vivo* studies, showing that lp(a) assembly occurs extracellularly (on the cell surface of the hepatocyte), and that approximately half of apo(a) secreted by monkey hepatocytes is present as free apo(a). Apo B-100 was primarily found in a

particle with the buoyant density of LDL. Only a small part was present in a VLDL particle, suggesting that during the 24 h incubation period most of the VLDL was converted into LDL. On the other hand, it is also possible that apo B-100 is secreted mainly as an LDL-like particle, as in HepG2 cells. Apo A-I was found in the lipid-poor or lipid-free fraction as well as in a lipoprotein particle with the buoyant density of HDL. These findings indicated that primary cultures of cynomolgus monkey hepatocytes are a good model for studying (apo)lipoprotein synthesis and secretion. The different studies in which we investigated and influenced the regulation of the synthesis of apo(a) and apo A-I are discussed in more detail below.

The effect of insulin on apo(a) synthesis and mRNA levels in primary cultures of cynomolgus monkey hepatocytes was studied, since data from literature indicated that insulin may have an effect on the plasma lp(a) concentration. Elevated lp(a) levels in patients with insulin-dependent diabetes mellitus (IDDM) were lowered by insulin therapy. However, data on lp(a) levels in noninsulin-dependent diabetes mellitus patients (NIDDM) are less consistent. The biochemical background of the changes in lp(a) was investigated, and apo A-I and apo B-100 synthesis were determined as control signals (chapter 2). Addition of insulin to the hepatocyte cultures had a mildly but significantly decreasing effect on apo(a) secretion (about 30%). Steady state apo(a) mRNA levels paralleled the decrease in apo(a) synthesis, indicating that the decreased synthesis is regulated at the (post)-transcriptional level. This study provides the first direct evidence that hepatic apo(a) synthesis is influenced by insulin. The concentrations we used (1-1000 nM) are higher than those found *in vivo*, but relevant considering the fact that hepatocytes degrade insulin extensively during the 24-h culture periods. Although the effect of insulin was not very strong, it was consistently seen in all independent cultures. A similar decrease in lp(a) plasma levels of about 30% was found in several *in vivo* studies in which IDDM patients with poor metabolic control were treated with insulin. Our results indicate that the regulation by insulin takes place at apo(a) synthesis level and not at the level of lp(a) clearance because the apo(a) mRNA was decreased by 30%, which suggests that the regulation occurs at the level of gene expression. Apo B-100 secretion, which was 30%-36% decreased when using 10-1000 nM in our study, compared well with several *in vitro* studies using primary human and rat hepatocytes and HepG2 cells which showed decreases of 30%-60%. As in the latter studies, apo B-100 mRNA did not change after incubation with insulin in our study. In contrast to the decreasing effect of insulin on apo(a) and apo B-100 secretion, apo A-I and albumin secretion and mRNA remained unchanged. These data may provide an explanation for the increased plasma levels of lp(a) as found in patients with insulin-dependent diabetes mellitus.

Our group has previously shown that retinoids stimulate apo A-I synthesis in cultured cynomolgus hepatocytes after a 24-h lag phase. We have investigated this in more

detail by studying the biochemical background of the slow response, the requirement for high retinoic acid concentrations and the involvement of different retinoid receptors (chapter 3). The time course of the effect of 10 µmol/L all-*trans* retinoic acid (at-RA) on apo A-I mRNA levels and protein secretion were comparable. By performing run-off assays we showed that, in contrast to apo A-I mRNA, apo A-I gene transcription was already increased after a 4-h incubation with at-RA. However, the transcription rate decreased after prolonged incubation (24 h) in the same medium. Furthermore, we showed that in the presence of cynomolgus hepatocytes at-RA disappeared rapidly from the medium: after 2 h of incubation 40% of the added amount was left and after 24 h only 2%. This suggests that at longer incubation times without renewing the medium, as was the case after a continuous 24 h-incubation period, retinoids may not be present at high enough levels for maximal induction of apo A-I gene expression. We think that apo A-I mRNA levels in our culture system are increased in a pulsatory way. The presence of a cellular pool of apo A-I mRNA, which is relatively stable and large with respect to the suggested small pulsatory increases, may explain the slow and time-dependent increase in apo A-I mRNA and apo A-I secretion.

We also investigated whether retinoids were able to affect the synthesis of apo(a) and the formation of lp(a) (chapter 4). In contrast to the increasing effect of retinoids on apo A-I synthesis, addition of at-RA to the medium of the simian hepatocytes resulted in a dose- and time-dependent decrease of *de novo* apo(a) synthesis (about 50%). Apo B-100 synthesis remained constant in all experiments, indicating that at-RA does not have a general effect on apolipoprotein synthesis in hepatocytes. Whereas retinol (Vitamin A) was not active, 9-*cis*-RA and 13-*cis*-RA, two natural isomers of at-RA, also inhibited the apo(a) synthesis although to a lesser extent than at-RA. Steady state apo(a) mRNA levels paralleled the decrease in apo(a) synthesis, indicating that the decreased synthesis is regulated at the (post)-transcriptional level. As in apo A-I synthesis, in apo(a) synthesis the effect was also delayed. No significant change was observed after 24 h of incubation with at-RA. This may indicate that as with apo A-I mRNA, a large and stable pool of apo(a) mRNA exists, which causes a retarded decrease of the apo(a) synthesis. Unfortunately, it was not possible to study the apo(a) gene transcription rate, because of the high sequence homology of apo(a) with plasminogen and other genes.

The effects of retinoids are mediated by two families of nuclear retinoid receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The two receptor types, each of which consists of 3 subclasses (α,ß and γ), are members of the thyroid/steroid receptor superfamily and function as ligand-dependent transcription factors. The retinoid receptors bind as RAR/RXR heterodimers or RXR/RXR homodimers to specific retinoid response elements in target genes. Both the apo A-I promoter and the apo(a) promoter contain a retinoid response element. In our primary

hepatocytes, transcriptional activity and mRNA of RARα, RARβ and RXRα were detectable (chapter 3). The involvement of these retinoid receptors in the increased gene transcription of apo A-I and the decreased apo(a) synthesis was investigated using receptor-specific ligands. The RXR-agonist (3M-TTNEB) did not have an effect on the apo(a) synthesis, whereas the RAR-agonist (TTNPB) resulted in a decreased apo(a) synthesis. This indicates that retinoids decrease apo(a) synthesis by the involvement of RARα and/or RARβ and not the RXRα-homodimer in cynomolgus hepatocytes. Both compounds induced the apo A-I synthesis (chapter 3 & 4).

Different effects of retinoids have been reported *in vitro* and *in vivo* with respect to the regulation of apo A-I and apo A-II and recently increased levels of lp(a) were found in two acute promyelocytic leukemia patients treated with at-RA. In view of these multiple and divergent effects of retinoids on apolipoprotein synthesis *in vivo* and *in vitro*, we decided to investigate the effect of retinoids on lp(a) levels *in vivo* in rhesus monkeys (chapter 4) and in an intervention study in volunteers (chapter 5). The monkeys received 1 mg/kg at-RA, the most potent retinoid in the *in vitro* study, per day or placebo orally for 4 weeks. This retinoid is used clinically in the treatment of leukemia. We chose a dose relevant to the human situation and used *in vivo* experiments addressing the pharmacokinetics and toxicology of RA *in vivo*. After supplementation of the monkeys with at-RA no significant change in plasma lp(a) level was observed when compared to the control group, whereas the plasma triglyceride level increased significantly in the treated group, indicating that the monkeys responded to the retinoid. Total cholesterol and apo B-100 levels were not changed during retinoid treatment when compared to the control group.

Isotretinoin (13-cis RA) was used in the intervention trial with human volunteers. It is known to be effective in the treatment of dermatological diseases like for instance acne. In the latter study we evaluated the short-term effect of isotretinoin on lp(a), apo A-I, apo A-II and the HDL subclasses lpA-I and lpA-I:A-II in a randomized, double-blind, placebo-controlled, cross-over study in 10 healthy male volunteers. Isotretinoin (80 mg per day for 5 days) had no effect on plasma levels of lp(a). In addition, no association was found between baseline lp(a) levels of the subjects and the change in lp(a) after isotretinoin treatment indicating that high initial lp(a) levels were also not affected by the drug. On the other hand, clear and significant changes in total cholesterol (+9%) and apo B-100 (+8%) were observed in the same period of this study, indicating that the retinoid was active. In contrast to our data, enhanced lp(a) levels in two patients with acute promyelocytic leukemia (APL) treated with at-RA were reported. It should be noted however, that APL patients are metabolically unstable which among other things is noticeable in the fluctuation of lp(a) values. In contrast, in another study in which patients with severe acne were treated with 0.5 mg/kg/day isotretinoin, decreased lp(a) levels were observed. The reason for the discrepancies between our *in vivo* data and

the latter studies is unclear. The differences may be related to the incomparable patient groups, different lp(a) baseline levels and duration of the studies.

We also found no change in lpA-I, lpA-I:A-II, apo A-I, apo A-II and HDL-cholesterol level, although the HDL composition appeared to be altered. These results do not necessarily contradict former studies in which an increase in apo A-I and apo A-II gene expression by retinoids was found in cultured hepatocytes. It is still conceivable that retinoids enhance apo A-I and apo A-II synthesis *in vivo*, which is not reflected in a raised apo A-I, apo A-II and HDL plasma level. No change or a slight decrease of the HDL level may partially be secondary to the reported increase in triglyceride-rich particles upon treatment with retinoids. Thus, for apo(a) as well as for apo A-I opposite effects of retinoids *in vivo* and *in vitro* are found. The reason for this is not completely clear but changes in the state of differentiation of cultured cells may underlie the discrepancy between data obtained in *in vivo* and *in vitro* experiments with retinoids. It is well known that retinoids are morphogens whose functions are most pronounced during development and cellular differentiation. Additionally, retinoids have been shown to have secondary effects on plasma lipid levels as exemplified by hypertriglyceridemia induced by retinoids, and unchanged plasma levels of lp(a), apo A-I and the HDL subclasses do not necessarily exclude changes occurring at the apolipoprotein synthesis level. Furthermore, a different metabolism of retinoids *in vivo* and *in vitro* can also not be excluded. We can conclude that the retinoids, which have been used so far, lead to an unfavorable lipoprotein profile and are not suitable for clinical use.

Data on the effect of fibrates on lp(a) levels in man are, like the data on insulin and retinoids, not consistent. Fibrates are a group of drugs which have been shown to decrease plasma levels of triglyceride-rich lipoproteins and LDL, and to increase HDL. We assessed the effect of fibrates on apo(a) and apo A-I synthesis, and investigated whether different fibrates, commonly used in clinical practice, have different effects on the synthesis of these apolipoproteins (chapter 6). The addition of gemfibrozil to cultures of monkey hepatocytes had no effect on apo(a) synthesis, but resulted in a dose- and time-dependent increase of apo A-I synthesis and mRNA. A similar stimulation of the apo A-I synthesis of about 2-fold was observed in human hepatocytes, whereas apo(a) synthesis remained unchanged as in monkey hepatocytes. Other fibrates like bezafibrate and clofibrate did not change the apo(a) synthesis either but they enhanced the synthesis of apo A-I, although less potently than gemfibrozil.

As stated above, data on the effects of fibrates on lp(a) levels *in vivo* are not conclusive. Decreased, increased and unchanged lp(a) serum levels are reported when hyperlipidemic subjects are treated with different fibrates. It is suggested that the divergent results are due to differences in the patient groups, in which the lp(a) concentrations are highly skewed and in which often no correction is made for the

genotype/phenotype. Furthermore, the duration of the intervention, the dose of fibrate administered, and the type of fibrate used might be responsible for the inconsistent results. The latter two suggestions are however not likely when our *in vitro* data are taken into account, since the type of fibrate and the concentrations we used are comparable to the *in vivo* situation. Because lp(a) levels are mainly regulated at the synthesis level of apo(a), which is not affected in our study in monkey and human hepatocytes, it is not likely that lp(a) levels *in vivo* are affected at the level of apo(a) synthesis.

Benzodiazepines are the most commonly prescribed tranquilizers worldwide, having anxiolytic, anticonvulsant and sedative effects. Some studies suggest that benzodiazepines may influence serum lipid levels, but the data are not consistent. Increased HDL-cholesterol levels in insulin-dependent diabetes mellitus patients treated with a benzodiazepine have been reported, but others reported lower plasma HDL levels in benzodiazepine users. So far, no studies were performed to evaluate the effect of benzodiazepines on lp(a) levels. We have investigated the effect of three triazolodiazepines, Ro 11-1464, U-34599 and U-51477 on the synthesis of apo A-I, apo(a) and apo B-100 in cultured cynomolgus monkey and human hepatocytes (chapter 7). Ro 11-1464, a thienotriazolodiazepine with no effect on behavior showed a dose- and time-dependent increase of apo A-I synthesis in simian hepatocytes. Induction of 3- to 4-fold was found after incubation with 100 µMol/L Ro 11-1464. Concomitantly, apo(a) synthesis was dose- and time-dependently suppressed, showing a 60-70% decrease when using 100 µMol/L Ro 11-1464. Apo B-100 and albumin synthesis remained constant, indicating that the compound does not have a general effect on protein synthesis in simian hepatocytes. Similar data to that obtained in simian hepatocytes were found using human hepatocytes. Although we used only two cultures, Ro 11-1464 seemed to be even more potent in human hepatocytes: apo A-I synthesis was increased about 7-fold after incubation with the compound, whereas the apo(a) synthesis decreased dramatically by about 80-90%. The structurally related benzotriazolodiazepines, U-34599 and U-51477, were equally active in stimulating apo A-I synthesis and in inhibiting apo(a) synthesis.

The triazolodiazepine concentrations used are comparable to peak plasma levels found *in vivo,* being between 3 and 10 µMol/L for triazolam, the most commonly prescribed triazolobenzodiazepine in the United States. The molecular mechanism by which triazolobenzodiazepines influence the synthesis of apo A-I and apo(a) is not understood at present. We have shown in cell culture experiments that the regulation of both processes is at the (post)-transcriptional level: Steady-state apo A-I mRNA levels were increased (about 2-3-fold), whereas the apo(a) mRNA level was decreased (-50%). This suggests that regulation takes place at gene expression level. However, no nuclear transcription receptors binding thieno- and benzotriazolodiazepines have been

described as far as we know. On the other hand the compounds may also affect the stability of the mRNAs for apo A-I and apo(a).

The induction of apo A-I synthesis and the down-regulation of the apo(a) synthesis together with unchanged apo B-100 synthesis may lead to a more favorable lipoprotein profile. Further *in vitro* studies should elucidate the exact mechanism by which triazolodiazepines regulate apo A-I and apo(a) synthesis. *In vivo* studies may establish whether this class of compounds can be used in the treatment of atherosclerosis in the future.

Future Perspectives

It is well established that patients with a high lp(a) plasma level (>20-30 mg/dl) in combination with an increased concentration of LDL-cholesterol have an enhanced risk of developing cardiovascular diseases. These patients may benefit from a decrease in lp(a) levels and this may also be true for patients who have an impaired fibrinolysis and high plasma levels of lp(a). At present there are very few clinically useful ways of lowering lp(a) levels in patients with high lp(a) plasma concentrations. Although some drugs are reported to lower lp(a) levels, the use of these compounds is limited in clinical practice due to side effects. So, developing new drugs is important and test models are needed to accomplish this. The availability of the primary human- and monkey hepatocytes described in this thesis, and the recent development of the human lp(a) and apo(a) YAC transgenic mice may be very valuable in this context, and can be used to search for and to develop potential new ways to lower lp(a) plasma levels.

It is of great interest to study the mechanism of action of compounds which are already described as influencing the lp(a) levels. Compounds such as nicotinic acid, growth hormone, acute phase mediators and estrogens are known to affect the lp(a) plasma levels, but the mechanism by which they act remains largely to be solved. The primary hepatocytes, and also instance promoter reporter studies may be very useful in explaining by which molecular mechanism the compounds act. Unravelling the molecular mechanism of action of the hormones and drugs may lead to new insights to affect the apo(a) synthesis and plasma lp(a) concentrations. Finally, *in vivo* studies in apo(a) and lp(a) transgenic mice should be performed to validate the *in vitro* findings, since discrepancies between *in vivo* and *in vitro* results may exist. To exclude species differences, key experiments in monkeys should determine whether new compounds may be useful in the treatment of atherosclerosis in the future.

In this thesis we suggest that triazolodiazepines may be promising compounds for reducing the risk of cardiovascular diseases. The compounds do not only decrease the apo(a) synthesis, but they also increase apo A-I synthesis in the primary hepatocytes. Experiments to reveal the mechanism of action of the triazolodiazepines at the molecular level are needed and the experiments should be validated *in vivo*.

Nederlandse Samenvatting

In Nederland overlijden ruim 50.000 mensen per jaar aan hart- en vaatziekten. Dit is bijna 40% van het totaal aantal sterfgevallen. Een belangrijke oorzaak van hart- en vaatziekten is atherosclerose, vaatvernauwing door cholesterol stapeling in de binnenwand van de slagaderen, verbindweefseling en proliferatie van cellen. Vernauwing van een slagader kan leiden tot zuurstof gebrek in delen van het lichaam, met name in het hart, hersenen en benen, door een afname van de bloedvoorziening. Als een slagader totaal is afgesloten, het weefsel geen bloed meer krijgt en afsterft spreekt men van een infarct. Bekende voorbeelden van slagader afsluiting zijn het hartinfarct en het herseninfarct. De afsluiting ontstaat doordat een stolsel zich vormt op de verdikte en beschadigde vaatwand. Belangrijke risicofactoren voor het krijgen van atherosclerose naast o.a. roken, hoge bloeddruk en diabetes mellitus (suikerziekte) is een verhoogde concentratie van cholesterol en triglyceride in het bloed. Hoewel te hoge cholesterol en triglyceriden concentraties de kans op hart- en vaatziekten vergroten en zelfs levensbedreigend kunnen zijn, zijn beide onmisbaar voor het goed functioneren van het lichaam. Cholesterol is een essentieel bestanddeel van de celwanden (celmembranen) van alle dierlijke en menselijke cellen. Bovendien gebruikt het lichaam cholesterol voor de vorming van hormonen en van galzuren, welke nodig zijn voor de opname van vetten uit de darm. Triglyceriden worden in het spierweefsel als energiebron en in vetweefsel als energieopslag gebruikt. Cholesterol en triglyceriden kunnen via de voeding worden opgenomen. Driekwart van het cholesterol wordt in ons lichaam door de lever gemaakt.

Cholesterol en triglyceriden zijn vetachtige stoffen die niet oplosbaar zijn in waterig milieu zoals bloed. Om deze vetachtige stoffen te kunnen transporteren via het bloed worden ze gebonden aan verschillende eiwitten. Deze lipide-eiwit complexen worden lipoproteinen genoemd. De eiwitten die deel uitmaken van deze lipoproteinen, de zogenaamde apolipoproteinen zijn van belang voor het vervoer van het cholesterol in het bloed en voor o.a. de synthese en klaring van lipoproteinen. Er worden verschillende lipoproteinen onderscheiden: de chylomicronen, het VLDL (zeer lage dichtheids-lipoproteine), het LDL (lage dichtheids-lipoproteine), het HDL (hoge dichtheids-lipoproteine) en het lp(a) (lipoprotein(a)). De lipoproteinen verschillen onderling in dichtheid, grootte, lipide- en eiwit samenstelling en hebben ook een verschillende functie.

Chylomicronen vervoeren lipiden die via de voeding worden opgenomen van de darm naar de lever en raken onderweg de triglyceriden kwijt. In de lever worden de opgenomen lipiden en het door de lever gemaakte cholesterol verpakt en als VLDL deeltje uitgescheiden in het bloed zodat het naar spier- en vetweefsel kan. Nadat het grootste gedeelte van de triglyceriden uit het deeltje zijn verwijderd ontstaat het

cholesterolrijke LDL-deeltje (het "slechte" cholesterol). Tweederde van het LDL wordt weer uit het bloed opgenomen door de lever met behulp van de zogenaamde LDL-receptoren, eenderde gaat naar andere weefsels en cellen voor groei en hormoon produktie. Als er niet genoeg receptoren aanwezig zijn, of als ze niet goed functioneren kan de LDL concentratie in het bloed te hoog worden. Een verhoogde LDL concentratie in het bloed leidt tot een grotere kans dat het LDL in de vaatwand terecht komt. LDL kan onder invloed van de cellen in de vaatwand geoxydeerd worden, waarna het opgenomen kan worden door zogenaamde macrofagen. Dit proces kan leiden tot de vorming van schuimcellen welke direct betrokken zijn bij het ontstaan van de vaatverdikking.

HDL wordt gemaakt in de lever en de darm. Het haalt het cholesterol weer op uit de weefsels en cellen van het lichaam en brengt het terug naar de lever zodat het omgezet kan worden en via de gal verdwijnt. HDL wordt ook wel het "goede" cholesterol genoemd.

Het lipoproteine deeltje lp(a) is een LDL deeltje waaraan een extra eiwit is gekoppeld, apolipoproteine(a). In 1963 is lp(a) voor het eerst beschreven en sindsdien is er veel onderzoek naar gedaan. De functie van dit lipoproteine is echter nog steeds niet duidelijk. Apolipoproteine(a) (apo(a)) is een bijzonder groot eiwit en toont sterke gelijkenis met plasminogeen, dat betrokken is bij het oplossen van bloedstolsels. De lp(a) concentraties verschillen sterk van persoon tot persoon en zijn voor een groot gedeelte genetisch bepaald. Apo(a) wordt door hepatocyten (een bepaald soort levercellen) gemaakt in de lever. Nadat het eiwit gesynthetiseerd is, wordt het getransporteerd naar de buitenkant van de hepatocyt. Daar blijft het hangen totdat er een LDL-deeltje voorbij komt. Dit lipoproteine deeltje zorgt ervoor dat apo(a) loskomt van de celwand en er een lp(a) deeltje ontstaat. Net zoals LDL is lp(a) een risicofactor voor het ontstaan van hart- en vaatziekten en wordt het gevonden in atherosclerotische plaques. Vooral mensen met een hoge lp(a) concentratie in het bloed (>20-30 mg/dl) in combinatie met een verhoogde LDL-cholesterol concentratie hebben een verhoogde kans op atherosclerose. De gelijkenis van apo(a) met plasminogeen zorgt er bovendien nog voor dat lp(a) in staat is het oplossen van bloedstolsels te remmen.

Wat kunnen we doen aan een te hoog LDL-cholesterol en lp(a) concentratie in het bloed? Mensen met een te hoog cholesterol gehalte moeten in eerste instantie hun dieet aanpassen. Daarnaast zijn er tegenwoordig verschillende medicijnen, zoals statines en fibraten, die het LDL cholesterolgehalte en de triglyceriden (m.n. fibraten) in het bloed doen dalen. Echter, deze medicijnen hebben nauwelijks of geen invloed op de lp(a) concentratie in het bloed. De behandelingsmethoden ter verlaging van een te hoge lp(a) concentratie zijn slecht te gebruiken in de kliniek omdat ze of te duur zijn (plasmaforese) of omdat ze gepaard gaan met veel bijwerkingen (nicotinezuur). Dus het is van groot belang medicijnen te ontwikkelen die de lp(a) synthese kunnen

remmen en zodoende de concentratie in het bloed kunnen verlagen. Toen wij met het in dit proefschrift beschreven onderzoek begonnen was er nog erg weinig bekend over de regulatie van de synthese van apo(a) en lp(a). Er waren wel een aantal hormonen en stoffen bekend die de lp(a) concentratie konden beinvloeden, maar via welk mechanisme dit ging was niet duidelijk. Lp(a) is moeilijk te bestuderen omdat het niet in alle dieren aanwezig is. Slechts in mensen, Oude Wereld apen en egels wordt dit lipoprotein gemaakt door de lever. Wij hadden in ons laboratorium de mogelijkheid primaire apenhepatocyten en primaire humane hepatocyten te kunnen gebruiken. De apen levers van de Java aap (Macaca fascicularis) waren afkomstig van het Rijksinstituut voor Volksgezondheid en Milieu waar de nieren van deze dieren worden gebruikt om poliovaccin mee te maken. De humane donor levers konden we gebruiken omdat ze door o.a. beschadiging niet geschikt waren voor transplantatie. Met behulp van deze hepatocyten konden we de regulatie van de apo(a) synthese bestuderen en naar mogelijkheden zoeken om de synthese snelheid te verlagen. Bovendien waren we in staat om gelijktijdig de twee meest voorkomende apolipoproteinen in het bloed te bestuderen, namelijk apo A-I, het belangrijkste eiwit op HDL en apo B-100, het enige eiwit op LDL. Apo B-100 wordt net zoals apo(a) alleen in lever gemaakt, terwijl apo A-I in zowel de darm als de lever wordt gesynthetiseerd. Verhoging van de apo A-I synthese en verlaging van de apo B-100 en apo(a) synthese moet in principe leiden tot een gunstige verandering in de concentratie van HDL, LDL en lp(a), waardoor het risico op atherosclerose kan worden verlaagd.

Om de experimenten te kunnen uitvoeren moesten we eerst de hepatocyten uit de levers isoleren. De cellen hebben we vervolgens gekweekt in kweekschaaltje met medium (voedsel voor de cellen). De apolipoproteinen werden door de hepatocyten gemaakt en gevonden in lipoprotein deeltjes met de dichtheden van HDL, LDL en lp(a). Apo(a) werd ook gevonden als een vrij deeltje, niet gebonden aan een LDL partikel.

Het effect van insuline op de apo(a) synthese en mRNA niveau's hebben we bestudeerd, omdat in de literatuur werd aangegeven dat insuline mogelijk een effect heeft op de plasma lp(a) concentratie. Verhoogde lp(a) concentraties in patiënten met diabetes mellitus type I kunnen worden verlaagd door middel van insuline therapie. Wij hebben de biochemische achtergrond van deze verlaging onderzocht en beschreven in hoofdstuk 2. Na toevoeging van insuline aan de hepatocyten zagen we een kleine maar significante verlaging van de apo(a) synthese en het apo(a) mRNA van 30%. Voor zover wij weten is dit de eerste studie die laat zien dat insuline een direct effect heeft op de apo(a) synthese in de lever. Onze *in vitro* (buiten het lichaam/in een celkweek systeem) resultaten komen goed overeen met de resultaten die gevonden worden *in vivo*, in mensen met suikerziekte. De plasma lp(a) concentratie is met ± 30% verlaagd in diabetes mellitus type I patiënten na behandeling met insuline. Apo B-

100 synthese was ook verlaagd in onze experimenten (30-36%) en dit kwam goed overeen met studies gedaan door andere onderzoeksgroepen waaruit een zelfde verlaging bleek. Hieruit konden we bovendien opmaken dat we een goed celsysteem gebruiken om het effect van insuline op de apo(a) produktie te onderzoeken.

Onze groep heeft in voorgaande studies laten zien dat retinoïden zoals Vitamine A-zuur (all-trans retinoic acid, at-RA) de apo A-I synthese kunnen stimuleren. Het doel van de studie beschreven in hoofdstuk 3 was om uit te zoeken welke receptoren betrokken zijn bij de stimulatie van apo A-I synthese. Bovendien wilden we weten waarom we zulke hoge retinoïd concentraties nodig hadden en waarom het even duurde voordat we een effect konden meten. Met behulp van de zogenaamde nuclear run off assays hebben we aangetoond dat de apo A-I gentranscriptie al na 4 uur was verhoogd. Echter, na 24 uur kweken (in hetzelfde medium) was de gentranscriptie weer terug op het basale niveau. Waarschijnlijk gebeurt dit omdat de hepatocyten de retinoïden heel snel omzetten tot inactieve componenten. Na 2 uur kweken was er nog maar 40% van de toegevoegde at-RA over en na 24 uur kweken nog maar 2%. Dit geeft aan dat de at-RA in de 24-uurs kweekperiodes niet meer in voldoende hoge concentraties aanwezig is om de apo A-I gentranscriptie maximaal te kunnen stimuleren. De aanwezigheid van de relatief grote en stabiele apo A-I mRNA pool, vergeleken met de pulserende mRNA toename, zou de verklaring kunnen zijn van de langzame en tijdsafhankelijk apo A-I mRNA en eiwit toename.

We hebben ook bekeken of retinoïden in staat zijn om de apo(a) synthese en de lp(a) formatie te beïnvloeden (hoofdstuk 4). In tegenstelling tot het stimulerende effect van retinoïden op de apo A-I synthese heeft toevoeging van retinoïden aan de hepatocyten een dosis en tijdsafhankelijk verlagend effect op de apo(a) synthese (50%), apo(a) mRNA en lp(a) vorming. Apo B-100 bleef gelijk in alle experimenten wat aangeeft dat retinoïden geen algemeen effect op de hepatocyt hebben. Naast at-RA waren 13-cis- en 9-cis retinoinezuur (isomeren van vitamine A-zuur) ook in staat de apo(a) produktie te verlagen. Retinol (vitamine A) daarentegen kon dit niet bewerkstelligen.

De effecten van retinoïden worden gemediëerd door twee in de celkern voorkomende retinoïd receptor families, de retinoïnezuur receptoren (RARs) en de retinoïd X receptoren (RXRs). Beide families zijn opgebouwd uit 3 subtypes (α,β en γ), behoren tot de thyroid/steroid superfamilie en functioneren als ligand afhankelijke transcriptiefactoren. De receptoren binden als RAR/RXR heterodimeren of als RXR homodimeren aan specifieke retinoïd receptor bindingsplaatsen (RAREs of RXREs) op de promoter regio's van bepaalde genen. Zowel het apo A-I gen als het apo(a) gen bevat zo'n bindingsplaats. Met receptor-specifieke liganden hebben we onderzocht welke receptoren betrokken zijn bij de beïnvloeding van de apo A-I en apo(a) synthese. In de apehepatocyten komen RARα, RARβ en RXRα tot expressie. Het RXR-

specifieke ligand bleek geen invloed te hebben op de apo(a) synthese, terwijl het RAR-specifieke ligand de apo(a) synthese reduceerde. Dit suggereert dat de RARα en/of de RARβ en niet de RXR-homodimeer in de apenhepatocyten betrokken zijn bij verlaging van de apo(a) synthese. Beide stoffen konden de apo A-I synthese verhogen (hoofstuk 3 en 4) wat aangeeft dat RARα en/of RARβ en RXRα betrokken zijn bij de activatie van de apo A-I expressie door retinoïden.

Verschillende *in vivo* (in proefdieren) en *in vitro* (in cellen) effecten van retinoïden zijn gerapporteerd wat betreft de regulatie van apo A-I, apo A-II en apo(a) synthese en de lp(a) plasma concentratie. Niet zo lang geleden is er een artikel verschenen waarin een verhoging van de lp(a) concentratie werd gevonden nadat leukemie patiënten waren behandeld met at-RA. Echter, in een andere publicatie werd een verlaging van de lp(a) plasma concentratie gerapporteerd nadat acné patiënten waren behandeld met isotretinoïne. Met het oog op deze uiteenlopende resultaten hebben we de effecten van retinoïden op lp(a) concentraties bestudeerd *in vivo* in rhesus apen (hoofdstuk 4) en in een interventie studie met vrijwilligers (hoofdstuk 5). De apen kregen 4 weken lang 1 mg/kg at-RA per dag, maar dit resulteerde niet in een verandering van de lp(a) concentratie. De triglyceriden waren verhoogd de na behandeling wat erop wijst dat de dieren wel hebben gereageerd op het retinoïd.

Isotretinoïne (13-cis vitamine A-zuur) is een retinoïd dat wordt gebruikt in de behandeling van dermatologische ziekten, zoals bijvoorbeeld acné. Wij hebben dit retinoïd in een placebo-gecontroleerde, cross-over studie gebruikt, waaraan 10 gezonde mannelijke vrijwilligers meededen. De mannen kregen 5 dagen lang 80 mg isotretinoine per dag toegediend. Na behandeling waren de lp(a) concentraties niet veranderd. Totaal cholesterol en apo B-100 concentraties waren toegenomen, wat aangeeft dat isotretinoïne opgenomen en actief was in het lichaam. We hebben ook geen verandering gevonden in de lpA-I, LpA-I:AII (HDL-subklassen), apo A-I, apo A-II en HDL-cholesterol concentraties, maar de samenstelling van het HDL deeltje leek veranderd te zijn. Hoewel de *in vivo* resultaten de *in vitro* resultaten tegen lijken te spreken hoeft dit toch niet het geval te zijn. De retinoïden zouden de genexpressie van zowel apo A-I als apo(a) kunnen beïnvloeden, terwijl dit op plasma niveau niet te zien is. Door de verhoogde triglyceride concentratie kan de samenstelling van het lp(a) deeltje veranderd zijn waardoor het anders (langzamer) geklaard wordt. Bovendien kan de verhoogde triglyceride concentratie een toename van de apo A-I genexpressie overschaduwen. Maar een ander metabolisme van retinoïden *in vitro* en *in vivo* kan ook niet uitgesloten worden. We kunnen concluderen dat de retinoïden die we tot nu toe gebruikt hebben een ongunstig lipoproteïne profiel tot gevolg hebben en niet geschikt zijn om in de kliniek te gebruiken.

Een groep medicijnen die al veel wordt voorgeschreven om triglyceriden en LDL-cholesterol te verlagen zijn de fibraten. Het effect van fibraten op de lp(a) concentratie

in patiënten is niet eenduidig. Wij hebben het effect van verschillende, veel voorgeschreven, fibraten op de apo(a) en apo A-I produktie in de humane- en apenhepatocyten getest (hoofdstuk 6). Geen van de geteste fibraten (gemfibrozil, clofibraat, clofibrinezuur en bezafibraat) was in staat de apo(a) synthese te veranderen in de humane- en apenhepatocyten. Daarentegen konden alle fibraten de apo A-I synthese en mRNA niveau's verhogen. Op grond van onze resultaten lijkt het niet voor de hand te liggen dat fibraten de lp(a) concentratie *in vivo* kunnen veranderen op apo(a) synthese niveau. Dit is bovendien het niveau waarop de plasma lp(a) concentratie voornamelijk wordt gereguleerd.

Het laatste hoofdstuk (7) van dit proefschrift beschrijft een studie met o.a. benzodiazepines, de meest voorgeschreven kalmerende middelen (hoofdstuk 7). Uit een aantal studies bleek dat kalmerende middelen de plasma lipiden concentraties konden beïnvloeden, maar de resultaten zijn niet eenduidig. Zowel verhoogde als verlaagde HDL concentraties zijn gerapporteerd. Wij hebben het effect van deze stoffen op de apo(a) synthese bestudeerd. Hiervoor hebben wij hebben drie verschillende triazolodiazepines gebruikt Ro 11-1464, U-34599 en U-51477. Ro 11-1464 een thienotriazolodiazepine zonder kalmerende werking, had een dosis en tijdsafhankelijk verhogend effect (3 tot 4 voudig) op de apo A-I synthese in de apenhepatocyten. De apo(a) synthese was daarentegen verlaagd tot ± 30-40% van de controle. De apo B-100 en albumine produktie veranderden niet. Dezelfde resultaten werden gevonden in de humane hepatocyten. Volgens welk mechanisme de diazepines de apo A-I synthese verhogen en apo(a) synthese verlagen is tot nu toe niet duidelijk. We hebben kunnen laten zien dat beide processen waarschijnlijk op (post)-transcriptioneel niveau gereguleerd worden omdat zowel het apo A-I als het apo(a) mRNA waren veranderd. Vervolg studies zullen het exacte mechanisme moeten ontrafelen.

De inductie van de apo A-I en de verlaging van apo(a) synthese samen met een onveranderde apo B-100 synthese zou kunnen leiden tot een gunstiger lipoprotein profiel. Met behulp van *in vivo* studies zou onderzocht kunnen worden of deze stoffen in de toekomst gebruikt kunnen worden in de behandeling van atherosclerose.

List of publications

Full papers

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Abbreviations

apo	apolipoprotein
at-RA	all-*trans* Retinoic Acid
ELISA	Enzyme Linked Immunosorbent Assay
FCS	Fetal Calf Serum
HDL	High Density Lipoprotein
HMGCoA	3-Hydroxy-3-Methylglutaryl-Coenzyme A
IDL	Intermediate Density Lipoprotein
LDL	Low Density Lipoprotein
Lp(a)	Lipoprotein(a)
LPDS	Lipoprotein Depleted Serum
3-Methyl-TTNEB	4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)-ethenyl] benzoic acid
PBS	Phosphate Buffered Saline
RA	Retinoic Acid
RAR	Retinoic Acid Receptor
RARE	Retinoic Acid Responsive Element
RXR	Retinoid X Receptor
RXRE	Retinoid X Responsive Element
SD	Standard Deviation
SEM	Standard Error of Mean
TG	Triglyceride
TTNPB	(E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid
VLDL	Very Low Density Lipoprotein

Dankwoord

Graag wil ik in dit nawoord alle mensen bedanken zonder wie dit proefschrift nooit was verschenen.
Allereerst Hans, veel dank voor alles wat jij mij over en van het onderzoek heb geleerd. Van de hepatocyten isolatie (niet één infectie) tot aan het schrijven van artikelen (vooral kort en bondig). Jouw precisie zal ik waarschijnlijk nooit krijgen, maar een poging heb ik zeker gedaan, het woord "futiliteit" staat in mijn geheugen gegrift. Mijn promotor, prof. Brakman wil ik graag bedanken voor zijn hulp, met name bij de afronding van dit proefschrift. Helen Richardson ben ik zeer erkentelijk voor de engelse correcties en Joke v/d Siepkamp, Wim Vos, Jan Staal en collega's van het RIVM in Bilthoven voor de isolatie van de apen levers. Studente, Natascha de Haas voor de bijdrage aan de insuline studie. Elly, het feit dat ik altijd bij je terecht kon met praktische vragen over het labwerk en je helpende hand tijdens de "kweekweekenden" heb ik zeer op prijs gesteld. Sabine, altijd was je een luisterend oor en maakte je tijd voor een bak koffie al had je het razend druk. Ontzettend fijn dat je mijn paranimf wilt zijn! Richard, geloof het of niet, maar sinds jij het Gaubius hebt verlaten werken wij allemaal 2x zo snel en is het aantal decibellen in onze kamer met een factor 5 gedaald! Maaike, gelukkig is onze samenwerking niet beperkt gebleven tot een paar kweken, veel dank voor je vriendschap. Miek, Wendy en Martine, mijn "congresmaatjes", mede dankzij jullie was de spanning voor een presentatie best dragelijk en heb ik bijzonder genoten van alle reizen. Verder wil ik alle andere collega's van het Gaubius bedanken die op directe of indirecte manier hebben bijgedragen aan dit proefschrift.
Mijn hele familie en alle vrienden wil ik graag bedanken voor hun belangstelling en speciaal Jolanda, dank je wel dat je me paranimf wilt zijn! Lieve Carla, bijna niemand was zo geïnteresseerd in mijn onderzoek als jij. Dank je wel, je was en bent een ontzettend grote stimulans. Liefste mama, al begreep je soms niet precies het hoe en waarom van mijn onderzoek je stond altijd en immer voor me klaar. Heel veel dank daarvoor en ik weet zeker dat papa ons, op welke manier dan ook, in de gaten houdt en net zo trots is als jij! Aangezien woorden toch niet voldoende zijn, allerliefste Goffe, voor jou een hele dikke kus!

Diana

Curriculum Vitae

Adriana Maria Neele werd geboren op 31 oktober 1968 in Zaandam. In 1988 behaalde zij haar VWO-diploma aan het Sint Michaël College te Zaandam. In augustus van datzelfde jaar werd begonnen met de studie Biologie aan de Universiteit van Amsterdam. De propedeuse Biologie werd behaald in augustus 1989, waarna vervolgens het doctoraal examen in de Medische Biologie werd afgelegd in juni 1994. In het kader van het docteraal examen werden twee onderzoeksstages gedaan waarvan één op de afdeling Kindergastro-enterologie en Voeding van het Academisch Medisch Centrum (AMC) te Amsterdam (Drs. E.H. Van Beers en Prof. Dr. H.A. Büller) en de ander op de afdeling Pediatric Gastroenterology and Nutrition van het New England Medical Center, Tufts University te Boston, USA (Dr. R.J. Grand en Dr. R.K. Montgomery). Voor het onderzoek gedaan in Boston ontving zij in oktober 1993 de Student abstract prize van de American Gastroenterological Association Foundation. De Parke-Davis prijs mocht zij in oktober 1994 in ontvangst nemen voor hetzelfde onderzoek.

Van juni 1994 tot juni 1998 was zij werkzaam als assistent in opleiding aan het Leids Universitair Medisch Centrum (LUMC) op de afdeling inwendige geneeskunde op een door de Nederlandse Hartstichting gesubsidieerd project (#92.328). Ze was gedetacheerd bij TNO Preventie en Gezondheid, Gaubius Laboratorium te Leiden. Gedurende deze periode werd onder leiding van Dr. Hans M.G. Princen het in dit proefschrift beschreven onderzoek verricht. Op basis van de resultaten beschreven in hoofdstuk 4 ontving zij in juli 1996 Young Investigator Award van het 66th Congress of the European Atherosclerosis Society.