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Retinoids Inhibit Primary Cynomolgus Monkey Hepatocyte Lipoprotein(a) Levels

Randy Ramharack,[1] Mark A. Spahr, and Catherine S. Sekerke
Department of Vascular and Cardiac Diseases, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, Ann Arbor, Michigan 48105

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Elevated plasma lipoprotein(a) [Lp(a)] independently contributes to a variety of vascular diseases; consequentially, factors that modulate its levels are of interest. Since Lp(a) is produced by a disulfide linkage between apolipoprotein(a) [apo(a)] and apolipoproteinB-100 (apoB-100) of low density lipoprotein (LDL) on the hepatocyte surface, modulation of either particle may be useful in lowering Lp(a). Using primary cynomolgus monkey hepatocyte cultures that endogenously express apo(a) and apoB-100, we showed that all-*trans* (retinol, retinal, retinoic acid) and 9-*cis* (retinal, retinoic acid) retinoids lower Lp(a) accumulation in the cell media, with the 9-*cis* derivatives being >10-fold more potent than the all-*trans* stereoisomers. Lp(a) lowering was related to decreases in apo(a) and its cognate transcript, but not to apoB-100. These results demonstrate that retinoids lower Lp(a) levels by decreasing apo(a) through its cognate mRNA. © 1997 Academic Press

Lipoprotein(a) [Lp(a)] is an independent risk factor for a variety of vascular diseases at plasma concentrations exceeding 20-30 mg/dl (1). Consequently, factors that lower Lp(a) may be useful in treating conditions related to its elevation. The Lp(a) particle consists of low density lipoprotein (LDL)-apolipoprotein (apo)B-100 covalently linked, through a single disulfide bond, to the plasminogen related apo(a) (1).

Both apo(a) and LDL are synthesized predominantly by the liver and assembled into Lp(a) on the hepatocyte surface (2) Therefore, inhibitors of hepatic apo(a) and/or LDL production may be effective in decreasing Lp(a) levels. Since HMG-CoA reductase inhibitors (statins) can potently lower LDL without decreasing Lp(a) levels (3,4), factors targeted at apo(a) synthesis may be more beneficial at lowering Lp(a).

Several agents such as niacin (5), estrogen (6-10), gemfibrozil (11,12) and alcohol (13-15) that upregulate high density lipoprotein (HDL)-apoAI, decrease Lp(a), suggesting a possible inverse relationship between HDL and Lp(a) regulation. HDL-apoAI levels are also increased by retinoids (16), however, the effect of these compounds on Lp(a) expression has not been investigated in a natural setting. To determine whether retinoids can modulate endogenous Lp(a) levels, they were tested in primary cultures of cynomolgus monkey hepatocytes that naturally express Lp(a).

METHODS

The retinoids (retinol, retinal, all-*trans*-retinoic acid and 9-*cis*-retinal) were purchased from Sigma, except for 9-*cis*-retinoic acid, which was synthesized by our chemistry department (Dr. H. T. Lee and J. A. Picard).

Hepatocytes were isolated from healthy cynomolgus monkeys (*Macaca fascicularis*), cultured and treated exactly as described (12). All animal experiments were approved by our Vertebrate Use Committee.

A commercially available (PerImmune) Lp(a) specific ELISA was used to measure Lp(a) levels in the hepatocyte culture media as documented earlier (12). Western blots were carried out exactly as described (17) using precast 4% (apo(a) and apoB-100) or 4-20% (apoAI) Novex Tris-glycine gels. The nitrocellulose blots were probed with goat anti-human Lp(a) (Biodesign) and sheep anti-human apoB-100 or apoAI (Boehringer Mannheim).

Total RNA was isolated from cells as previously reported (12). Cynomolgus monkey apo(a) mRNA levels were measured, in total RNA samples, by a specific ribonuclease protection assay (12,17). This assay uses the apo(a) kringle IV type-2 sequence as a probe. The type-2 sequence is the identically repeated kringle in the apo(a) mRNA and its use results in an amplification of the apo(a) mRNA. Amplification of the signal is necessary to detect the apo(a) transcript in hepatocyte total RNA. The ribonuclease protection assay employs

[1] Corresponding author: Department of Vascular Cardiac and Diseases, Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105. Fax: (313) 996-3135. E-mail: ramharr@aa.wl.com.

Abbreviations used: apo, apolipoprotein; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein; monkey, *Macaca fascicularis;* RA, all-*trans*-retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; 9-*cis*-RA, 9-*cis*-retinoic acid; 9-*cis*-RL, 9-*cis*-retinal.

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an internal standard to normalize each sample, therefore, differences detected between samples represent real changes and not assay artifacts.

RESULTS

The effect of retinoids on Lp(a)/apo(a) levels in primary monkey hepatocyte cultures. To determine the effect of retinoids on Lp(a), primary cultures of cynomolgus monkey hepatocytes that endogenously express and secrete Lp(a) into their culture media were used. First, the all-*trans*-retinoids, retinoic acid (RA), retinal and retinol, were tested for their ability to modulate Lp(a) levels in two independent experiments. These compounds inhibited Lp(a) levels in the monkey hepatocyte culture media by 65-75%, relative to control values, and were equipotent (Fig. 1).

Next, the 9-*cis* retinoids, 9-*cis*-RA and 9-*cis*-retinal (9-*cis*-RL), were investigated. In two independent experiments, 9-*cis*-RL inhibited Lp(a) levels by 74% and 78%, compared to untreated cells by (Fig. 1). However,

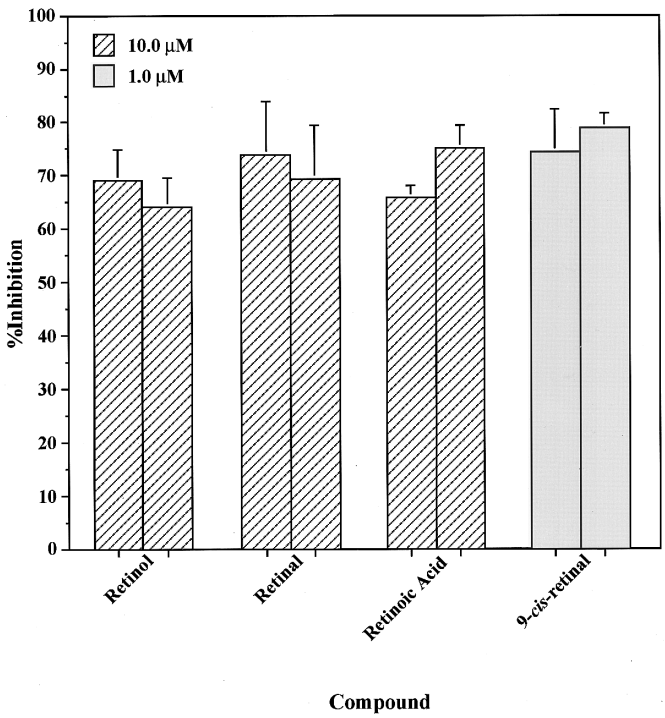

FIG. 1. Effect of retinoids on Lp(a) accumulation in the culture media of primary cynomolgus monkey hepatocytes. Hepatocytes were isolated and cultured as described in Methods. Each retinoid was tested in six separate wells (n=6) per experiment. Cells were treated with the retinoid for a total of four days, with fresh media containing retinoid added daily. At the end of the fourth day, media were harvested, frozen at −90°C, and assayed for Lp(a) by ELISA within one month. The two bars for each retinoid represent results from two separate experiments carried out on different days. Results are expressed as percentage inhibition of control culture (treated with vehicle alone) values. Error bars are standard errors of the mean (SEM).

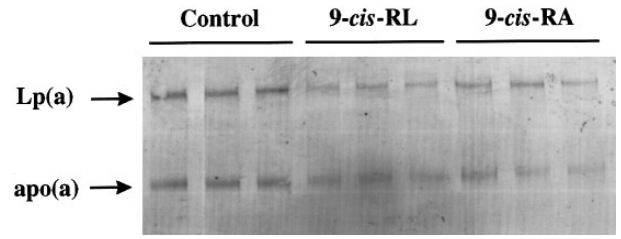

FIG. 2. Comparison of 9-*cis*-retinal and 9-*cis*-retinoic acid on Lp(a) and apo(a) levels in primary cynomolgus monkey hepatocyte cultures. Hepatocytes were treated with the retinoids as described in Fig. 1. After treatment, Lp(a) and apo(a) were analyzed in the media from triplicate wells by western blots using a monospecific, polyclonal, goat-anti human Lp(a) antibody. The positions of Lp(a) and apo(a) are indicated on the figure. 9-*cis*-RT, 9-*cis*-retinal and; 9-*cis*-RA, 9-*cis*-retinoic acid.

9-*cis*-RL was effective at a 10-fold lower concentration, 1μM, than RA or its precursors. As observed for the all-*trans* stereoisomers, western blot analysis of hepatocyte culture media demonstrated that 9-*cis*-RL and 9-*cis*-RA were equally effective at lowering Lp(a) levels when tested at 1μM (Fig. 2). In addition, these blots showed that the retinoids lowered Lp(a) levels by decreasing apo(a) (Fig. 2, apo(a)). Western blot analysis of the above samples also indicated that, in contrast to their effect on Lp(a)/apo(a), retinoid treatment moderately *increased* apoB-100 and apoAI levels (data not shown). The ability of retinoids to inhibit Lp(a) levels was seen in hepatocytes isolated from monkeys expressing different Lp(a) plasma levels (Fig. 3). In these and all future experiments, 9-*cis*-RL was used because it was equally potent to 9-*cis*-RA and more readily available.

Dose response inhibition of Lp(a). The previous results indicated that the 9-*cis* retinoid stereoisomer was the most active at inhibiting Lp(a)/apo(a) levels. To further determine the potency of 9-*cis* retinoid mediated Lp(a) inhibition, dose-response curves were performed using 9-*cis*-RL. The retinoid was tested at 0.1, 0.3, 1 and 3 μM. Lp(a) levels were inhibited 20% at 0.1 μM with a maximum of 68% inhibition at 0.3 μM (Fig. 4). Further increases in the concentration of 9-*cis*-RL, up to 3 μM, did not result in further inhibition of Lp(a) levels suggesting saturation of the response. Based on these results, the IC_{50} for 9-*cis*-RL inhibition of Lp(a) levels in the monkey hepatocyte culture media was approximately 0.2 μM.

Effect of retinoids on apo(a) mRNA expression in primary cynomolgus monkey hepatocytes. To determine whether the retinoids were lowering Lp(a) by decreasing apo(a) through changes in its transcript, total RNA was isolated from retinoid treated cells and apo(a) mRNA was measured using a previously

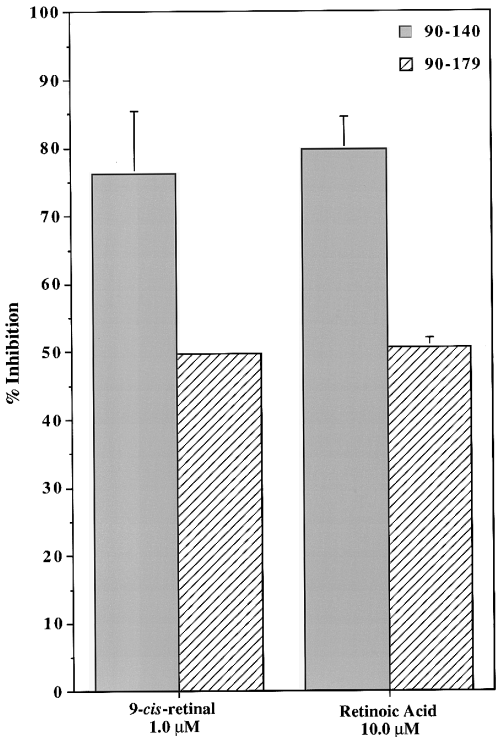

FIG. 3. Effect of retinoids on Lp(a) levels in two different primary cynomolgus monkey hepatocyte preparations. Hepatocytes were isolated from animal that had Lp(a) levels of 26 mg/dl (Mk# 90-179) and 17 mg/dl (Mk# 90-140). Cells were cultured, treated, and analyzed as described in Fig. 1.

described, highly specific, ribonuclease protection assay (12,17). A representative example of the apo(a) mRNA ribonuclease protection assay is shown in Fig. 5. This experiment demonstrated that 9-*cis*-RL treatment decreased apo(a) mRNA. The equal intensity of the internal standard band in control and treated lanes indicated that apo(a) mRNA lowering was not due to assay variability or gel loading artifacts. RA treatment also decreased the apo(a) mRNA, however, as with its effect on Lp(a)/apo(a) levels it was 10-fold less potent than the 9-*cis* stereoisomer (data not shown).

DISCUSSION

The equal potency of retinol, retinal and RA at lowering Lp(a) levels indicated that these retinoids are taken-up by the monkey hepatocytes and converted to a common form before effecting Lp(a). Since RA is thought to be the most potent of the latter three all-*trans*-retinoids, it is likely that during incubation with the hepatocytes, retinol and retinal are converted from their respective alcohol and aldehyde form to RA. These observations indicate that the necessary enzymatic pathways needed to process precursor forms of active retinoids are functional in the monkey hepatocyte cultures. The use of precursor retinoids more closely mimics *in vivo* conditions where retinoids, obtained through the diet as retinol (vitamin A), are taken up by the liver and reversibly converted to retinal-the direct precursor of RA. However, the high concentration of the all-*trans*-retinoids (10 μM) needed to see an effect on Lp(a) levels hinted that they may not be the principal hormones but instead precursors of the active retinoid. Our finding that the 9-*cis*-retinoid stereoisomers, 9-*cis*-RL and 9-*cis*-RA, are at least 10-fold more potent than the corresponding all-*trans* isomers suggested that they are the active agents modulating Lp(a) and apo(a) mRNA levels. Since 9-*cis*-RA can be generated by RA isomerization (18) its is possible that the Lp(a) lowering activity of RA is due to its conversion to 9-*cis*-RA by the hepatocytes.

In contrast to our study, in which endogenous Lp(a) expression was investigated, a report using HepG2 cells transfected with a small part of the apo(a) promoter, linked to a luciferase reporter gene, showed increased transcriptional activity with high concentrations of RA (19). The construct used in the latter study extended only to −442 of the apo(a) promoter. Computer scanning of this region did not identify a consensus retinoid response element (RRE) that is usually found in retinoid responsive genes (20-22). Therefore, it is difficult to reconcile the increased transcriptional affect of RA in the HepG2 model with the absence of a RRE, unless the apo(a) promoter uses a cryptic RRE in the 442 nucleotide region. Furthermore, HepG2 cells do not ex-

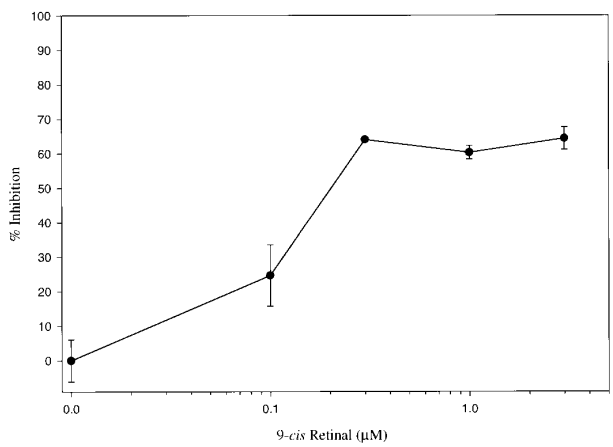

FIG. 4. Dose response inhibition of 9-*cis*-retinal on Lp(a) accumulation in primary cynomolgus monkey hepatocyte cultures. Hepatocytes were cultured, treated with 9-*cis*-retinal, and analyzed for Lp(a) levels as described in Fig. 1, except that four wells (n=4) were used per concentration.

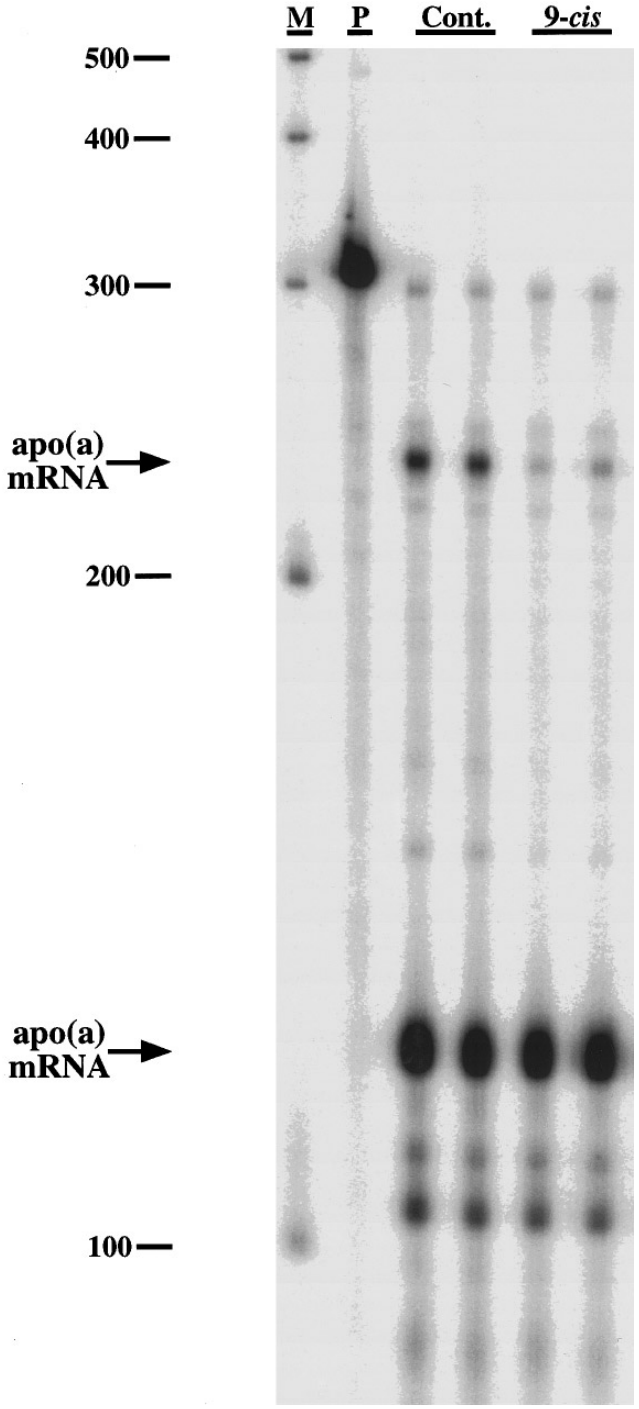

FIG. 5. Representative example of the effect of retinoids on apo(a) mRNA levels in primary cynomolgus hepatocyte cultures. Cells were cultured and treated exactly as described in Fig. 1. At the end of dosing, total RNA was isolated from control and retinoid treated cells by the direct addition of RNAzol to the culture plate after media removal and two PBS washes. Apo(a) mRNA was assayed in total RNA by a ribonuclease protection assay as described in Methods. M, RNA standards (nucleotides); P, cynomolgus monkey apo(a) riboprobe; Cont., control; 9-*cis*, 9-*cis*-retinal (1 μM); apo(a) Std., apo(a) internal standard.

press Lp(a) (23) and their lack of retinoid X receptors (RXR) (24,25), which, together with retinoic acid receptors (RAR) are required to generate a RA response (26,27), suggests that they may not be the best cell model to investigate retinoid mediated apo(a) promoter expression.

In conclusion, we have used primary monkey hepatocyte cultures, that endogenously express Lp(a), to demonstrate that retinoids decrease Lp(a) by lower apo(a) through changes in its cognate transcript. Therefore, development of compounds targeted at modulating apo(a) levels may be effective Lp(a) lowering therapies.

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