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Inhibition of Apolipoprotein(a) Synthesis in Cynomolgus Monkey Hepatocytes by Retinoids via Involvement of the Retinoic Acid Receptor

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ABSTRACT. We have shown previously that retinoids induce apolipoprotein (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 apolipoprotein(a) (apo(a)) synthesis in cultured hepatocytes was investigated. The addition of all-*trans* retinoic acid (at-RA) to the medium of the hepatocytes resulted in a dose- and time-dependent decrease in apo(a) synthesis. Maximal inhibition was 54% after 72 hr 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 72 hr, 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 apo(a) synthesis, whereas retinol was not active. To investigate which retinoid receptors are involved in the inhibition of apo(a) synthesis, specific retinoid X receptor (RXR) and retinoic acid receptor (RAR) ligands were used. 4-[1-(3,5,5,8,8-Pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)-ethenyl] benzoic acid (3-methyl-TTNEB), a specific RXR agonist, did not have an effect on apo(a) synthesis, whereas incubation with (E)-4-[2-(5,5,8,8-tetramethyl-5,6,7,8-tetrahydro-2-naphthalenyl)-1-propenyl] benzoic acid (TTNPB), a specific RAR agonist, resulted in a decrease of 34%. Steady-state apo(a) mRNA levels were decreased by 42% and 33% after the cells were incubated for 48 hr with 10 μmol/L at-RA and TTNPB, respectively, indicating that the decreased synthesis is regulated at the (post)transcriptional level. We conclude that retinoids down-regulate apo(a) synthesis and mRNA via involvement of RAR and not the RXR homodimer in cynomolgus hepatocytes. BIOCHEM PHARMACOL **58**;2: 263–271, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. apolipoprotein(a); retinoids; RAR; RXR; cynomolgus monkey hepatocytes

Lp(a)† is a lipoprotein particle in which apo(a) is attached to LDL via a disulfide bridge. Lp(a) plays an important role in atherogenesis, and high plasma levels of lp(a) are considered to be a risk factor for vascular diseases (for review see [1]). Apo(a) synthesis is the major level at which lp(a) concentrations *in vivo* are regulated [2, 3]. Down-regulation of apo(a) synthesis, therefore, may be a way to decrease lp(a) levels, thereby reducing the risk of developing cardiovascular diseases. 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 [4, 5]. Nicotinic acid and (sex) steroids 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 of fibrates on lp(a) levels and apo(a) synthesis [6, 7] have been reported, but decreased [8–10] as well as increased lp(a) serum levels have been observed [11]. Similarly, data on the effects of retinoids on lp(a) levels and apo(a) synthesis are inconsistent. Recently, decreased lp(a) levels were reported in acne patients treated with 0.5 mg/kg/day isotretinoin [12]; no effect of short-term treatment with isotretinoin on lp(a) levels in healthy men was found [13]; and increased levels of lp(a) were reported in two acute promyelocytic leukemia patients treated with at-RA [14]. *In vitro* studies show similar discrepancies: At-RA stimulated apo(a) promoter activity when a promoter–reporter construct was transfected in HepG2 cells [14], but suppression of apo(a) synthesis in simian hepatocytes was also reported [15]. In view of these divergent effects of retinoids, we investigated the effect of retinoids on the synthesis of apo(a) in cultured cynomolgus hepatocytes.

Primary cultures of monkey hepatocytes are one of the few cell models which synthesizes and secretes apo(a) and in which the effect of modulation by drugs and hormones can be studied [6, 15, 16]. We used primary hepatocyte cultures from cynomolgus monkey (*Macaca fascicularis*),

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† *Abbreviations:* apo, apolipoprotein; apo(a), apolipoprotein(a); at-RA, all-*trans* retinoic acid; FBS, fetal bovine serum; HDL, high-density lipoprotein; LDL, low-density lipoprotein; lp(a), lipoprotein(a); RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; 3-methyl-TTNEB, 4-[1-(3,5,5,8,8-pentamethyl-5,6,7,8-tetrahydro-2-naphthyl)-ethenyl]benzoic acid; 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; and RARE, retinoic acid responsive element.

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because these cells were shown to be a good model for studying regulation apo A-I, apo B-100, and apo(a) synthesis [6, 15, 17–19].

The effects of retinoids are mediated by two families of nuclear retinoid receptors, the RARs and the RXRs. These 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 either as heterodimers of RXR with RAR or another nuclear receptor or as RXR/RXR homodimers to specific retinoid X and retinoic acid responsive elements (RXRE's and RAREs) in target genes (for reviews see 20–22).

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, [23, 24] we were able to demonstrate that the RAR and not the RXR homodimer is involved in the suppression of apo(a) synthesis.

MATERIALS AND METHODS

Materials

At-RA, retinol, and 13-*cis*-RA were purchased from Sigma Chemical Co. 9-*cis*-RA was a generous gift from Mr. P. Weber and Dr. F. Schneider, Hoffmann-La Roche Ltd. 3-Methyl-TTNEB and TTNPB were kindly provided by Dr. S. Karathanasis, American Cyanamid Company and by Dr M. Issandou, Laboratories Glaxo, Centre de Recherches. Since retinoids are light-sensitive, the stock solutions, which were prepared in DMSO and preserved at −20° in the dark, were diluted in culture medium just before it was used to renew the medium of the cells. All incubations, i.e. 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 [6, 17–19].

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.) *ad lib.* with one or two pieces of additional fruit per day and were fasted overnight before killing. The isolation procedure was performed as described previously [6, 17–19]. The cells were seeded on culture dishes at a density of 1.5×10^5 viable cells per square cm and were maintained for the first 24 hr in Williams E medium supplemented with 10% heat-inactivated (30 min at 56°) FBS (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° in a 5% CO_2/95% air atmosphere. After 14–16 hr 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 hr. At the end of each incubation period, the medium was collected and after the last incubation, the cells were also collected for determination of cellular protein.

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

Total apo(a) concentrations were determined using the TintElize Lp(a) (Biopool AB) as described previously [6, 17]. This ELISA uses polyclonal antibodies to human apo(a) both as catching and as detecting antibodies and thus detects 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). 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-I and apo B-100 concentrations in the medium were measured using a sandwich ELISA with polyclonal antibodies to human apo A-I or human apo B-100, respectively, both as catching and detecting antibodies as described previously [17–19]. Analysis of apo(a)-, apo A-I-, and apo B-100-containing lipoproteins secreted by primary hepatocytes was performed as previously described by Kaptein *et al.* [25]. Briefly, culture medium was collected at the end of a 48-hr incubation period with simian hepatocytes cultured in lipoprotein-depleted medium. Lipoprotein-depleted serum was prepared from heat-inactivated FBS by density gradient ultracentrifugation. After the medium was harvested, protease inhibitors were added and density gradient ultracentrifugation was performed. Subsequently, the gradient was fractionated and the density was measured. The fractions were dialyzed, and the lp(a) (Innotest ELISA), apo(a) (TintElize Lp(a), Biopool), apo A-I, and apo B-100 concentrations in the fractions were determined by the ELISA procedure.

RNA Isolation and Hybridization

Total RNA was isolated from cynomolgus hepatocytes as described previously [17–19]. Equal amounts of total RNA (10 μg) from different incubations were fractionated by electrophoresis in a 0.8% agarose gel containing 0.27 mol/L

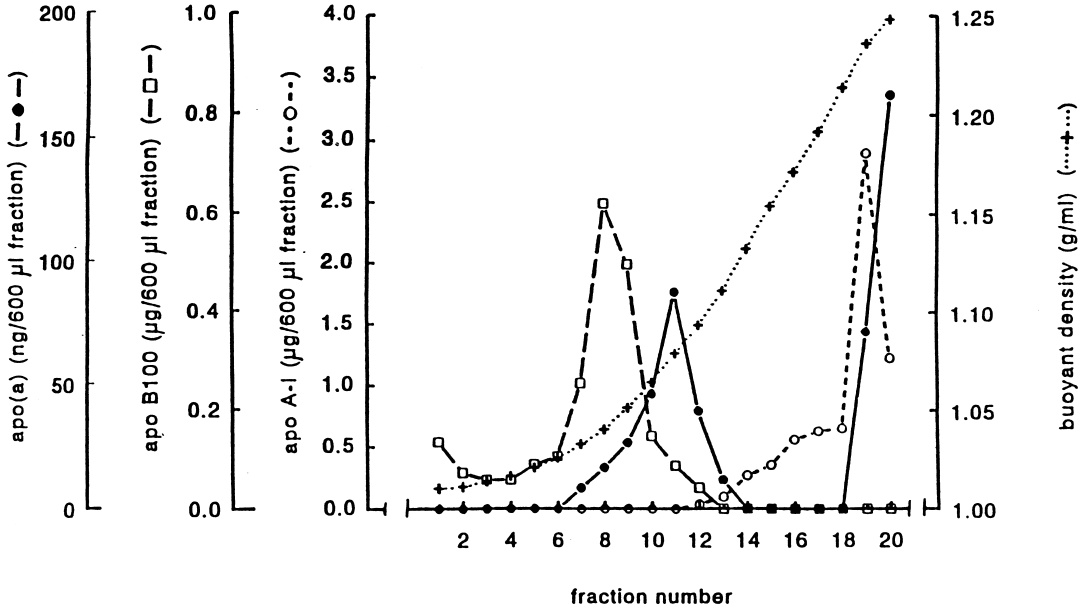

FIG. 1. Density gradient pattern of apo(a)-, apo B-100-, and apo A-I-containing lipoproteins in the culture medium of cynomolgus monkey hepatocytes. Cynomolgus monkey hepatocytes were cultured for 48 hr in standard medium with lipoprotein-deficient FBS, after a 48-hr preincubation in the same medium. Medium (4 mL) 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.

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 [17–19]. Apo(a) mRNA(s) were detected using a kringle IV synthetic double-stranded probe of 75 nucleotides with the sense sequence: GGGAATTC-GAACCTGCCAAGCTTGGTCATCTATGACACCA-CACTCGCATAGTCGGACCCCAGAATAAAGCT-TGGG, based on the sequence published by McLean *et al.* [26]. 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 2× saline-sodium citrate (SSC/1%SDS) and twice with 1 × SSC/1% SDS for 30 min at 65° (1 × SSC = 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 hr. 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

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. Correlation coefficients were determined using Pearson's correlation analysis.

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 Materials and Methods section), 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-hr culture period and 56 ± 6% in the second 48-hr culture period for 4 independent cultures. The total apo(a) production in control medium during the first incubation period was 53 ± 18 ng/24 hr per milligram of cell protein (mean ± SD), 80 ± 55 during the second period, and 124 ± 70 (range, 68–210 ng/24 hr 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 that most apo A-I was found in the lipid-poor or lipid-free fraction. Most apo B-100 was present in a lipoprotein particle with the buoyant density of LDL

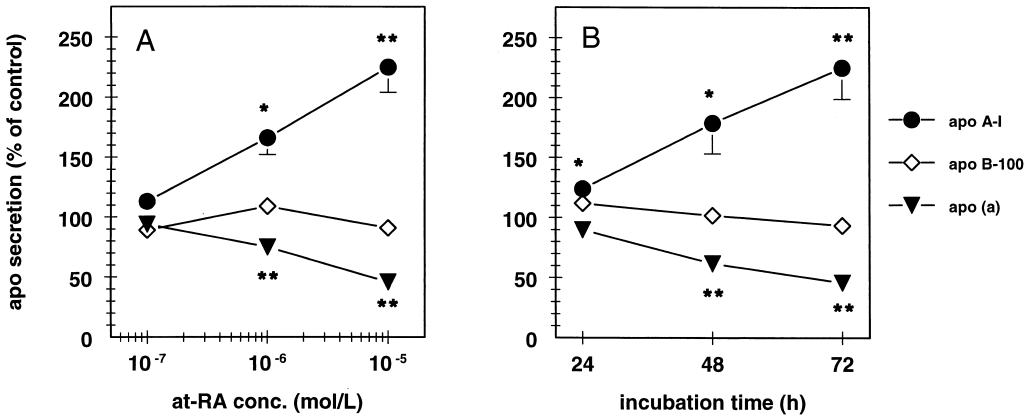

FIG. 2. Dose and time dependency of the effect of at-RA on the apolipoprotein synthesis of cynomolgus monkey hepatocytes. After a 24-hr attachment and recovery period, hepatocytes were cultured for 24, 48, or 72 hr with or without different concentrations of at-RA. Medium was renewed every 24 hr. Apo(a), 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. Some error bars are too small to be visible in the figure. (A) Hepatocytes were incubated for 72 hr with various concentrations of at-RA. Values are means ± SEM of duplicate incubations of hepatocytes from 4–7 independent hepatocyte isolations. (B) Hepatocytes were incubated for 24, 48 and 72 hr with 10 μmol/L at-RA. Values are means ± SEM 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.

(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-hr incubation period (from 72–96 hr of culture age) for 7 independent hepatocyte cultures was 1542 ± 809 (range, 795–2888) and 555 ± 251 (range, 289–1015) ng/24 hr 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.

Dose Dependency and Time-Course of the Effect of at-RA on Apo(a) Synthesis

In Fig. 2A, the dose dependency of the effect of at-RA on apo(a) synthesis by cynomolgus monkey hepatocytes is shown. The effects of at-RA on the synthesis of apo A-I and apo B-100 [18] are presented for comparison. The addition of increasing amounts of at-RA to the medium resulted in a dose-dependent decrease in apo(a) synthesis. The effect was significant after the addition of 1 μmol/L at-RA for 72 hr (−25%). Maximal suppression of 54% was observed after incubation with 10 μmol/L at-RA for 72 hr. The lp(a) concentration in the medium was decreased to the same extent (−42%) as the total amount of apo(a). Metabolic labeling experiments showed that *de novo* synthesis of apo(a) was decreased to a similar extent as apo(a) secretion (data not shown). 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-hr incubation period. After 48 hr of incubation, apo(a) synthesis was decreased by 38%, and the effect of at-RA was even more pronounced after 72 hr of incubation (−54%). Apo A-I synthesis was slightly, but significantly increased (+24%) after 24 hr with 10 μmol/L at-RA, and by 79% and 125% after 48 and 72 hr of incubation, respectively. Apo B-100 synthesis did not change significantly during the incubation periods.

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. The results are shown in Table 1.

TABLE 1. 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 hr 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 ± SEM 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.

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 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%.

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 [23, 24]. Figure 3 shows the effects of the RXR-specific ligand 3-methyl-TTNEB and of the RAR-specific ligand TTNPB on apo(a) synthesis. Apo(a) synthesis was inhibited by 34% when the hepatocytes were incubated for 72 hr with 10 μmol/L of the RAR agonist. Interestingly, incubation with 10 μmol/L of the RXR agonist did not inhibit 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 in accordance with a previous report [19]. 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.

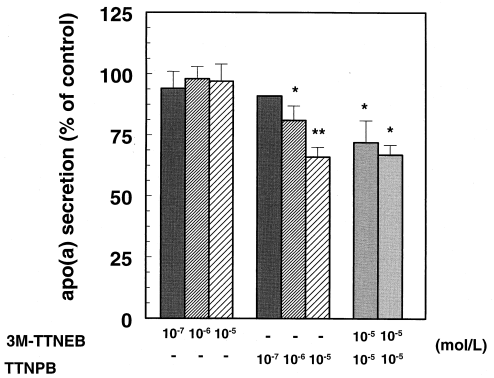

FIG. 3. Effect of retinoid receptor-specific ligands on apo(a) synthesis. Primary cynomolgus monkey hepatocytes were incubated for three consecutive 24-hr periods with increasing concentrations of TTNPB (RAR agonist) and 3-methyl-TTNEB (RXR agonist), and with a combination of both compounds. Apolipoprotein(a) synthesis was determined in the last 24-hr 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 ± SEM 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.

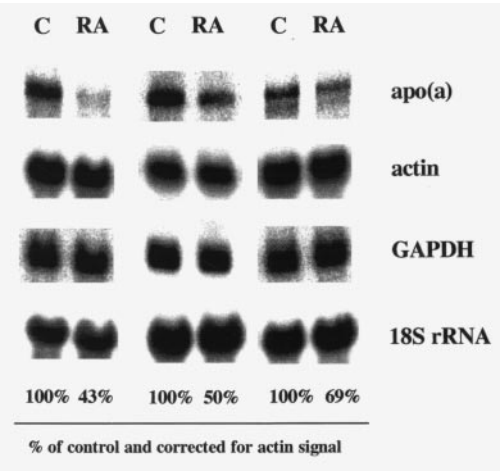

FIG. 4. Effect of at-RA on apo(a) mRNA levels in cynomolgus monkey hepatocytes. Total RNA was isolated from hepatocytes incubated for 48 hr with 10 μmol/L at-RA or control medium. Phosphor Imager Scan of three representative Northern blots hybridized with ^{32}P-labeled probes for apo(a), actin, GAPDH, and 18S rRNA is shown. All mRNA bands were quantified and the apo(a) signals were corrected for the actin, GAPDH, and 18S signals, which were used as internal standards.

Effect of at-RA on Apo(a) mRNA Levels

mRNA levels were determined to investigate at which level retinoids inhibit apo(a) synthesis. 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 represent transcripts of different apo(a) alleles (Fig. 4). 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, $P < 0.05$, $N = 4$) after incubation of the hepatocytes for 48 hr with 10 μmol/L at-RA. A similar decrease (33 ± 7%, $P < 0.05$, $N = 3$) was found with 10 μmol/L TTNPB. The apo B-100 mRNA level remained constant in these experiments (data not shown).

DISCUSSION

In this study, we showed that retinoids inhibit the synthesis of apo(a) in a dose- and time-dependent way in cynomolgus monkey hepatocytes and that regulation takes place at the (post)transcriptional level. By using receptor-specific reti-

noids, 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. 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 [27, 28]. After density gradient ultracentrifugation, about 50% of the total amount of apo(a) was found as a free protein, whereas apo B-100-containing lipoproteins, mainly LDL, were present in excess. Four to five times more apo B-100 than apo(a) was synthesized in the third culture period (72–96 hr), indicating that not all of the synthesized apo(a) associates with LDL to form an lp(a) particle in hepatocyte cultures. This is in agreement with other cell culture studies which showed that approximately half the apo(a) secreted by monkey hepatocytes is present as free apo(a) [16]. It has been demonstrated that lp(a) assembly occurs extracellularly [16, 29, 30]. Newly synthesized apo(a) binds to the hepatocyte surface where apo(a) comes into contact with apo B-100 and is released from the cell surface as a lipoprotein particle [31]. Here, 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, which suggests that during the 24-hr incubation period the VLDL may be 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 [32]. 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.

When primary cynomolgus monkey hepatocytes were incubated with at-RA, a dose- and time-dependent decrease in apo(a) secretion was observed. Lp(a) concentration in the medium was lowered to the same extent as the apo(a) concentration. We found no correlation (Pearson) between apo(a) production in untreated cells and the change in production induced by 10 μmol/L at-RA, indicating that the effect of retinoids is independent of factors influencing basal apo(a) synthesis. 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 indicates that the decrease in apo(a) secretion is the result of a decreased mRNA level. The finding that apo(a) synthesis was decreased after a lag phase of 24 hr suggests that the half-life of apo(a) mRNA is relatively long, as also reported for apo A-I [19]. Our previous observation that gene expression of the retinoid receptors relevant for regulation of apo(a) synthesis (RARα, RARβ, and RXRα) remains constant during culture [19] and the fact that the concentration of the precursor of the ligands for these receptors (retinylacetate) in the culture medium is the same throughout culture suggests that the retinoid receptors are not (directly) involved in the time-dependent increase in apo(a) synthesis. This makes it unlikely that the inhibition of apo(a) synthesis by retinoids is only a blocking of the increase in apo(a) synthesis during culture time. 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 in 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 hr of incubation, approximately 60% of the added at-RA has disappeared from the medium, whereas after 24 hr only 2% of at-RA is left [19]. Thus, the high concentrations of retinoids applied in this study are necessary to maintain a sufficiently high concentration during the 24-hr 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 [33–35]. We have compared the effect of these natural retinoids. Retinol was not active in the suppression of apo(a) synthesis, although apo A-I synthesis was induced in the same experiments, 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 or in transactivation of gene transcription and that the compound first has to be metabolized to at-RA to become active [36]. 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 naturally occurring retinoids to each other in these metabolically active hepatocytes. Moreover, 9-*cis*-RA, which has been shown to be the natural ligand for the RXR, is also able to activate the RAR, whereas at-RA is only able to activate the RAR [34, 35, 37]. To establish which retinoid receptors are involved in the suppression of apo(a) synthesis, retinoid receptor-specific ligands were applied [23, 24]. 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 were observed. In contrast, the RAR-specific ligand, TTNPB, decreased apo(a) synthesis and mRNA dose dependently. These results indicate that the RXR homodimer is not involved in the decrease in 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 [21, 22, 38–40]. Our data suggesting that RXR can act as a transcriptionally silent partner [22] are in line with the finding that RXR ligand binding is not necessary for the RXR/RAR dimer to be active [22, 41]. We conclude that only RAR is actively involved in decreased apo(a) synthesis. In contrast, both RAR-selective (TTNPB) and RXR-selective (3-methyl-TTNEB) agonists induced apo

A-I synthesis indicating that both RAR and RXR are involved in the activation of apo A-I gene expression [19].

The apo(a) mRNA level was found to be decreased after retinoid treatment with both at-RA and TTNPB. This finding, together with the fact that RARs act as nuclear transcription factors, suggests that regulation takes place at the transcription level. This contention is supported by the presence of several half-sites of the RARE sequence, AG G/T TCA (for review see [20]), in the 1.4 kb 5′ flanking region of the human apo(a) gene and the baboon apo(a) gene as described by Wade *et al.* [42] and Hixson *et al.* [43], respectively. Recently, Ramharack *et al.* [44] identified a retinoid response element at position −1036 in the human apo(a) promoter, which interacted with RAR/RXR heterodimers in electrophoretic mobility shift assays.

In line with our results, Ramharack *et al.* observed a similar suppression of apo(a) synthesis by retinoids using only two cultures of cynomolgus hepatocytes [15]. However, no experiments were performed to reveal which retinoid receptors were involved in the reported decrease in apo(a) synthesis. In contrast to the latter study and our study, Azuma *et al.* [14] reported an increased apo(a) promoter activity when a relatively short human apo(a) promoter–reporter construct (−444 to −3 bp) was transiently transfected in HepG2 cells and incubated with at-RA. The reason for this discrepancy is unclear, but may be due to several factors. First, transfected HepG2 cells are not comparable with primary hepatocytes. Then, differences in the expression of retinoid receptors may influence the results: retinoic acid receptors are essential for the decreasing effect of retinoids on apo(a). Furthermore, only 442 bp of the apo(a) promoter were used for the transfection, although it has been suggested that longer fragments of the apo(a) promoter must be used in order to analyze the transcriptional regulation of the apo(a) gene [45, 46]. As stated above, a RARE was found to be located further upstream [44], and functional direct repeats of the nuclear hormone receptor half-site AGGTCA were found in two enhancer regions located far upstream in the human apo(a) promoter [45]. In addition, no control experiments using mutation (deletion or substitution) constructs were performed by Azuma *et al.* [14].

With respect to the influence of retinoids on lp(a) plasma levels, data of *in vivo* studies are inconsistent. Recently, decreased lp(a) levels were reported in acne patients treated with 0.5 mg/kg/day isotretinoin [12]. Azuma *et al.* [14] showed enhanced lp(a) levels in two patients with acute promyelocytic leukemia (APL) treated with at-RA, and no effect of isotretinoin on lp(a) levels in healthy volunteers was reported by our group [13]. The various results may be related to differences in patient groups and treatment period. Azuma *et al.* treated only two APL patients. These patients are metabolically unstable which, among other things, is noticeable in the fluctuation of lp(a) values [14]; moreover, elevated serum lp(a) levels have been reported in leukemia [47]. The possibility that the disease influences the effects of retinoids on lipoproteins and that the increase in lp(a) is secondary to other changes cannot, then, be excluded. The discrepancy between our data [13], and the lp(a) decrease reported by Georgala *et al.* [12] 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 [13].

In conclusion, although at the moment the effect of retinoids on lp(a) plasma levels is not completely clear, we have clearly shown here that retinoids decrease apo(a) synthesis in primary cultures of cynomolgus monkey hepatocytes and that activation of the RAR is a prerequisite for this.

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