rapid communication

# Administration of a PPARα agonist increases serum apolipoprotein A-V levels and the apolipoprotein A-V/apolipoprotein C-III ratio

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**Abstract** **Apolipoprotein A-V (apoA-V) first gained attention as a regulator of triglycerides through transgenic mouse studies. Furthermore, peroxisome proliferator-activated receptor α (PPARα) agonists such as fenofibrate increase apoA-V mRNA expression. Our group recently developed the first assay to quantitate serum apoA-V levels. Therefore, we sought to determine whether administration of a PPARα agonist would increase circulating apoA-V. Cynomolgus monkeys were dosed for 14 days with 0.3 mg/kg/day LY570977 L-lysine, a potent and selective PPARα agonist. Blood samples were drawn throughout the treatment period and after a 2 week washout. Administration of the PPARα agonist caused a 50% decrease in triglycerides that reversed at washout. Serum apoA-V concentrations increased 2-fold, correlated inversely with triglycerides, and were reversible at washout. The apoA-V/apoC-III ratio increased >2-fold, with this increase also reversible at washout. These data demonstrate for the first time that a PPARα agonist increases circulating apoA-V protein levels and the apoA-V/apoC-III ratio.—** Schultze, A. E., W. E. Alborn, R. K. Newton, and R. J. Konrad. **Administration of a PPARα agonist increases serum apolipoprotein A-V levels and the apolipoprotein A-V/apolipoprotein C-III ratio. *J. Lipid Res.* 2005.** 46: **1591–1595.**

**Supplementary key words** triglycerides • cholesterol • enzyme-linked immunosorbent assay • lipids • cynomolgus monkeys • peroxisome proliferator-activated receptor α

Apolipoprotein A-V (apoA-V) is now recognized as a key regulator of serum triglyceride levels (for a complete review, see 1, 2). The gene for this novel apolipoprotein was originally identified in experiments seeking new open reading frames in the ApoA1-ApoC3-ApoA4 gene cluster located on human chromosome 11q23 and at the molecular level in animal studies (3, 4). What emerged from this work was a new gene coding for an apolipoprotein with greatest homology to ApoA4; the new protein was named apoA-V (3, 4).

When the human gene for apoA-V was expressed in transgenic mice, triglyceride levels decreased by 50–70%, and when the mouse ApoA5 gene itself was knocked out, triglyceride levels increased by ~4-fold (4, 5). These data suggested that apoA-V expression may be highly and inversely correlated with triglyceride levels. In addition, polymorphisms in the human ApoA5 gene correlate with increased triglyceride levels (6–11).

Our group recently developed the first assay to measure apoA-V protein and demonstrated that apoA-V is present in human serum in specific lipoprotein particles (12). It was also shown recently that apoA-V mRNA expression is upregulated by peroxisome proliferator-activated receptor α (PPARα) agonists such as fenofibrate, suggesting that increased apoA-V mRNA expression may contribute to decreases in serum triglycerides (13, 14). In light of these data and our ability to measure actual serum apoA-V protein levels, we sought to determine whether the administration of a potent and selective PPARα agonist would increase circulating levels of apoA-V protein.

## MATERIALS AND METHODS

### Animals

Young adult, male, cynomolgus monkeys (*Macaca fasciularis*) weighing ~2.5–5.0 kg were obtained from Charles River BRF, Inc. Monkeys were housed individually in aluminum cages with suspended floors under conditions of controlled temperature (22.2 ± 4.4°C), humidity (20–80%), and light cycle (12/12 h light/dark). Monkeys were offered ~8–20 biscuits (Certified Primate Diet 5048; PMI Nutrition International, Inc.) daily, with fresh fruit provided three times weekly and water supplied ad libitum.

### Study design and institutional compliance statement

Six monkeys were given 0.3 mg/kg LY570977 L-lysine in a 2 ml/kg dose volume by nasogastric gavage daily for 14 consecutive days, followed by 14 days without treatment. Fasting blood samples were collected on days 0 (baseline), 1, 3, 7, 14, and 28 (washout). The $EC_{50}$ of LY570977 L-lysine for human PPARα is 1,082 nM. No activity is measurable for PPARγ or PPARδ. Based on these data

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as well as previous experience, a dose of 0.3 mg/kg LY570977 L-lysine was selected. Monkeys were housed in facilities at Eli Lilly and Co. (Greenfield, IN) that are accredited by the American Association for the Accreditation of Laboratory Animal Care, and all protocols were approved by the Eli Lilly Institutional Animal Care and Use Committee.

### Blood sample collection and hematologic analysis

Monkeys were restrained in a chair and blood was drawn from the femoral vein into tubes containing EDTA for hematologic analysis or into tubes containing no anticoagulant for clinical chemistry analysis. EDTA anticoagulated blood samples were analyzed using Bayer Advia 120 (Bayer Corp., Tarrytown, NY). Morphology comments and differential leukocyte counts were made by direct microscopy of Modified Wright-stained blood smears.

### Clinical chemistry, apolipoprotein, and lipoprotein analysis

Concentrations of all chemistry analytes, including triglycerides and apoA-I and apoB, were measured using Hitachi Chemistry Systems (Roche Diagnostics, Indianapolis, IN). Serum apoC-III levels were measured on a Roche Hitachi 717 instrument using reagents manufactured by Wako Chemicals. Lipoproteins were analyzed using NMR technology (Liposcience, Inc., Raleigh, NC).

### Measurement of apoA-V levels

Recombinant apoA-V standard and anti-apoA-V antibodies were prepared as described previously (12). An apoA-V ELISA was used to measure apoA-V levels as described previously with minor modifications (12). Briefly, wells were coated overnight (Pierce carbonate-bicarbonate coating buffer, pH 9.40) with anti-N-terminal apoA-V antibody at a concentration of 5 μg/ml. The next day, wells were aspirated, washed three times with assay buffer (50 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 5 mM EGTA), and blocked for 1 h with TBS-casein blocking buffer (Pierce). Wells were washed three times with assay buffer. Next, 100 μl of human serum or recombinant apoA-V standard (varying concentrations of recombinant protein with 1.0% BSA as a carrier protein) diluted 1:10 in assay buffer was added to the wells and incubated for 2 h at room temperature. After aspiration, wells were washed six times with assay buffer, and 100 μl of a 1:1,000 dilution of conjugate antibody (HRP-labeled anti-C-terminal apoA-V antibody; 1 mg/ml) in assay buffer supplemented with 0.1% BSA was added to the wells for a 1 h incubation at room temperature. After aspiration, wells were washed six times with assay buffer. After the last aspiration, 100 μl of TMB development substrate (BioFX Laboratories) was added to the wells and allowed to incubate for 30 min at room temperature. The reaction was stopped with an equal volume of 2 N phosphoric acid, and plates were read at 450 nm.

### Data analysis

SigmaPlot version 8.0 was used for fitting of the calibration curves. Data are expressed as means ± SEM using version 2.98 of the program FigP (Biosoft, St. Louis, MO). Statistical analysis was performed using the same program. Data were analyzed by one-way ANOVA followed by comparisons between the means using the least significant difference test. $P < 0.05$ was considered to indicate statistical significance.

## RESULTS

No important compound-related alterations in complete blood counts occurred (data not shown). Minor compound-related chemistry alterations consisted of slight time-dependent decreases in the activity of alkaline phosphatase and γ-glutamyltransferase (**Table 1**). All compound-related changes normalized at completion of the reversibility phase. None of the minor compound-related alterations was considered adverse to the monkeys' health.

As **Fig. 1A** shows, decreases in the serum triglycerides began on day 1 and were sustained throughout the treatment period with LY570977 L-lysine, a potent and selective PPARα agonist. The mean baseline triglyceride level was 60.8 ± 5.3 mg/dl. On day 1 of dosing, triglycerides decreased to 27.3 ± 2.8 mg/dl ($P < 0.05$ compared with baseline, $P < 0.05$ compared with washout). This was followed by triglyceride levels of 26.8 ± 2.3 mg/dl ($P < 0.05$ compared with baseline, $P < 0.05$ compared with washout), 24.0 ± 2.1 mg/dl ($P < 0.05$ compared with baseline,

TABLE 1. Routine chemistry laboratory values for cynomolgus monkeys dosed for 14 days with LY570977 L-lysine, a potent and selective PPARα agonist

| Analyte | Day 0 (Baseline) | Day 1 | Day 3 | Day 7 | Day 14 | Day 28 (Washout) |
|---|---|---|---|---|---|---|
| Blood urea nitrogen (mg/dl) | 19 ± 4 | 18 ± 2 | 19 ± 3 | 20 ± 3 | 19 ± 2 | 24 ± 5 |
| Creatinine (mg/dl) | 0.9 ± 0.1 | 0.8 ± 0.1 | 0.9 ± 0.1 | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 |
| Bilirubin (mg/dl) | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 |
| Alkaline phosphatase (IU/l) | 802 ± 165 | 738 ± 134 | 686 ± 118 | 638 ± 122 | 564 ± 147 | 661 ± 143 |
| γ-Glutamyl transpeptidase (IU/l) | 190 ± 53 | 178 ± 53 | 171 ± 50 | 163 ± 56 | 142 ± 49 | 164 ± 48 |
| Alanine aminotransferase (IU/l) | 56 ± 15 | 51 ± 12 | 50 ± 16 | 50 ± 14 | 57 ± 18 | 54 ± 19 |
| Aspartate aminotransferase (IU/l) | 35 ± 13 | 42 ± 11 | 32 ± 4 | 34 ± 7 | 36 ± 10 | 40 ± 8 |
| Creatine kinase (IU/l) | 378 ± 212 | 389 ± 426 | 173 ± 99 | 345 ± 413 | 252 ± 164 | 530 ± 497 |
| Sodium (mmol/l) | 146 ± 2 | 146 ± 2 | 146 ± 2 | 146 ± 2 | 148 ± 3 | 149 ± 6 |
| Potassium (mmol/l) | 4.4 ± 0.6 | 4.3 ± 0.2 | 4.2 ± 0.6 | 3.9 ± 0.4 | 4.5 ± 0.5 | 4.7 ± 0.5 |
| Chloride (mmol/l) | 105 ± 2 | 107 ± 2 | 107 ± 2 | 107 ± 2 | 104 ± 2 | 105 ± 3 |
| Calcium (mmol/l) | 9.9 ± 0.1 | 10.0 ± 0.2 | 10.0 ± 0.2 | 9.9 ± 0.1 | 10.2 ± 0.4 | 10.5 ± 0.6 |
| Phosphorus (mg/dl) | 4.9 ± 0.7 | 4.6 ± 0.4 | 5.2 ± 0.4 | 5.6 ± 0.6 | 5.7 ± 0.7 | 5.5 ± 0.7 |
| Total protein (g/dl) | 7.8 ± 0.3 | 7.5 ± 0.4 | 7.7 ± 0.4 | 8.0 ± 0.4 | 7.7 ± 0.5 | 7.9 ± 0.5 |
| Albumin (g/dl) | 4.5 ± 0.3 | 4.3 ± 0.1 | 4.4 ± 0.2 | 4.6 ± 0.2 | 4.3 ± 0.4 | 4.4 ± 0.3 |
| Glucose (mg/dl) | 90 ± 20 | 70 ± 11 | 75 ± 9 | 81 ± 17 | 83 ± 14 | 106 ± 39 |
| Cholesterol (mg/dl) | 119 ± 19 | 106 ± 12 | 123 ± 14 | 131 ± 19 | 132 ± 33 | 129 ± 15 |
| Globulins (mg/dl) | 3.4 ± 0.3 | 3.2 ± 0.4 | 3.2 ± 0.4 | 3.4 ± 0.4 | 3.4 ± 0.4 | 3.5 ± 0.4 |

PPARα, peroxisome proliferator-activated receptor α.

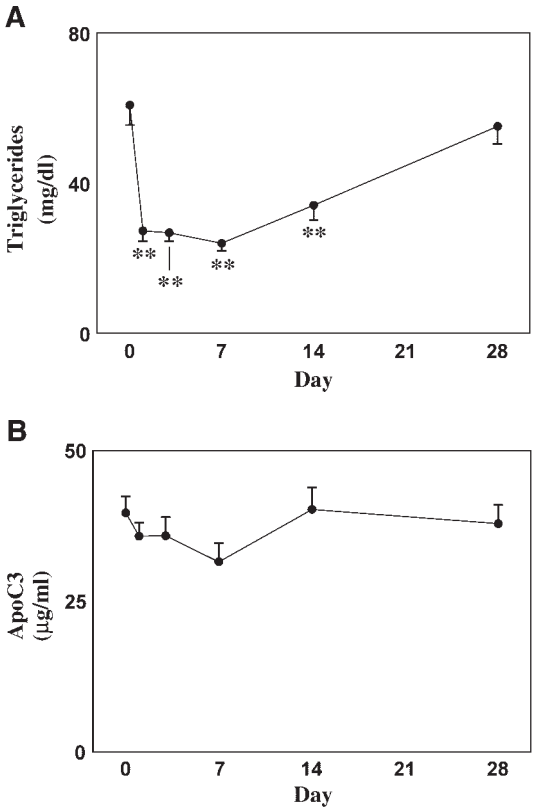


**Fig. 1.** Effect of a potent and selective peroxisome proliferator-activated receptor α (PPARα) agonist on triglycerides and apolipoprotein C-III (apoC-III) levels. A: Serum triglyceride levels in cynomolgus monkeys are plotted versus duration of dosing with LY570977 L-lysine, a potent and selective PPARα agonist. Day 0 represents baseline, days 1–14 represent the dosing period, and day 28 is the end of the 2 week washout. Results are shown as means ± SEM for all six cynomolgus monkeys (** $P < 0.05$ vs. baseline and $P < 0.05$ vs. washout). B: ApoC-III assays were performed to determine apoC-III serum levels in cynomolgus monkeys at the time points corresponding to those in A. Serum apoC-III levels are plotted versus duration of dosing with LY570977 L-lysine. Day 0 represents baseline, days 1–14 represent the dosing period, and day 28 is the end of the 2 week washout. Results are shown as means ± SEM for all six cynomolgus monkeys.

$P < 0.05$ compared with washout), and 34.1 ± 4.0 mg/dl ($P < 0.05$ compared with baseline, $P < 0.05$ compared with washout) on days 3, 7, and 14 of dosing, respectively. On day 28, after a 14 day washout of the compound, triglyceride levels were 55.1 ± 4.7 mg/dl.

ApoC-III levels were measured at the same time points used for triglyceride measurements. As Fig. 1B shows, moderate decreases in serum apoC-III levels were observed. None of these decreases was statistically significant compared with either baseline or washout values. The mean baseline apoC-III level was 39.6 ± 2.7 μg/ml. On day 1 of dosing, apoC-III levels decreased slightly to 35.8 ± 2.5 μg/ml. This was followed by apoC-III levels of 35.9 ± 3.1 μg/ml, 31.6 ± 3.0 μg/ml, and 40.2 ± 3.6 μg/ml on days 3, 7, and 14 of dosing, respectively. On day 28, after a 14 day washout, apoC-III levels were 37.9 ± 3.1 μg/ml.

Serum apoA-V levels were next measured via a dual-antibody sandwich ELISA (12) at corresponding time points. **Figure 2A** shows raw results from a representative apoA-V ELISA in which all samples were clearly on the standard curve and no sample required additional dilution for repeat analysis. This ELISA permits the measurement of apoA-V levels ranging from ~10 to 1,000 ng/ml.

As Fig. 2B demonstrates, increases in serum apoA-V levels were detected beginning on day 1 and were sustained throughout the treatment period with the PPARα agonist. The mean baseline apoA-V level was 34.3 ± 4.6 ng/ml. On day 1 of dosing, there was an increase in serum apoA-V levels to 43.0 ± 6.0 ng/ml ($P = 0.2$ compared with baseline, $P < 0.05$ compared with washout). This was followed by apoA-V levels of 77.8 ± 21.0 ng/ml ($P = 0.07$ com-

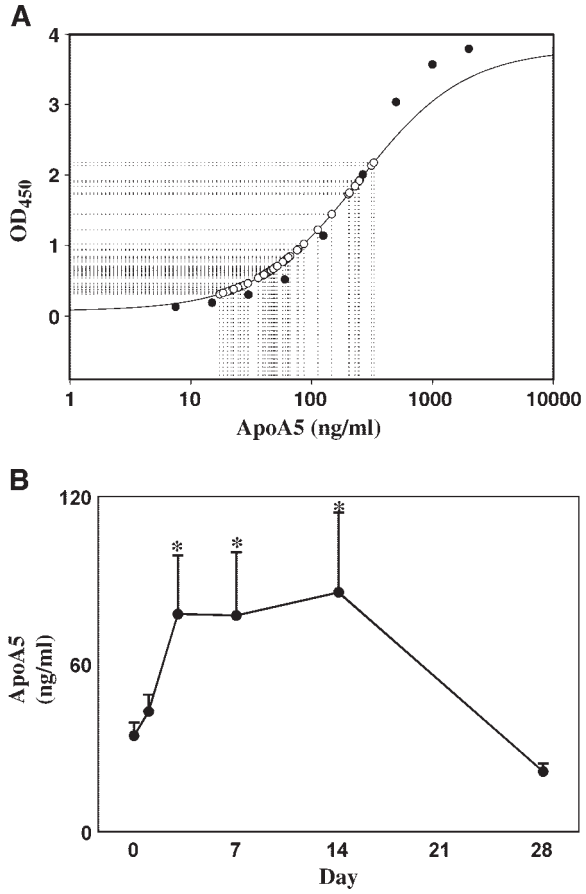


**Fig. 2.** Effect of a potent and selective PPARα agonist on serum apoA-V levels. A: ApoA-V assays were performed on each sample using the ELISA described in Materials and Methods. Serum samples (open circles) were run on the ELISA at a 1:10 dilution, with optical densities ($OD_{450}$) plotted on the standard curve (closed circles) shown. All ELISA results were well on the standard curve, with no sample requiring additional dilution for repeat testing. Results shown are representative of two independent assays. B: ApoA-V ELISAs were performed as in A to determine apoA-V serum levels in cynomolgus monkeys at time points corresponding to those in Fig. 1. Serum apoA-V levels are plotted versus duration of dosing with LY570977 L-lysine, a potent and selective PPARα agonist. Day 0 represents baseline, days 1–14 represent the dosing period, and day 28 is the end of the 2 week washout. Results are shown as means ± SEM for all six cynomolgus monkeys. For each individual monkey sample, the serum apoA-V level represents an average of two determinations from independent assays (* $P < 0.05$ vs. washout).

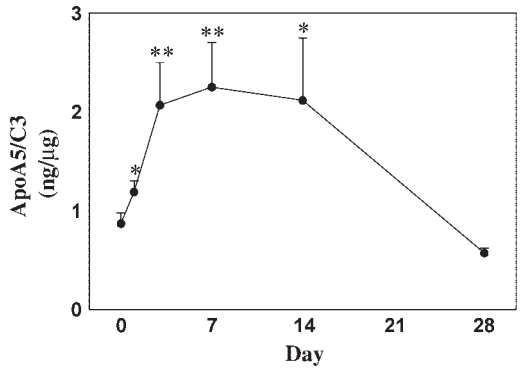


**Fig. 3.** Effect of a potent and selective PPARα agonist on the apoA-V/apoC-III ratio. For time points corresponding to those in Fig. 1, the apoA-V/apoC-III ratio (ng/μg) was calculated to determine the effect of LY570977 L-lysine, a potent and selective PPARα agonist, on the ratio. Day 0 represents baseline, days 1–14 represent the dosing period, and day 28 is the end of the 2 week washout. Results are shown as means ± SEM for all six cynomolgus monkeys (* $P < 0.05$ vs. washout, ** $P < 0.05$ vs. baseline and $P < 0.05$ vs. washout).

pared with baseline, $P < 0.05$ compared with washout), 77.3 ± 22.6 ng/ml ($P = 0.09$ compared with baseline, $P < 0.05$ compared with washout), and 85.7 ± 28.6 ng/ml ($P = 0.09$ compared with baseline, $P < 0.05$ compared with washout) on days 3, 7, and 14 of dosing, respectively. On day 28, after a 14 day washout of the compound, apoA-V levels were 21.5 ± 2.9 ng/ml.

An apoA-V/apoC-III ratio was also calculated for each data point. **Figure 3** shows the changes that occurred in this ratio over time. The apoA-V/C-III ratio increased beginning on day 1, and this increase was sustained throughout the dosing period. The mean baseline apoA-V/C-III ratio was 0.86 ± 0.10 ng/μg. On day 1 of dosing, the ratio increased to 1.18 ± 0.11 ng/μg ($P = 0.06$ compared with baseline, $P < 0.05$ compared with washout). This was followed by apoA-V/C-III ratios of 2.07 ± 0.43 ng/μg ($P < 0.05$ compared with baseline, $P < 0.05$ compared with washout), 2.25 ± 0.44 ng/μg ($P < 0.05$ compared with baseline, $P < 0.05$ compared with washout), and 2.11 ± 0.62 ng/μg ($P = 0.07$ compared with baseline, $P < 0.05$ compared with washout) on days 3, 7, and 14 of dosing, respectively. On day 28, after a 14 day washout of the compound, the apoA-V/C-III ratio was 0.57 ± 0.06 ng/μg.

Routine lipoprotein measurements were performed at all time points, with data shown in **Table 2**. Small changes in HDL, HDL particle size, HDL-cholesterol, and apoA-I were observed. Minimal changes were also noted in LDL, LDL particle size, and apoB. VLDL-triglyceride was decreased significantly during dosing of the PPARα agonist ($P < 0.05$ compared with baseline, $P < 0.05$ compared with washout at days 3, 7, and 14). Decreases in VLDL particle number were also significant and essentially paralleled those observed for VLDL-triglyceride. Minimal changes were observed in VLDL particle size.

## DISCUSSION

Our results demonstrate that administration of a potent and selective PPARα agonist results in increased levels of serum apoA-V protein, which correlate inversely with decreased triglycerides, VLDL, and VLDL-triglyceride. The apoA-V/C-III ratio was also increased with administration of the PPARα agonist. Upon washout of the compound, these changes were reversible, with apoA-V levels decreasing and serum triglycerides, VLDL, and VLDL-triglyceride increasing. Modest decreases were observed in serum levels of apoC-III, whose expression has been reported to decrease with PPARα agonists (15–18).

We previously described the first assay to measure apoA-V levels and found that apoA-V circulates at much lower concentrations than other apolipoproteins (12). These data explained why apoA-V was not discovered in serum, as were related apolipoproteins coded for on the human chromosome 11q23 locus, but was instead identified at the DNA level via a search for open reading frames (3, 4). Our current observations confirm this and are particularly important because they represent the first demonstration that administration of a PPARα agonist causes increases in circulating levels of apoA-V protein.

As we reported previously, the lipoprotein particle distribution of apoA-V is most similar to that of apoC-III, an interesting observation in light of recent data suggesting that apoC-III and apoA-V influence triglyceride levels in opposite directions (1, 19). In the case of apoC-III, it is thought that the protein is an inhibitor of lipoprotein lipase. The mechanism by which apoA-V decreases triglyc-

TABLE 2. Lipoprotein measurements for cynomolgus monkeys dosed for 14 days with LY570977 L-lysine, a potent and selective PPARα agonist

| Analyte | Day 0 (Baseline) | Day 1 | Day 3 | Day 7 | Day 14 | Day 28 (washout) |
|---|---|---|---|---|---|---|
| HDL particles (μmol/l) | 38 ± 2 | 41 ± 2 | 42 ± 2 | 45 ± 2 | 44 ± 4 | 35 ± 2 |
| HDL size (nm) | 9.7 ± 0.2 | 9.4 ± 0.1 | 9.2 ± 0.1 | 9.1 ± 0.1 | 9.2 ± 0.1 | 10.0 ± 0.2 |
| HDL-cholesterol (mg/dl) | 87 ± 5 | 84 ± 6 | 77 ± 5 | 78 ± 5 | 81 ± 7 | 93 ± 4 |
| LDL particles (nmol/l) | 686 ± 63 | 463 ± 71 | 565 ± 90 | 650 ± 93 | 768 ± 54 | 604 ± 46 |
| LDL size (nm) | 22 ± 1 | 21 ± 1 | 21 ± 1 | 21 ± 1 | 21 ± 1 | 22 ± 1 |
| VLDL particles (nmol/l) | 3.2 ± 1.0 | 2.6 ± 1.3 | 0.3 ± 0.1 | 0.2 ± 0.1 | 0.2 ± 0.1 | 1.2 ± 0.7 |
| VLDL size (nm) | 72 ± 8 | 86 ± 9 | 82 ± 16 | 125 ± 10 | 78 ± 24 | 95 ± 18 |
| VLDL-triglyceride (μmol/l) | 8.3 ± 0.8 | 5.7 ± 1.2 | 1.6 ± 0.5 | 1.2 ± 0.4 | 0.9 ± 0.4 | 4.7 ± 1.8 |
| Apolipoprotein A-I (mg/dl) | 137 ± 11 | 127 ± 8 | 126 ± 7 | 130 ± 8 | 132 ± 12 | 152 ± 7 |
| Apolipoprotein B (mg/dl) | 28 ± 3 | 23 ± 3 | 28 ± 3 | 32 ± 3 | 33 ± 4 | 25 ± 2 |

eride levels is unclear, although it has been demonstrated to inhibit VLDL production and to stimulate lipoprotein lipase-mediated VLDL-triglyceride hydrolysis (20, 21). Our current data suggest that decreases in VLDL-triglyceride observed in this study may have been attributable more to VLDL particle number reduction than to decreased VLDL particle size. What is clear, however, is that serum levels of apoA-V are minuscule compared with those of apoC-III. When differences in the molecular weights of the two proteins are taken into account, there is ~4,000–5,000-fold more apoC-III than apoA-V in serum on a molar basis.

There is significant current interest in better understanding the role of apoA-V in the regulation of serum lipids, with additional recent reports of apoA-V polymorphisms associated with hypertriglyceridemia (22, 23), with hyperlipidemia in patients with apoE2/2 phenotype (24), and of apoA-V mRNA expression being regulated via insulin (25). In light of our data describing increased serum levels of apoA-V in response to administration of a PPARα agonist, it is very likely that assays for serum levels of apoA-V will emerge as important biomarkers for PPARα agonists. We continue to anticipate that apoA-V measurement will be increasingly used in the selection and development of PPAR agonists as well as other types of small and large molecules developed to treat dyslipidemia.

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