Apolipoprotein A-V Deficiency Results in Marked Hypertriglyceridemia Attributable to Decreased Lipolysis of Triglyceride-Rich Lipoproteins and Removal of Their Remnants

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Apolipoprotein A-V Deficiency Results in Marked Hypertriglyceridemia Attributable to Decreased Lipolysis of Triglyceride-Rich Lipoproteins and Removal of Their Remnants


Objective—ApoAV, a newly discovered apoprotein, affects plasma triglyceride level. To determine how this occurs, we studied triglyceride-rich lipoprotein (TRL) metabolism in mice deficient in apoAV.

Methods and Results—No significant difference in triglyceride production rate was found between apoa5−/− mice and controls. The presence or absence of apoAV affected TRL catabolism. After the injection of 14C-palmitate and 3H-cholesterol labeled chylomicrons and 125I-labeled chylomicron remnants, the disappearance of 14C, 3H, and 125I was significantly slower in apoa5−/− mice relative to controls. This was because of diminished lipolysis of TRL and the reduced rate of uptake of their remnants in apoa5−/− mice. Observed elevated cholesterol level was caused by increased high-density lipoprotein (HDL) cholesterol in apoa5−/− mice. VLDL from apoa5−/− mice were poor substrate for lipoprotein lipase, and did not bind to the low-density lipoprotein (LDL) receptor as well as normal very-low-density lipoprotein (VLDL). LDL receptor levels were slightly elevated in apoa5−/− mice consistent with lower remnant uptake rates. These alterations may be the result of the lower apoE-to-apoC ratio found in VLDL isolated from apoa5−/− mice.

Conclusions—These results support the hypothesis that the absence of apoAV slows lipolysis of TRL and the removal of their remnants by regulating their apoproteins content after secretion. (Arterioscler Thromb Vasc Biol. 2005;25:2573-2579.)

Key Words: Apoa5 ▪ hypertriglyceridemia ▪ knockout ▪ lipolysis ▪ triglyceride-rich lipoproteins

The level of triglycerides in the blood has been correlated with the risk of atherosclerosis in a variety of studies. Apoproteins of triglyceride-rich lipoproteins (TRL) play an important role in triglyceride transport. In particular, ApoCs and apoE3,4 strongly affect TRL metabolism and thus plasma triglyceride levels. ApoAV is a newly discovered apoprotein, which was identified independently by 2 groups. Disruption of the apoa5 gene in mice resulted in hypertriglyceridemia, whereas overexpression led to decreased plasma triglyceride concentrations, thus establishing an important role for this protein in triglyceride homeostasis. One explanation for apoAV’s lack of earlier identification is its low plasma concentration (≈ 1 μg/mL), which may be in part caused by the observation that during its synthesis, it is largely retained in the endoplasmic reticulum and does not traffic to the Golgi. Furthermore, the low concentration of apoAV in plasma suggests that either this protein does not exert its effect in plasma or that it has a very potent effect on lipoprotein particle composition and/or metabolism. Several studies in humans revealed strong associations between APOA5 polymorphisms and triglyceride levels. Most striking was the association between the minor APOA5 haplotypes found in 25% to 50% of whites, blacks, and Hispanics, and increased plasma triglyceride levels. To better understand the specific mechanisms by which altered apoAV exerts its effect on triglyceride levels in humans and mice, we studied the metabolism of TRL in vivo in mice lacking apoAV.

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ApoAV could influence triglyceride levels through alterations in the hepatic triglyceride secretion rate, or alternatively in the rate of catabolism of triglycerides within plasma. We report that the presence or absence of apoAV does not affect triglyceride production. The absence of apoAV leads to
a reduction in the rate of removal of triglycerides with the accumulation of larger than normal very-low-density lipoprotein (VLDL) particles in the plasma. These particles are paradoxically worse substrates for lipoprotein lipase than normal size VLDL particles. In addition, there are lower than normal lipoprotein lipase (LPL) levels in the knockout mice. Additionally, chylomicron remnant removal is delayed in apoAV deficient mice because of decreased uptake of even normal remnant particles. These alterations in lipoprotein physiology may be caused by the ability of apoAV to regulate the ratio of apoE-to-apoC of TRL. Subsequent to our original submission, 2 reports of metabolic effects of apoAV appeared. Schaap et al suggested apoAV has effects on triglyceride but not apoB secretion and on LPL activity, whereas Fruchart-Najib et al also observed an enhanced LPL activity with apoAV overexpression, along with the expected metabolic effects of this.\textsuperscript{11}

### Methods

#### Animals

Mice heterozygous for the apoA5 gene, as previously described, were mated to yield progeny apoA5\textsuperscript{+/−} mice and wild-type littermate controls having intact apoA5. Mice were maintained at the Palo Alto Medical Foundation Research Institute Animal Facility (Palo Alto, Calif). All procedures were in accordance with institutional guidelines of the Palo Alto Medical Foundation. Male mice were used at 3 to 5 months of age when they weighed 25 to 35 grams. Body weight increased as the animals aged, but there were no differences with genotype.

#### Plasma Lipid and Lipoprotein Analysis

Blood was drawn from study mice in the morning after overnight fast. Aliquots for lipid measurement were immediately frozen at −70°C and levels of triglyceride and cholesterol were determined within 2 weeks. VLDL, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) fractions were separated by sequential ultracentrifugation of pooled plasma samples from 8 to 15 mice. Concentrations of cholesterol and triglyceride were measured using enzymatic kits (#TR13421, Thermo DMA Ltd., and TR0100; Sigma, respectively). Electron microscopy of isolated VLDL was determined by isopropanol precipitation, so only apoB removal was studied. The data were modeled by a single pool or 2-pool model for TRL kinetics. The 2-pool model was used only if it improved the fit of the data.

#### Preparation of Chylomicron and Chylomicron Remnants

Chylomicrons were obtained from the lymph of lymph duct-cannulated rats as previously described.\textsuperscript{15} Chylomicron remnants were prepared in functionally hepatectomized rats.\textsuperscript{14} Chylomicrons and chylomicron remnants had, in various batches, a triglyceride to cholesterol ratio of 15±2.3 and 5±0.8, respectively. In the chylomicrons, an average of 16% of the cholesterol was unesterified. In some experiments, chylomicrons were labeled by adding either 0.3 mCi \textsuperscript{13}C-labeled palmitate or 0.3 mCi \textsuperscript{14}C-labeled palmitate and 0.5 mCi \textsuperscript{3}H-labeled cholesterol (ARC, St Louis, Mo) to the duodenal infusion.\textsuperscript{15}

#### Radiolabeling of Chylomicron Remnants

Chylomicron remnants were labeled with carrier-free Na\textsuperscript{125}I (ARC) by a modification\textsuperscript{16} of the method described by McFarlane.\textsuperscript{17} The distribution of the radioactivity between lipid and protein fell in all batches within the range reported: 71.64±4.64%, on apoB\textsubscript{48} (determined by isopropanol precipitation), 11.05±2.12 on lipid (chloroform-methanol extraction), and the remainder on non-apoB proteins (apoE, apoAs).\textsuperscript{18}

#### In Vivo Removal of Labeled Chylomicrons and Chylomicron Remnants

Mice were injected with 10 μg protein of \textsuperscript{3}H-, \textsuperscript{14}H-, and \textsuperscript{13}C-labeled chylomicrons, or \textsuperscript{125}I-labeled chylomicron remnants. Measurement of plasma clearance of the respective isotopes followed the protocol of de Faria et al.\textsuperscript{15} In a separate group of experiments, \textsuperscript{125}I CPM were determined after isopropanol precipitation, so only apoB removal was studied. The data were modeled by a single pool or 2-pool model for TRL kinetics. The 2-pool model was used only if it improved the fit of the data.

#### Purified Lipoprotein Lipase Assay

VLDL from apoA5\textsuperscript{+/−} mice and controls containing 10, 20, 30, and 40 μg triglyceride were incubated for 12 minutes with 14.5 ng LPL (Sigma L-2254) to assay lipolysis by a modification of the method described by de Man\textsuperscript{20} for lipoprotein lipase in solution. In our hands, the release of free fatty acids was linear from 6 to 20 minutes.

#### Determination of Lipoprotein Lipase and Hepatic Lipase Activities

Measurement of lipoprotein lipase and hepatic lipase activities in post-heparin plasma of apoA5\textsuperscript{+/−} mice and controls followed the protocol of Siri et al.\textsuperscript{21}

#### Glucose and Insulin Tolerance Tests

Glucose and insulin tolerance tests were conducted after an overnight fast exactly as described by Siri et al.\textsuperscript{21}

#### LDL Receptor Binding Assay

The binding and internalization of VLDL via the LDL receptor was assayed using a modification\textsuperscript{12,22} of the competition method of Goldstein and Brown\textsuperscript{23} using \textsuperscript{125}I-labeled human LDL. Values for inhibitory concentration 50 (IC50) for VLDL was derived from incremental plasma triglyceride concentrations and individual mouse blood volume (body weight × 0.05).

#### Liver LDL Receptor Expression Level Using Real-Time Polymerase Chain Reaction

The primers and probe for LDL receptor were designed using Primer Express 1.5 (Applied Biosystems, Foster City, Calif). Primers corresponded to nucleotides +461 to +481 and +572 to +589 of mouse LDLR gene. DNA sequences from +483 to +504 were used as the specific probe. This technique measures the absolute amount of RNA present. In all samples, GAPDH mRNA levels were measured to provide an internal standard.

#### Statistical Analysis

The means and standard deviations (unless stated otherwise) are presented. Statistically significant differences (ie, \(P<0.05\); 2-tailed) in mean values between the 2 groups were assessed by Student’s t test.
Plasma Lipid and Lipoprotein Concentration in apoa5<sup>−/−</sup> and Control Mice

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*Number of pools, each contains 8 to 15 mice. Trig indicates triglyceride; Chol, cholesterol. Values are expressed as mmol/L±SD.

Results

ApoAV Deficiency Increases Plasma Triglycerides, Cholesterol, and Alters Lipoprotein Profile

Total plasma triglyceride and cholesterol levels were significantly increased in apoa5<sup>−/−</sup> mice (Table). This confirmed previous reports of altered triglycerides in these mice. The triglyceride increase was mainly because of a 4.6-fold increase within the VLDL fraction (<i>P</i>&lt;0.001). In the LDL fraction, triglyceride levels were 1.5-fold higher (<i>P</i>=0.001) relative to controls. In HDL fraction, cholesterol and triglyceride levels were 41% (<i>P</i>=0.01) and 38% (<i>P</i>=0.006) higher, respectively, relative to controls. Similar differences were present at 4 hours postprandial (data not shown).

Alterations in VLDL Particle Size

To determine whether increased triglyceride content affected VLDL particle size, we next examined these particles by electron microscopy. VLDL particles were on average 30% larger in the apoa5<sup>−/−</sup> mice relative to controls (55.9 nm ±23 versus 43.8 ±16 nm, <i>P</i>&lt;0.001) (Figure I, available online at http://atvb.ahajournals.org).

Triglyceride Secretion Is Normal in Apoa5<sup>−/−</sup> Mice

To determine whether the alteration in triglyceride level was the result of altered hepatic secretion or reduced peripheral and hepatic removal each process was studied separately. After inhibition of triglyceride lipolysis by Triton WR 1335 no differences were found in the rate of appearance of triglyceride in apoa5<sup>−/−</sup> mice relative to controls (531 ±81.3 mg · [180 minutes]<sup>−1</sup> versus 486.7 ±43.2 mg · [180 minutes]<sup>−1</sup>, n=7, <i>P</i>=0.36).

Apoa5<sup>−/−</sup> Mice Have Abnormal Removal of TRL

After injection of rat chylomicrons containing triglycerides labeled with <sup>14</sup>C-palmitate into normal and apoa5<sup>−/−</sup> mice <sup>14</sup>C disappearance was significantly slower in apoa5<sup>−/−</sup> mice relative to controls (7.8 ±4.9 versus 15.5 ±4.5 pools · h<sup>−1</sup>, <i>P</i>=0.01) (Figure 1A; Table I, available online at http://atvb.ahajournals.org). To examine whether the effect was only on triglyceride removal or whether cholesterol removal was also affected, <sup>14</sup>C-palmitate and <sup>3</sup>H-cholesterol double-labeled chylomicrons were injected. Again, clearance of <sup>14</sup>C-palmitate was slower in the apoa5<sup>−/−</sup> mice than in the controls (6.1 ±2.4 versus 11.7 ±5.5 pools · h<sup>−1</sup>, <i>P</i>=0.03). <sup>3</sup>H-cholesterol clearance was also slower in apoa5<sup>−/−</sup> mice relative to controls (7.0 ±0.9 versus 9.2 ±1.7 pools · h<sup>−1</sup>, <i>P</i>=0.03) (Figure 1B; Table I).

Absence of ApoAV Delays Chylomicron Remnant Removal

These results suggested that there might be an effect of apoAV on both remnant formation (lipolysis) and removal. To differentiate effects on lipolysis from those on remnant removal we first examined the rate of removal of <sup>125</sup>I-labeled chylomicron remnants. After injection of labeled remnants, removal of <sup>125</sup>I was slower in apoa5<sup>−/−</sup> mice than controls (1.66 ±0.6 versus 3.16 ±0.53 pools · h<sup>−1</sup>, <i>P</i>=0.007; Figure 2A; Table I). In a separate group of experiments <sup>125</sup>I CPM were determined after isopropanol precipitation so only apoB removal was studied. The curves were similar although the absolute removal rate was somewhat slower. To further evaluate whether this was caused by an alteration of hepatic remnant removal, the removal of <sup>125</sup>I-labeled rat chylomicron remnants by isolated perfused mouse liver was measured. The area under the uptake curve was 21% lower in livers of apoa5<sup>−/−</sup> mice compared with controls (6.21 ±1.13 and 7.88 ±0.94 arbitrary units, <i>P</i>=0.02; Figure 2B).

Reduced remnant removal could be caused by reduced LDL receptor levels. There is a strong correlation between...
LDL receptor mRNA and protein level. Accordingly we measured LDL receptor mRNA. It was found to be 37% higher in apo5−/− mice compared with controls (0.44±0.03 versus 0.32±0.02 intensity units compared with GAPDH, n=3, P=0.004).

**ApoAV-Deficient VLDL Has Lower Affinity to the LDL Receptor**

To learn whether the alterations of VLDL affected its binding to the LDL receptor, we evaluated the affinity of VLDL of apo5−/− mice and controls for the LDL receptor by determining its competition with human LDL for uptake by hepatoma cells. IC50 for apo5−/− mice VLDL was 1.53-fold higher compared with control mice (13.3±1.01 μg of apo5−/− VLDL per well versus 8.7±0.34 μg of control, number of experiments=4, P=0.004). Thus, the absence of apoAV decreases the ability of TRL to serve as a ligand for the LDL receptor.

**Effect of ApoAV on Triglyceride Hydrolysis**

To establish the basis of the effect of apoAV on triglyceride hydrolysis, we measured lipolysis by purified LPL of VLDL in vitro. The absence of apoAV in VLDL from apo5−/− mice inhibited LPL-mediated lipolysis by 10-fold compared with normal VLDL (Kcat 7.5·105±5.6·104 versus 7.9·105±2.4·104, n=3, P=0.007). To better understand whether defects in lipolysis is a cause of hypertriglyceridemia, we conducted experiments to measure LPL and HL activities after the intravenous administration of heparin. Apo5−/− mice had significantly lower LPL (3.39±0.82 versus 26.65±4.9 μmol FFA·mL−1·h−1, P=0.013) and hepatic lipase (1.53±0.21 versus 2.91±0.32 μmol FFA·mL−1·h−1, P=0.034) activity compared with controls.

**Apoa5−/− Mice Are Insulin-Resistant**

To evaluate insulin sensitivity in our model of interest, apo5−/− mice were subjected to glucose and insulin tolerance tests. Apo5−/− mice were significantly impaired in their ability to clear exogenously administered glucose compared with control mice (Figure IIA, available online at http://atvb.ahajournals.org). Insulin tolerance tests further revealed a greater resistance to insulin in apo5−/− mice compared with control mice (Figure IIB).

**Apoproteins on VLDL of Apo5−/− Mice**

The apoprotein composition of the VLDL was determined by SDS-PAGE (Figure 3) and IEF electrophoresis. Densitometric scanning revealed that in the apo5−/− mice the apoE-to-apoB ratio was 40% that of the littermate controls (control=4.5±1 versus apo5−/−=1.9±0.2 arbitrary units, n=3, P=0.015) and the apoC-to-apoB ratio was increased >2-fold (control=0.75±0.2 versus apo5−/−=1.64±0.08 arbitrary units, n=3, P<0.01) causing a reduction in the apoE:apoC ratio to ≈20% of that of littermates expressing the apo5 gene. IEF gels showed that apoCII of VLDL from apo5−/− mice was 16% of the sum of apoCII plus apoCIII compared with 20% in control mice (apo5−/−=0.16±0.01 versus control=0.207±0.01 arbitrary units, n=3, P=0.04).

**Discussion**

The present investigation examined TRL metabolism in mice that lacked apoAV and contrasted this to strain matched controls. We observed that apoAV does not significantly affect the production of triglycerides but has substantial effects on removal of TRL. We found that the absence of the apoprotein affects both the lipolysis of TRL as well as the removal of their remnants. Together these results provide the basis for the effect of apoAV on triglyceride levels in vivo.
The effect on LPL is consistent with two recent reports of the effects of apoAV overexpression. In our studies the absence of apoAV did not significantly affect triglyceride secretion in 7 experiments, although its overexpression did in one of the reports. Neither report examined its effect on remnant removal.

Four factors contributed to elevation of triglyceride level in the apoa5/H11002 mice. First, VLDLs from apoa5/H11002 mice were poor substrates for LPL. Second, LPL activity was lower in plasma form apoa5/H11002 mice. Third, VLDLs from apoa5/H11002 mice were poor ligands to LDL receptor. Fourth, HL activity was lower in plasma from apoa5/H11002 mice.

The slower rate of removal of TRL particles in apoa5/H11002 mice resulted in the presence of more triglyceride-rich particles as well as in VLDL of larger particle size. If the only difference in the particles were their triglyceride content, then one would expect that the larger VLDL from these mice should be better substrates for LPL. They were, however, inferior to normal particles as a substrate for LPL. This suggests that either apoAV activates LPL or that the VLDL of targeted mice have a different complement of apoprotein cofactors. In other transgenic mouse models in which apoC content of VLDL has been altered, this has resulted in marked hypertriglyceridemia. Indeed, apoa5/H11002 mice have altered apoC content relative to both apoB and apoE.

The removal of both chylomicrons and their remnants was slowed in the apoa5/H11002 mice. Humans have little difference between the metabolism of chylomicrons and VLDL except that chylomicrons are the preferred substrate for LPL. This has not been clearly shown in mice. Because rodents differ from humans in the rates of clearance of chylomicron and VLDL triglycerides it is possible that high VLDL triglycerides in apoa5/H11002 mice could explain, at least in part, the slower clearance of chylomicron triglycerides by competing for LPL. However, the somewhat larger pool size of VLDL remnants should not affect chylomicron remnant removal because the remnant pool size is well below saturation, and VLDL remnants are poor competitors for chylomicron remnant removal.

Chylomicrons have only apoB48, whereas VLDL in mice normally has either apoB48 or apoB100. Both species, however, can acquire E and C apoproteins. In humans and transgenic mice with larger amounts of apoB100 VLDL there is conversion of some of these particles to LDL but there has not been any evidence that the initial steps in the metabolism of these particles are different. Thus, it is reasonable to think that the studies with chylomicrons likely also reflect the behavior of VLDL.

The slower rate of removal of remnants, once formed, contribute to the higher cholesterol levels in apoa5/H11002 mice, but most of the elevation is in HDL. The mechanism for elevated HDL cholesterol in these mice is, however, not fully understood. Lower activity of HL in apoa5/H11002 mice could hinder selective uptake of HDL cholesteryl ester by the liver as well as diminish hydrolyzes of HDL triglycerides and phospholipids that in turn may reduce clearance of HDL by the kidney. Whether apoAV on the HDL directly plays a part in its catabolism which may be slowed in the absence of this apolipoprotein awaits further elucidation.

There was also a remarkably lower level of LPL in apoa5/H11002 mice, which should also contribute to a slower rate of remnant formation. LPL activity decreased to a much greater extent (87%) relative to another mouse model of elevated triglycerides ie, heterozygous LPL deficient mice, that had <40% less enzyme activity and 1.8-fold to 3.1-fold increase in triglyceride levels. Whereas the mechanism for lower enzyme activity in LPL deficient mice is obvious, the one operative in apoa5/H11002 mice is unknown. It has been suggested that LPL is directly activated by apoAV. This is consistent with our observation that pure LPL has lower affinity to VLDL from apoa5/H11002 mice but could also be explained by the lower content of apoCII on these particles observed in our study. The most logical explanation for the lower LPL level is, however, that the hypertriglyceridemia caused by other differences eventually leads to a metabolic syndrome like pattern with reduced LPL. This is supported by the observed significantly impaired response to glucose loading and greater resistance to insulin in the apoa5/H11002 mice relative to controls. Together, the slower lipolysis of the particles both because of their composition and because of the lower LPL level explains the slower rate of triglyceride hydrolysis in vivo and why VLDL accumulates in these animals.

Consistent with the postulate that apoAV rapidly alters the composition of TRL was the effect on remnant removal. Reduced remnant removal could be caused by reduced LDL receptors, a change in the affinity of the particle for the receptor or alteration in the cofactors for uptake by the LDL receptor-related protein (LRP). Changes in the LRP level are uncommon and most have not been associated with altered lipoprotein metabolism. LDL receptor levels were not reduced in the apoa5/H11002 mice and in fact were slightly increased. This is consistent with a reduced baseline uptake of remnants and perhaps other lipoproteins by the livers of these mice.

The findings that removal of normal remnants by the isolated livers is delayed argues strongly that in the absence of apoAV the composition of TRL is rapidly altered in the hepatic sinusoid in a manner that affects the ability of the particles to serve as ligands for the removal process. Indeed, studies of LDL binding with cultured cells revealed that VLDLs from apoa5/H11002 mice were poor competitors for binding to the LDL receptor as compared with VLDL from normal mice. The weak binding could be caused by the larger VLDL size, as well as by possible difference in apoE to C apoprotein ratios. The latter could reflect the size difference of VLDL particle between the apoa5/H11002 mice and the controls or be a direct effect of the absence of apoAV. Because the same chylomicron remnants were used in the perfused liver experiments in both control and knockout mice, only the latter reason can explain the slower removal of these particles. This suggests that the apoprotein content is likely to be rapidly altered at the hepatocyte surface. Lastly, the lower level of HL in apoa5/H11002 may also lead to reduced (or delayed) capture of remnant particles.
The hydrolysis of triglyceride in a particle by lipoprotein lipase is determined in part both by the ratio of apoCII to apoCIII and the amount of apoC and E per particle. The ratio of apoCII to apoCIII (practically measured as apoCII: apoCIII) was only slightly altered in our studies thus the change in C and E apoproteins content may explain the differences in the behavior of the different particles to serve as a substrate for LPL. Support for this is provided by works showing that besides the reduced capacity of TRL to bind LPL as a result of ApoC enrichment, apoC and apoE inversely modulate the binding of TRL to hepatic proteoglycans, thus potentially impeding and facilitating, respectively, their hydrolysis by hepatic proteoglycan-bound lipoprotein lipase.

The rate of remnant removal depends on the amount of apoE per particle and the ratio of apoE:apoC and the alterations in this ratio in apoA5-/- mice correlate nicely with the changes in remnant removal. Based on the results of the perfused liver experiments, however, it is reasonable to speculate that changes in particle composition occur at the level of the hepatocyte. In a number of studies from this laboratory, it has become established that the apoprotein milieu at the hepatocyte surface can rapidly affect the behavior of particles entering the liver. This is best described for apoE, but has been seen with apo(a) fragments (Tabas et al, unpublished data, 2003) and thus could occur with the C apoproteins.

The mechanism whereby hypertriglyceridemia predisposes to atherosclerosis is not known. VLDLs themselves are not atherogenic but hypertriglyceridemia is associated with a number of pro-atherogenic and pro-inflammatory changes. In apoA5-/- mice the reduced rate of remnant removal, however, could result in prolonged residence of these particles in the circulation and this is established to be an atherogenic factor. Also, in humans lower expression of apoAV could have atherogenic effect by increasing the residence time of remnants in the circulation. As this is elucidated, the potential of apoAV as a therapeutic target will be clarified.

Acknowledgments
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