Gemfibrozil significantly lowers cynomolgus monkey plasma lipoprotein[a]-protein and liver apolipoprotein[a] mRNA levels

Randy Ramharack,[1] Mark A. Spahr, Gary W. Hicks, Karen A. Kieft, David W. Brammer, Laura L. Minton, and Roger S. Newton

Parke-Davis Pharmaceutical Research, Division of Warner-Lambert Company, 2800 Plymouth Road, Ann Arbor, MI 48105

Abstract Eight male cynomolgus monkeys (*Macaca fascicularis*) on a normal chow diet were orally administered gemfibrozil daily using a weekly rising dose protocol for 3 weeks (50, 125, and 200 mg/kg per day). At these drug doses, Lp[a] levels were reduced: 83.7% ± 3.2 (SEM), ($P < 0.024$); 63.7% ± 4.1 ($P < 0.013$); and 36.2% ± 1.1 ($P < 0.002$), respectively, of pretreatment values. Lp[a] reduction was directly related to blood gemfibrozil concentration (range 36–428 μM, $r = 0.969$) and occurred without concomitant changes in apolipoprotein B. Three weeks posttreatment Lp[a] levels returned to pretreatment values. A specific ribonuclease protection assay demonstrated that liver apolipoprotein[a] (apo[a]) mRNA expression was decreased in all animals to an average of 19.1% ± 3.0 ($P < 0.0026$), of pretreatment values after the 200 mg/kg treatment, whereas, albumin, apolipoprotein A-I, apolipoprotein E, and glyceraldehyde-3-phosphate dehydrogenase mRNAs were unchanged. Lp[a] levels were unaffected by gemfibrozil in HepG2 cells permanently transfected with an apo[a] 10-kringle cDNA construct containing partial 5'- and 3'-untranslated sequences and under control of a constitutive CMV promoter. However, both Lp[a] and apo[a] mRNA in primary cynomolgus monkey hepatocytes were coordinately lowered in a dose-dependent fashion by gemfibrozil. Thus, Lp[a] can be regulated by gemfibrozil at the level of apo[a] mRNA expression.—**Ramharack, R., M. A. Spahr, G. W. Hicks, K. A. Kieft, D. W. Brammer, L. L. Minton, and R. S. Newton.** Gemfibrozil significantly lowers cynomolgus monkey plasma lipoprotein[a]-protein and liver apolipoprotein[a] mRNA levels. *J. Lipid Res.* 1995. **36:** 1294–1304.

Supplementary key words apo[a] • transfected HepG2 cells • primary monkey hepatocytes • CMV promoter

Elevated plasma levels of lipoprotein[a] (Lp[a]) is an independent risk factor for coronary heart disease (1–10), intermittent claudication (11), stroke (12–18), and restenosis after vein bypass surgery (19, 20) and balloon angeoplasty (21, 22). The involvement of Lp[a] in these disease processes may be related to its unique composition that allows it to act as both a lipoprotein and an inhibitor of plasminogen activity (23, 24). Lp[a] is formed through a disulfide bond (25, 26) between apolipoprotein B-100 (apoB-100) of low density lipoprotein (LDL), and the liver-derived (27) plasminogen-related glycoprotein, apolipoprotein[a] (apo[a]) (28–30). Apo[a] is highly polymorphic; 34 different isoforms have been detected in human plasma ranging from 280 to 775 kDa (31, 32). The size of a particular apo[a] isoform is directly related to the size of its cognate mRNA (33). The apo[a] size variation is due to differences in copy number of a sequence highly related to plasminogen kringle IV (KIV) (28–30). Single copies of plasminogen kringle V and protease domain are also present in apo[a] in a position C-terminal to the last KIV repeat (30). However, no serine protease activity has been demonstrated for apo[a] (34) and it may therefore function as a plasminogen competitor (35, 36).

Lp[a] levels vary considerably in the population ranging from < 0.1 mg/dl to > 100 mg/dl with a distribution skewed to the lower concentrations in most ethnic groups (37). Several studies have demonstrated that plasma Lp[a] levels are determined by its production rate and not its catabolism (38–40). Consistent with this finding is the observation that the apo[a] locus accounts for greater than 90% of plasma variation in Lp[a] concentration (28, 41). Investigations using cynomolgus monkeys (42) have shown a correlation between hepatic apo[a] mRNA abundance and Lp[a] levels that accounted for a significant proportion of the plasma Lp[a] variability in these animals. This observation indicates that apo[a] gene transcription and/or apo[a] mRNA stability may play a role

Abbreviations: Lp[a], lipoprotein[a]; LDL, low density lipoprotein; PCR, polymerase chain reaction; RT, reverse transcription; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; VLDL, very low density lipoprotein; HDL, high density lipoprotein.

[1]To whom correspondence should be addressed.

in determining plasma Lp[a] levels. A similar correlation between human apo[a] mRNA transcript abundance and Lp[a] levels was not observed; however, this study was complicated by a very small sample size and concerns about the integrity of the RNA (43). In addition to possible control at the level of apo[a] mRNA abundance, certain apo[a] isoforms are regulated by post-translational processing in baboon hepatocytes (44, 45). Taken together these observations suggest that transcription as well as post-transcriptional mechanisms maybe involved in determining basal Lp[a] levels.

Very little is known concerning factors that regulate Lp[a] levels and the mechanism(s) by which they exert their effect(s). Sex steroids (46–48), a niacin/neomycin combination (49), nicotinic acid (50), and the fibric acid derivatives bezafibrate (51) and gemfibrozil (52) have been shown to lower Lp[a] levels in humans. Because the Lp[a] attenuating effect of these drugs was observed in individuals that were under treatment for primary diseases such as cancer (46, 47) or dyslipidemia (51, 52), it was uncertain whether these agents were directly influencing Lp[a] or whether Lp[a] was being lowered due to reversal of the disease state. We were therefore interested in determining whether Lp[a] could be modulated without a preexisting condition.

The cynomolgus monkey has been shown to be a good model to study the basal expression of Lp[a] and apo[a] mRNA (42). Therefore, normal cynomolgus monkeys on a chow diet were used to investigate Lp[a] protein and apo[a] mRNA regulation by gemfibrozil. Gemfibrozil was used as a tool in these studies for two main reasons: *1)* preliminary data indicated that it was capable of lowering Lp[a] in a small group of type IIa hyperlipidemic patients after only 3 months of therapy at the normal clinical dose of approximately 17 mg/kg per day (600 mg B.I.D.) (52), whereas 6 months of treatment with sustained-release bezafibrate was required for significant Lp[a] lowering (51); and *2)* no toxicological effects of gemfibrozil were observed in rhesus monkeys treated at a dose of 300 mg/kg per day for 3 months (53).

MATERIALS AND METHODS

Animals

Cynomolgus monkeys (*Macaca fascicularis*) were individually housed in an AAALAC accredited facility according to recommendations (54). Base line Lp[a] levels were determined in eight animals 3, 2, and 1 weeks before initiating drug treatment and found not to differ significantly (see Lipoprotein and apolipoprotein analysis). Due to variation in base line Lp[a] levels among the monkeys (10–50 mg/dl), each animal was used as its own control. Animals were dosed by oral gavage with gemfibrozil (Lopid) as described (53). Control experiments in several animals indicated that oral gavage did not influence Lp[a] levels. A 3-week rising dose protocol in which the animals were dosed every day was used followed by a wash out period. The drug was administered daily as follows: week 1, 50 mg/kg; week 2, 125 mg/kg; and week 3, 200 mg/kg. Blood was collected immediately after the first 125 mg/kg dose, immediately after the first 200 mg/kg dose, and immediately after the last 200 mg/kg dose. One and 3 weeks after the last 200 mg/kg dose, six animals were also bled. Blood was drawn from anesthetized animals into 5-ml tubes containing EDTA. Samples were immediately placed on ice, plasma was collected by centrifugation at 4°C, divided into 500-μl aliquots while on ice, and immediately frozen at −90°C. Lp[a] levels were determined within 1 month of drawing blood on plasma samples that were thawed from −90°C only once. The percent change in Lp[a] levels relative to control (pretreatment) values (100% level) was calculated for each animal and these values for all animals were used to obtain the percent change in Lp[a] levels at each gemfibrozil dose and during the two recovery periods.

Liver biopsy samples were obtained from the animals 1 week before initiating drug administration and the last day of the 200 mg/kg dose.

In this study, monkeys were treated with gemfibrozil at significantly higher concentrations (50–200 mg/kg per day) than those used clinically. This dose range was chosen because non-human primates in general have a resistance to hypolipidemic drugs when administered at clinical doses (55–57). As examples, 20- to 50-times (20–50 mg/kg per day) the human dose of CS-514 (pravastatin) was need to observe significant lipid-lowering effects in cynomolgus monkeys (55) while 3-times (50 mg/kg per day) and 35-times (1000 mg/kg per day) the clinical dose of gemfibrozil (57) and clofibrate (56), respectively, were required to observe changes in rhesus monkey plasma lipids. The maximum dose of gemfibrozil used was 200 mg/kg per day for 1 week to ensure that the present investigation was well within the drug's safety margin (53).

Gemfibrozil plasma levels were determined by Pharmaco (Richmond, VA) using a validated GLP analytical method. Gemfibrozil concentrations in plasma from control animals were below the quantitation level of this method.

Primary monkey hepatocytes

Hepatocytes were isolated from a single male cynomolgus monkey of approximately 6.5 kg exactly as described (58) except that the final cell suspension was purified by Percoll (Pharmacia) density gradient centrifugation. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.2% bovine serum albu-

min (BSA). Triplicate wells were used for each drug or vehicle (DMSO) dose. Cells were treated for a total of 3 days with medium containing drug or vehicle being replaced daily. Medium was collected, frozen at −90°C, and analyzed within 1 week for Lp[a] levels. Total RNA was extracted by directly adding RNAzol (Biotecx) to the culture plate.

Lipoprotein and apolipoprotein analysis

Lp[a] levels were measured using a specific ELISA (Capell) that crossreacted with cynomolgus monkey Lp[a], and was independent of apo[a] isoform size, but did not crossreact with LDL or plasminogen (59). Experiments in which gemfibrozil was added to control plasma at 450 μM demonstrated no effect on the ELISA measurement of Lp[a]. As pooled human plasma was used in this ELISA as a standard, the Lp[a] values determined in monkey plasma did not represent absolute Lp[a] levels. Lp[a] levels in cell culture medium were determined by the same ELISA except that the medium was assayed undiluted. Lipoprotein cholesterol was measured using high performance gel filtration chromatography (60). Triglycerides were measured by an Abbott automated analyzer. ApoB was analyzed by ELISA. A goat anti-human Lp[a] antibody (Biodesign) and a rabbit anti-human apoB antibody (Boehringer Mannheim) were used in the Western blot analysis. The Lp[a] and apoB antibodies were detected on the Western blots using a biotin/strepavidin-phosphatase amplification detection system (Kirkegaard and Perry).

Molecular biology

A human apo[a] cDNA construct containing 10 KIV repeats (K-10) was obtained from Dr. M. Koschinsky (Queen's University, Kingston, Canada). Relative to the published apo[a] cDNA (30), K-10 contains: 24 nucleotides of 5'-untranslated sequence; leader sequence; KIV 1-3 spliced to KIV 31-37; kringle V; protease domain; and the first 67 bases of the 201 nucleotide 3'-non-coding sequence. This construct was cloned into the eukaryotic expression vector pcDNA-Amp (In Vitrogen) that contains the cytomegalovirus (CMV) immediate early gene enhancer/promoter and SV40 transcription termination and polyadenylation signals. The 10-kringle apo[a] expression construct was co-transfected with pRc/CMV (In Vitrogen), which contains the neomycin resistance gene, into 50% confluent HepG2 cells grown in DMEM containing FBS (10%). Three clonal cell lines expressing different levels of Lp[a] were obtained using G418 selection.

Monkey liver cDNA was synthesized using random hexamers and amplified using an upstream primer that represents the most 5' end of the published human cDNA (nucleotide 1–25) and a downstream primer that represents the 3'-end of the first kringle in the human cDNA (nucleotide 411–435) (30). Control RT-PCR experiments using these primers but omitting RNA from the reactions resulted in no PCR products. Addition of RNA to the latter reaction resulted in amplification of the expected 435 bp fragment that was cloned pBluescript II SK(+) (Stratagene). Both strands of three independently isolated inserts were sequenced completely. Complete agreement was obtained between the sequence from both strands of all three clones. This monkey apo[a] cDNA clone was designated pMK-1.

A solution hybridization assay was used to measure monkey apo[a] mRNA levels as described (61) with the following modifications. Hybridization was carried out for 15 h in the absence of high molecular weight DNA and ribonuclease A and TI were used to digest unhybridized RNA molecules (62, 63). This assay was linear over time (8–24 h) and total RNA concentration (1–50 μg). A 340 nucleotide anti-sense cRNA ^{32}P-labeled probe that protects the last 240 nucleotides of the first apo[a] mRNA kringle was generated from StyI-digested pMK-1. A 365 nucleotide sense strand cRNA was generated from SpeI-digested pMK-1 and approximately 100 pg was included in the solution hybridization assay. Hybridization of the

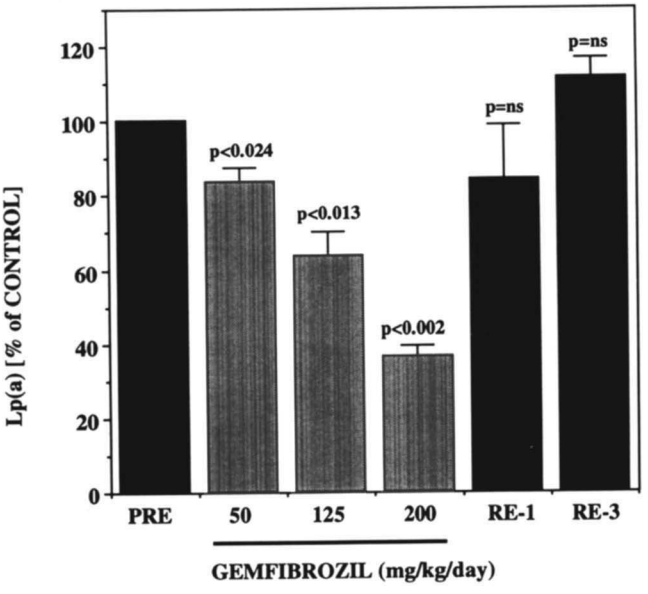

Fig. 1. Effect of gemfibrozil on cynomolgus monkey Lp[a] plasma levels. Baseline Lp[a] levels (range 10–50 mg/dl) were determined using an ELISA that was unaffected by LDL, plasminogen, and gemfibrozil, in eight healthy male animals on a normal chow diet. Gemfibrozil was administered daily by oral gavage as a liquid suspension, in a weekly rising dose protocol for 3 weeks (50, 125, and 200 mg/kg per day) followed by a 3-week washout period (recovery). Lp[a] was measured at the end of each week and each animal was used as its own control. The percent change in Lp[a] levels relative to control values (100% level) was calculated for each animal and the average of these values is represented in the figure: PRE, pre-treatment; RE-1, 1 week recovery; RE-3, 3 week recovery.

sense strand with the anti-strand probe generates a 123 nucleotide protected fragment that serves as an internal control. Gels were transferred to developed XAR-5 film (Kodak), exposed to a phosphor screen, and analyzed using a PhosphorImager (Molecular Dynamics).

Human albumin and apoA-I and apoE cDNAs were obtained from ATCC. A 1.1-kb purified G3PDH cDNA fragment was purchased from Clontech. All probes were labeled by nick-translation (Promega) using [α-^{32}P]dATP (3,000 Ci/mmol, Amersham) to a specific activity of approximately 10^9 cpm/μg.

RNA was isolated using the RNAzol method (Biotecx). Northern and slot blots were carried out under stringent conditions as described (64). The slot blot was sequentially probed and stripped. Overnight exposure of the stripped slot blot to a phosphor screen indicated that in all cases the probe had been completely removed. Probes were used in the following order: albumin, apoA-I, apoE, and G3PDH. Control experiments indicated that these probes hybridized to a single mRNA of the correct size on northern blots.

Statistical analysis

A paired two-tail, two-sample for means *t*-test at the 95% confidence level was used for all statistical analyses.

RESULTS AND DISCUSSION

Effect of gemfibrozil on cynomolgus monkey plasma Lp[a] levels

In the present study, gemfibrozil was used as a tool to determine whether Lp[a] could be lowered in normal chow-fed cynomolgus monkeys without any known preexisting conditions. Due to the resistance of monkeys to

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MAPOAK1   - ctgggattgggacacactttctgggcactgctggccagtcccata ATGGA -50
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HAPOAK1   - ctgggattgggacacactttctggacactgctggccagtcccaaa ATGGA -50

MAPOAK1   - ACATAAGGAAGTGGTTCTTCTACTTCTTTTATTTCTGAAATCAGCACTAC -100
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HAPOAK1   - ACATAAGGAAGTGGTTCTTCTACTTCTTTTATTTCTGAAATCAGCAGCAC -100

MAPOAK1   - CTGAGCAAAGCCATGTGGTCCAGGATTGCTACCATGGTGATGGACAGAGT -150
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HAPOAK1   - CTGAGCAAAGCCATGTGGTCCAGGATTGCTACCATGGTGATGGACAGAGT -150

MAPOAK1   - TATCAAGGCACATCCTCCACCACTGTCACAGGAAGGACCTGCCAAGCTTG -200
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HAPOAK1   - TATCGAGGCACGTACTCCACCACTGTCACAGGAAGGACCTGCCAAGCTTG -200

MAPOAK1   - GTCATCTATGGAACCACATCAGCATAATAGAACCACAGAAAACTACCCAA -250
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HAPOAK1   - GTCATCTATGACACCACATCAACATAATAGGACCACAGAAAACTACCCAA -250

MAPOAK1   - ATGCTGGCTTGATCAGGAACTACTGCAGGAATCCAGATCCTGTGGCAGCC -300
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HAPOAK1   - ATGCTGGCTTGATCATGAACTACTGCAGGAATCCAGATGCTGTGGCAGCT -300

MAPOAK1   - CCTTATTGTTATACGATGGATCCCAATGTCAGGTGGGAGTACTGCAACCT -350
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HAPOAK1   - CCTTATTGTTATACGAGGGATCCCGGTGTCAGGTGGGAGTACTGCAACCT -350

MAPOAK1   - GACACGATGCTCAGATGCAGAAGGGACTGCCGTCGCACCTCCGAATGTCA -400
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HAPOAK1   - GACGCAATGCTCAGACGCAGAAGGGACTGCCGTCGCGCCTCCGACTGTTA -400

MAPOAK1   - CCCTGGTTCCAAGCCTAGAGGCTCCTTCCGAACAA -435
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HAPOAK1   - CCCCGGTTCCAAGCCTAGAGGCTCCTTCCGAACAA -435
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Fig. 2. Comparison of cynomolgus monkey and human apo[a] cDNA sequence. A monkey apo[a] cDNA was cloned by RT-PCR using primers that, based on the published human apo[a] cDNA sequence (30), flank the 5'-untranslated region and first kringle IV (Type 1). Alignment of the cloned monkey apo[a] cDNA with the corresponding human sequence is shown. Sequence identity is indicated by a vertical line. MAOPAK1, monkey apo[a] cDNA; HAPOAK1, human apo[a] cDNA; lowercase letters, 5'-untranslated region; italic uppercase letters, leader sequence, uppercase letters, first kringle IV (Type 1) sequence. The monkey clone was designated as pMK-1.

lipid modulating agents (55–57), their high tolerance to these drugs (53), and not knowing the concentration of gemfibrozil needed for Lp[a] lowering, the drug was administered at oral doses 3- to 12-times those used clinically. The results from this experiment demonstrate that monkey plasma Lp[a] levels can be significantly lowered by the direct action of gemfibrozil in a dose-dependent fashion over a short time period. At gemfibrozil doses of 50, 125, and 200 mg/kg, the mean Lp[a] plasma concentration was reduced to: 83.7% ± 3.2 (SEM), ($P < 0.024$); 63.7% ± 4.1 ($P < 0.013$); and 36.2% ± 1.1 ($P < 0.002$), respectively, of pretreatment values (**Fig. 1**). One week after stopping drug administration, Lp[a] levels rapidly increased to 83.95% (not significant) of pretreatment values, and by 3 weeks posttreatment Lp[a] concentrations had returned to normal in the six animals that were tested (Fig. 1). Western blots showed the identical pattern of Lp[a] inhibition as observed using the Lp[a] ELISA (data not shown).

To ascertain whether Lp[a] reduction was related to circulating levels of the drug, blood gemfibrozil concentrations were determined at the end of each week's dose. This analysis indicated that there was a direct relationship between blood gemfibrozil levels and Lp[a] reduction (range 36–428 μM gemfibrozil, $r = 0.969$).

Effect of gemfibrozil on apoB levels

Lp[a] consists of apo[a] linked to apoB through an interchain disulfide bond (25, 26). Therefore, it was possible that the gemfibrozil-mediated decrease in Lp[a] plasma levels was due to apo[a] and/or apoB reduction. To address the latter possibility, apoB levels were assayed in control, gemfibrozil-treated, and recovery plasma by both a specific ELISA and Western blot analysis. Both the ELISA and Western blots demonstrated no significant changes in apoB levels between pretreatment, treatment, and posttreatment samples (data not shown) indicating that changes in apoB could not account for the decrease in Lp[a] observed with gemfibrozil treatment. Pretreatment total (119.74 ± 13.27 mg/dl), VLDL (7.12 ± 1.46 mg/dl)-, LDL (37.11 ± 7.13 mg/dl)-, and HDL (59.10 ± 10.0 mg/dl)-cholesterol, apoA-I protein (387.38 ± 45.56 mg/dl), and triglycerides (93.78 ± 21.93 mg/dl) were determined before initiating drug treatment. These parameters remained unchanged during the drug dosing period except that LDL-cholesterol was slightly increased after the second week of treatment (48.69 ± 6.61 mg/dl, $P < 0.04$) and triglyceride levels were transiently elevated after the third week of treatment (183.16 ± 47.04 mg/dl $P < 0.03$).

Gemfibrozil influences apo[a] mRNA levels

As apoB levels were unaffected during treatment, the possibility existed that Lp[a] levels were being influenced by an effect of gemfibrozil on apo[a] levels and/or a decrease in apo[a]/apoB coupling efficiency. There are several possible means by which gemfibrozil could decrease the plasma concentration of Lp[a] by influencing apo[a] levels. Gemfibrozil may modulate apo[a] by: enhancing its catabolism, decreasing its synthesis/secretion from hepatocytes, and/or decreasing the level of its mRNA.

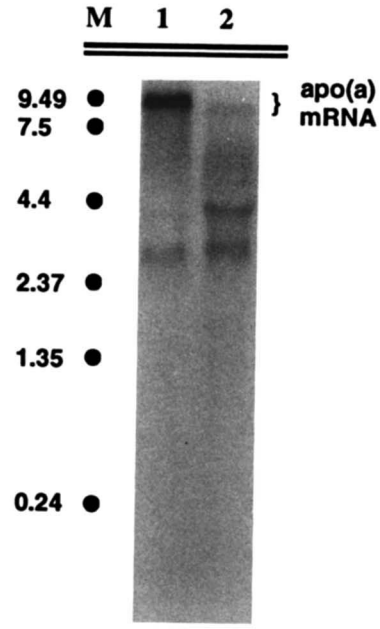

Fig. 3. Northern Blot analysis of monkey apo[a] mRNA. Northern blot analysis of total monkey liver RNA (20 μg/lane) was carried out as described in Materials and Methods. The blot was probed with ^{32}P-labeled nick-translated gel purified insert form pMK-1 described in Fig. 2. Blots were hybridized, washed under stringent conditions, and exposed to X-ray film (XAR-5, Kodak) with an intensifying screen at −90°C for 24 h. apo[a], apolipoprotein[a] mRNA; m, RNA size marker (Kb).

To determine whether gemfibrozil treatment was influencing apo[a] mRNA levels, the 5'-end of monkey liver apo[a] mRNA was cloned and used as a probe. The region between the 5' non-coding and 3'-end of the first apo[a] mRNA kringle was amplified by RT-PCR of monkey liver total RNA cloned and sequenced. Alignment of the monkey (pMK-1) and human sequence is shown in **Fig. 2.** The percent nucleotide sequence identity between the two primate sequences is 93.7%. Both monkey and human apo[a] cDNAs are also organized identically, containing a 5'-untranslated region, followed by a leader sequence and unique kringle IV Type-1 sequence (Fig. 2). This structure is different from plasminogen in which the leader sequence is followed by a kringle I sequence (65).

Northern blot analysis of monkey total mRNA using the pMK-1 probe revealed that it hybridized to a high molecular weight band that was variable in size and intensity in different animals (**Fig. 3**). Other lower molecular weight bands could be observed on the blot (Fig. 3). These hybridizing species most likely represent other kringle-

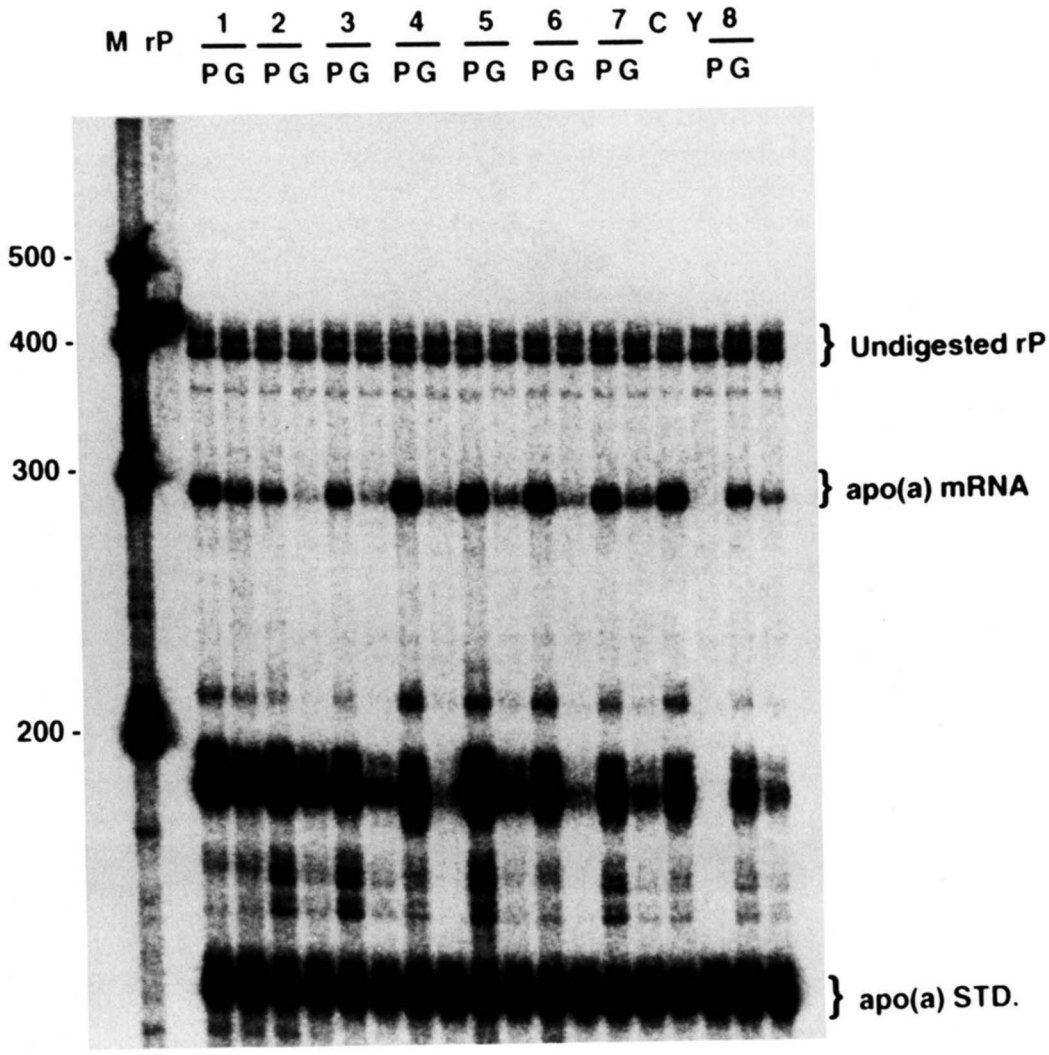

Fig. 4. Ribonuclease protection assay of apo[a] mRNA. Total RNA (5 μg) isolated from monkey liver biopsy samples before (pre-treatment) and at the end of the 200 mg/kg gemfibrozil dosing period was analyzed by a specific ribonuclease protection assay as detailed in Materials and Methods. Numbers (1–8) above each lane represent individual monkeys; P and G, below these numbers represent RNA from pre-treatment and gemfibrozil treatment, respectively; m, RNA size markers (nucleotides); rP, riboprobe; C, apo[a] mRNA positive control monkey liver RNA sample; Y, yeast t-RNA; apo[a] mRNA, represents apo[a] present in the monkey liver samples; apo[a] std, represents synthetic apo[a] mRNA added to the assay.

containing sequences related to apo[a] mRNA. The difference in size of the apo[a] mRNA bands between the animals correlated with different apo[a] protein isoform sizes (data not shown). However, the intensity of the mRNA band did not compare with the plasma Lp[a] level in these particular animals (monkey 1, 33.64 mg/dl and monkey 2, 25.86 mg/dl). This observation may indicate a lack of correlation between apo[a] mRNA abundance and plasma Lp[a] levels; alternatively, large mRNAs such as apo[a] are inefficiently transferred to solid support membranes which could possible lead to variable Northern blot results.

To circumvent the transfer problems associated with high molecular weight mRNAs and to accurately measure apo[a] mRNA, a ribonuclease protection assay was developed. A riboprobe complementary to monkey apo[a] mRNA was generated from pMK-1 that spans nucleotide 195 to 482 (Fig. 2). Based on the human cDNA (30), sequence analysis of the probe region protected by the apo[a] mRNA indicated that only the first apo[a] kringle VI (Type-1) was completely complementary to the probe. The sequence of all other apo[a] kringles (30), and kringle-containing sequences such as prothrombin (66, 67) plasminogen (65), tissue type plasminogen activator (68), and hepatocyte growth factor (68) showed substantial mismatches and/or deletions compared to the K-1 sequence. Therefore, the protected region of this probe represents only the first kringle IV of the apo[a] mRNA.

As the protected probe sequence is unique, hybridization analysis will not be influenced by the number of kringle IV repeats but will be directly proportional to the apo[a] mRNA abundance. The apo[a] mRNA ribonuclease protection assay, carried out on total RNA before treatment and at the end of the 200 mg/kg gemfibrozil dosing period, is shown in **Fig. 4**. The larger protected fragment of 287 nucleotides that is decreased in treated samples represents the apo[a] mRNA and the smaller species of 123 nucleotides represents the protected synthetic sense strand. Differences in apo[a] mRNA levels in the treated samples was not related to hybridization conditions or variability in gel loading as the apo[a] standard was constant in all lanes (Fig. 4, apo(a) STD.). The hybridizing species between the apo[a] mRNA and the apo[a] synthetic strand likely represent cross-hybridization with other apo[a] kringles due to their uniform repression by gemfibrozil. The observed decrease in apo[a] mRNA levels could not be due to sample degradation or differences in total RNA concentration because measurement of 18s rRNA in 1.0 μg of total RNA showed that it was the same in all samples except for one (monkey 2, gemfibrozil treated) which was slightly less than the other samples (data not shown).

Quantitation of the protected apo[a] fragment represented in Fig. 4 showed that gemfibrozil treatment caused a marked and substantial reduction in apo[a] mRNA in all eight animals relative to pretreatment levels (**Fig. 5**). Pair-wise comparison of the change in Lp[a] and apo[a] mRNA at the 200 mg/kg gemfibrozil dose for each monkey demonstrated that in all animals the levels of both molecules were reduced. In all cases the apo[a] transcript was attenuated to a greater degree than plasma Lp[a]. This indicates that reduction in apo[a] mRNA is reflected by decreased plasma Lp[a] levels.

To determine whether the lowering of apo[a] mRNA by gemfibrozil was specific, the levels of albumin, apoA-I, apoE, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNAs were measured in the control and experimental samples. The latter mRNAs were assayed using a slot blot due to the small amount of total RNA isolated from biopsy samples and the use of human cDNA probes. Apo[a] mRNA levels were normalized to the expression of these four mRNAs which resulted in essentially the same degree of inhibition observed before normalization (data not shown). The apo[a] mRNA inhibition observed when the four normalized values were averaged was 23.9% of control levels verses 19.1% for the

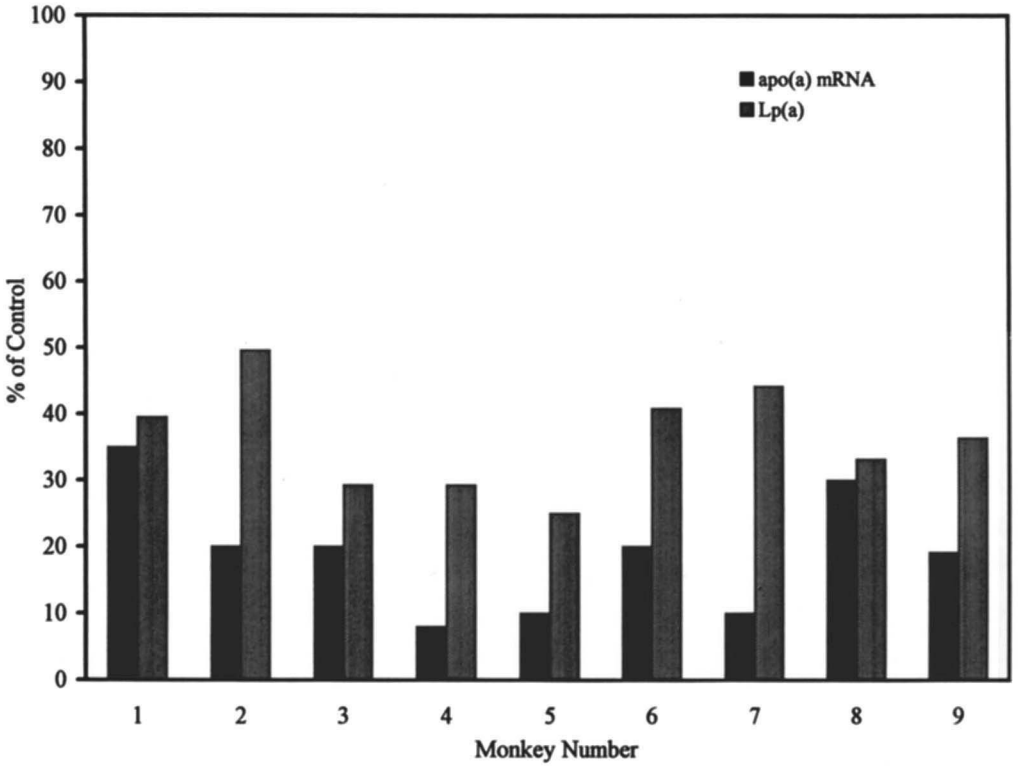

Fig. 5. Pair-wise comparison of the change in plasma Lp[a] and liver apo[a] mRNA levels. Changes in plasma Lp[a] and apo[a] mRNA levels in each monkey are compared at the end of the 200 mg/kg gemfibrozil dosing period. Plasma Lp[a] was determined by ELISA as described in Fig. 1. Apo[a] mRNA was measured by quantitating the bands in Fig. 4 using a PhosphorImager. Numbers 1-8 represent the individual monkeys depicted in Fig. 4 and number 9 represents the average value from all eight animals.

unadjusted value. These results indicate that gemfibrozil specifically inhibits apo[a] mRNA levels relative to albumin, apoA-I, apoE, and G3PDH transcript expression. However, the data do not exclude the possibility that other transcripts are regulated by gemfibrozil.

In this study we found no association between apo[a] protein isoform size and either apo[a] transcript abundance or the magnitude of apo[a] mRNA down-regulation by gemfibrozil. The former observation is in agreement with previous monkey studies in which apo[a] transcript length was found not to correlate with liver mRNA levels (42).

Gemfibrozil regulates apo[a] mRNA in cell culture

To determine whether gemfibrozil was capable of attenuating Lp[a] levels in vitro through a mechanism that involves changes in apo[a] mRNA, two cell-based systems were used. The first consisted of HepG2 cells permanently transfected with a 10-kringle apo[a] cDNA construct driven by a constitutive CMV promoter. Therefore, apo[a] transcription in this stable cell line cannot be specifically regulated. Treatment of these cells with gemfibrozil (1–1000 μM) for 5 days demonstrated that the drug did not change the levels of Lp[a] at all doses used at any time during the 5-day period **(Fig. 6)**. These results indirectly suggest that Lp[a] is modulated at the level of apo[a] mRNA expression and not at the translational or post-translational levels by gemfibrozil.

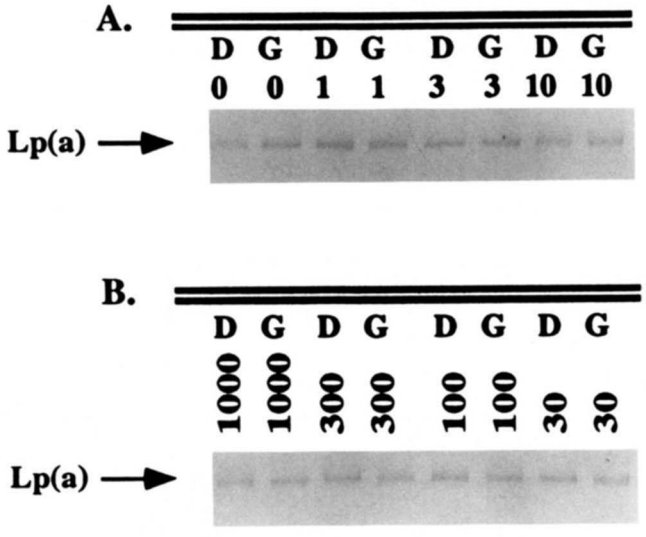

Fig. 6. Effect of gemfibrozil on Lp[a] expression in HepG2 cells permanently transfected with an apo[a] 10-kringle cDNA expression construct under control of the constitutive CMV promoter. Transfected HepG2 cells were grown in DMEM supplemented with FBS (10%) and treated with gemfibrozil (1–1000 μM) or the DMSO vehicle alone each day for a total of 5 days with the media being changed daily. The figure represents Lp[a] expression after 4 days of treatment. Lp[a] expression was analyzed by Western blots in non-reduced samples using a specific Lp[a]/apo[a] antibody. Lp[a] was not detected in HepG2 cells transfected with the 10-kringle construct in the anti-sense orientation (data not shown). The size of the Lp[a] band in the figure is approximately 700 kDa which is consistent with the expected size of a particle formed between apoB-100 (500 kDa) and the recombinant apo[a] (200 kDa). Reduction of the samples results in the appearance of a single band of 200 kDa on Western blots (data not shown). Panel A, gemfibrozil (0–10 μM); panel B, gemfibrozil (30–1000 μM); D, DMSO control; G, gemfibrozil.

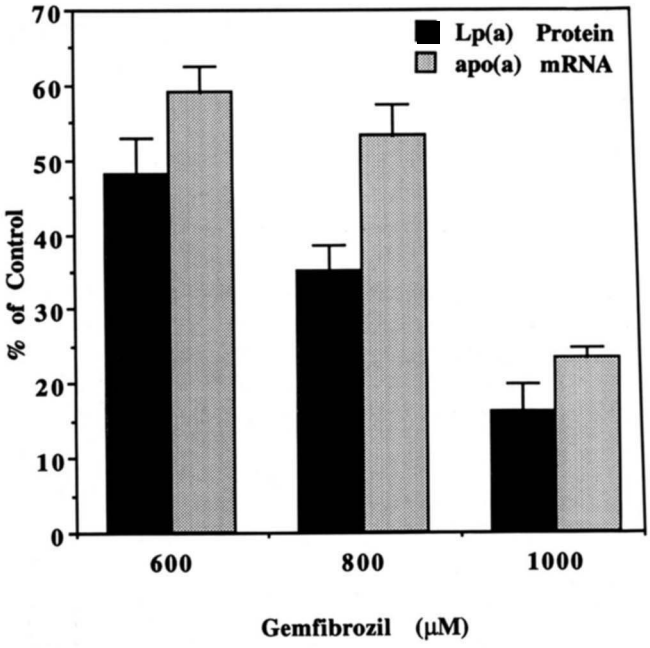

Fig. 7. Influence of gemfibrozil of the expression of Lp[a] and apo[a] mRNA in primary cynomolgus monkey hepatocytes. Monkey hepatocytes were treated with gemfibrozil as detailed in Materials and Methods. Lp[a] levels in the culture media were analyzed by ELISA and apo[a] mRNA was measured as described in Fig. 4.

The use of primary monkey hepatocyte cultures, which have a functional apo[a] gene, provided more direct evidence for apo[a] mRNA regulation by gemfibrozil. Treatment of the hepatocytes with gemfibrozil at concentrations ranging from 600 to 1000μM attenuated both Lp[a] and apo[a] mRNA expression. Gemfibrozil at 600 μM reduced Lp[a] levels to 50% of control values, while a decrease to 20% of control was observed at a drug concentration of 1000 μM (**Fig. 7**). There was also a corresponding decrease in apo[a] mRNA levels in the treated cells (Fig. 7).

Both the in vivo and in vitro data indicate that gemfibrozil can affect Lp[a] levels by decreasing the concentration of apo[a] mRNA. Changes in mRNA steady state levels can be due to alterations in gene transcription, mRNA stability, or a combination of both factors. The data presented in this investigation cannot unequivocally distinguish between the latter regulatory pathways due to the nature of the apo[a] expression construct used. This construct contains partial apo[a] mRNA 5'- and 3'-untranslated regions that may have resulted in deletion of *cis*-acting elements that could potentially be involved in regulating the transcript's half-life by gemfibrozil. The

presence of these putative regulatory elements could explain the drug's ability to lower apo[a] mRNA levels in monkey liver and hepatocyte cultures. However, the human apo[a] mRNA 3'-untranslated region does not contain any A-U rich elements that regulate the half-life of certain mRNAs (64) possibly making the apo[a] transcript an unlikely candidate for regulation at the level of mRNA stability. Furthermore, gemfibrozil, which is a well-established peroxisomal proliferator (69), has been shown in co-transfection assays to mediate its action by activating the peroxisome proliferator activated receptor (PPAR) transcription factor (70). Therefore, it is likely that the compound acts primarily as a transcriptional modulator rather than being involved in mRNA turnover. Interestingly, fatty acids, which have been suggested as the endogenous ligands for PPAR (70), can modulate apo[a] mRNA expression in some monkeys (71), further supporting a role for transcription in regulating apo[a] mRNA levels.

The data presented in this study show that Lp[a] levels can be regulated at the level of apo[a] mRNA expression. This observation suggests that therapies targeted at factors that regulate apo[a] transcript abundance may be an effective means of lowering plasma Lp[a] levels.

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