Antisense Oligonucleotide Directed to Human Apolipoprotein B-100 Reduces Lipoprotein(a) Levels and Oxidized Phospholipids on Human Apolipoprotein B-100 Particles in Lipoprotein(a) Transgenic Mice

Esther Merki, PhD; Mark J. Graham, BS; Adam E. Mullick, PhD; Elizabeth R. Miller, BS; Rosanne M. Crooke, PhD; Robert E. Pitas, PhD; Joseph L. Witztum, MD; Sotirios Tsimikas, MD

Background—Lipoprotein (a) [Lp(a)] is a genetic cardiovascular risk factor that preferentially binds oxidized phospholipids (OxPL) in plasma. There is a lack of therapeutic agents that reduce plasma Lp(a) levels.

Methods and Results—Transgenic mice overexpressing human apolipoprotein B-100 (h-apoB-100 [h-apoB mice]) or h-apoB-100 plus human apo(a) to generate genuine Lp(a) particles [Lp(a) mice] were treated with the antisense oligonucleotide mipomersen directed to h-apoB-100 mRNA or control antisense oligonucleotide for 11 weeks by intraperitoneal injection. Mice were then followed up for an additional 10 weeks off therapy. Lp(a) levels [apo(a) bound to apoB-100] and apo(a) levels [“free” apo(a) plus apo(a) bound to apoB-100] were measured by chemiluminescent enzyme-linked immunoassay and commercial assays, respectively. The content of OxPL on h-apoB-100 particles (OxPL/h-apoB) was measured by capturing h-apoB-100 in microtiter wells and detecting OxPL by antibody E06. As expected, mipomersen significantly reduced plasma h-apoB-100 levels in both groups of mice. In Lp(a) mice, mipomersen significantly reduced Lp(a) levels by $75\%$ compared with baseline ($P<0.0001$) but had no effect on apo(a) levels or hepatic apo(a) mRNA expression. OxPL/h-apoB levels were much higher at baseline in Lp(a) mice compared with h-ApoB mice ($P<0.0001$) but decreased in a time-dependent fashion with mipomersen. There was no effect of the control antisense oligonucleotide on lipoprotein levels or oxidative parameters.

Conclusions—Mipomersen significantly reduced Lp(a) and OxPL/apoB levels in Lp(a) mice. The present study demonstrates that h-apoB-100 is a limiting factor in Lp(a) particle synthesis in this Lp(a) transgenic model. If applicable to humans, mipomersen may represent a novel therapeutic approach to reducing Lp(a) levels and their associated OxPL.

(Circulation. 2008;118:743-753.)

Key Words: antibodies ■ arteriosclerosis ■ atherosclerosis ■ lipoproteins ■ pharmacology

Lipoprotein(a) [Lp(a)] is lipoprotein composed of apolipoprotein(a) [apo(a)], which is covalently bound to low-density lipoprotein (LDL) via a single disulfide bond on apoB-100. Unlike other apolipoproteins, apo(a) is carbohydrate rich and hydrophilic and therefore projects into the aqueous phase when bound to apoB-100. Apo(a) is composed of unique loop structures called kringles (K), which are each stabilized by 3 disulfide bonds. The apo(a) gene encodes for KIV, KV, and a protease-like domain that is catalytically inactive. Apo(a) KIV is composed of 10 subtypes, of which KIV-2 is present in a variable number of copies (from 3 to $>40$), resulting in markedly different apo(a) sizes among individuals. Furthermore, most subjects (≈80%) have 2 distinct apo(a) alleles that also may vary significantly in size. Apo(a) expression is restricted to humans and Old World apes and monkeys, but an unrelated version containing only KIII repeats is present in the European hedgehog. Lp(a) levels are largely determined genetically, may vary by 1000-fold (<0.1 to $>250$ mg/dL), and are generally inversely associated with the size of the smaller apo(a) allele.

Clinical Perspective p 753

In clinical studies, Lp(a) is recognized as an independent risk factor for myocardial infarction, stroke, and peripheral arterial disease. As with most genetic risk factors that initiate risk at birth, it is a stronger cardiovascular risk factor in young patients with highly elevated Lp(a) levels and those with additional atherogenic risk factors, particularly elevated LDL cholesterol. Lp(a) has many potential atherogenic properties resulting from its LDL moiety. Additionally,
apo(a) may confer proinflammatory, proatherogenic, and prothrombotic properties, although most of these putative atherogenic attributes have not been conclusively demonstrated in vivo or in humans. The pathophysiologic role of Lp(a) and the underlying mechanisms through which it contributes to cardiovascular disease are unknown. One hypothesis suggests that apo(a) promotes thrombosis by competitively inhibiting the conversion of plasminogen to plasmin. Although Lp(a) inhibits thrombolysis in vitro, there is little evidence to support such a role in vivo. We have recently proposed a novel hypothesis that part of the atherogenicity of Lp(a) may be due to its unique ability to strongly bind and transport proinflammatory and proatherogenic oxidized phospholipids (OxPL). OxPL present on apoB-100 (OxPL/apoB) can be measured by the murine monoclonal antibody E06, which binds to the phosphocholine head group of OxPL but not native PL. This observation is supported by experimental studies showing that Lp(a) is the preferential carrier of PC-containing OxPL in human plasma and by clinical studies showing a strong correlation between plasma Lp(a) levels and OxPL/apoB. However, differences exist between Lp(a) mass and OxPL/apoB within different apo(a) isoform classes (K repeats), with this correlation being weakest for the largest isoforms and strongest for the lowest number of KIV repeats (>29 repeats, r = 0.66; 23 to 29 repeats, r = 0.88; and <22 repeats, r = 0.93; P = 0.001 each). In line with these data, a mouse model overexpressing both human apoB-100 and apo(a) (Lp(a) mice), to generate authentic Lp(a) particles at very high plasma levels, was recently described and characterized. The Lp(a) particles in these mice carry most of the E06-detectable OxPL in plasma, similar to Lp(a) particles in humans.

Apob-100 and apo(a) are expressed primarily in hepatocytes. Effective therapeutic modalities to reduce Lp(a) levels in humans are lacking. Lp(a) levels can be lowered by niacin by 15% to 30%, but niacin is associated with high risk of side effects (severe flushing and glucose intolerance) that limit its clinical use. The recent development of antisense oligonucleotides (ASOs) that can target a specific mRNA and suppress the translation of its protein in the liver has opened up a novel therapeutic window for reducing levels of atherogenic lipoproteins. Mipomersen (ISIS 301012), an antisense inhibitor directed to human apoB-100, was recently documented in a mouse model overexpressing both human apoB-100 and cholesterol levels in humans. The generation of human apoB-100 transgenic mice (h-apoB mice), which express only apoB-100 but not apoB-48, and apo(a) mice were recently described. Briefly, wild-type h-apoB cDNA encoding KIV-1, 1 copy of KIV-2, a fusion of KIV-3 and KIV-5, KIV-6 to KIV-10, and the protease-like domain was inserted into a liver cDNA expression vector containing the apoE hepatic control region (LE6). The 8.6-kb apo(a) expression vector [containing the apoE promoter, ApoE intron 1, the apo(a) cDNA, and LE6] was excised from pLvHa8 with SacII, purified, and microinjected into C57BL/6xSJL zygotes. To generate Lp(a) mice, hemizygous mice expressing apo(a) were crossed with hemizygous mice expressing h-apoB-100. Lp(a) mice were then mated with wild-type C57BL/6 mice to generate additional Lp(a) mice. Mice were genotyped by polymerase chain reaction from tail biopsies. DNA was isolated with the Qiagen DNAeasy tissue kit. Primers for apo(a) were 5′ primer GACGGGAGACAGAGT-GAACGC and 3′ primer TACCTAAACACGGCAGGAC. Primers for apoB-100 transgene were 5′ primer GAAGAATTCCTCG-GAGAATTCGCAT and 3′ primer CTCTTAGCCCCATTTAGCTCTGAC. All mice were weaned at 28 days of age, housed in a barrier facility with a 12-hour light/12-hour dark cycle, and fed normal mouse chow containing 4.5% fat (Harlan Teklad, Madison, Wis).

### Methods

#### Antisense Oligonucleotides

The ASOs used in this study were previously described in detail. Mipomersen is targeted to human apoB-100 and is a 20-nucleotide phosphorothioate oligonucleotide composed of five 2′-O-(2-methoxyethyl)-modified ribonucleosides (2′-MOE) at the 3′ and 5′ ends with ten 2′-deoxynucleosides in between. The sequence of mipomersen is 5′-GCCCTAGTCTGTTGCGACC-3′, where the italicized bases are 2′-MOE-modified ribonucleosides, and all cytosines are methylated at the 5′ position. It does not bind to murine apoB-100 mRNA. ISIS 141923 (5′-CCCTCCCCGAGGTTCC-3′), which is the same chemical class as mipomersen but does not hybridize to either human or mouse apoB-100 mRNA, was used as a control ASO.

#### Transgenic Mice

The generation of human apoB-100 transgenic mice (h-apoB mice), which express only apoB-100 but not apoB-48, and apo(a) mice were recently described. Briefly, wild-type h-apoB cDNA encoding KIV-1, 1 copy of KIV-2, a fusion of KIV-3 and KIV-5, KIV-6 to KIV-10, and the protease-like domain was inserted into a liver cDNA expression vector containing the apoE hepatic control region (LE6). The 8.6-kb apo(a) expression vector [containing the apoE promoter, ApoE intron 1, the apo(a) cDNA, and LE6] was excised from pLvHa8 with SacII, purified, and microinjected into C57BL/6xSJL zygotes. To generate Lp(a) mice, hemizygous mice expressing apo(a) were crossed with hemizygous mice expressing h-apoB-100. Lp(a) mice were then mated with wild-type C57BL/6 mice to generate additional Lp(a) mice. Mice were genotyped by polymerase chain reaction from tail biopsies. DNA was isolated with the Qiagen DNAeasy tissue kit. Primers for apo(a) were 5′ primer GACGGGAGACAGAGT-GAACGC and 3′ primer TACCTAAACACGGCAGGAC. Primers for apoB-100 transgene were 5′ primer GAAGAATTCCTCG-GAGAATTCGCAT and 3′ primer CTCTTAGCCCCATTTAGCTCTGAC. All mice were weaned at 28 days of age, housed in a barrier facility with a 12-hour light/12-hour dark cycle, and fed normal mouse chow containing 4.5% fat (Harlan Teklad, Madison, Wis).

#### Treatment Protocol

Three studies were performed in this set of experiments. In study 1, the baseline values of the various lipoprotein and OxPL parameters were determined in 8 h-apoB mice and 8 Lp(a) mice (Table 1). The therapeutic experiments were then performed in studies 2 and 3; study 2 was used to obtain data on the effects of mipomersen on lipoprotein parameters and apo(a) liver mRNA expression, and study 3 was used to assess the effects of mipomersen on Lp(a) and OxPL parameters in h-apoB mice and Lp(a) mice.

In study 2, 4 Lp(a) mice were treated for 4 weeks with twice-weekly intraperitoneal injections of mipomersen (25 mg/kg), and 4 Lp(a) mice were treated with control ASO 141923 (25 mg/kg IP). Blood was collected at baseline and 2 and 4 weeks. After 4 weeks of treatment, the mice were perfused with ice-cold PBS for 5 minutes, and liver tissue was harvested. Plasma at each time point was used for measurement of total cholesterol, triglycerides, Lp(a), and apo(a).

In study 3, h-apoB mice and Lp(a) mice 5 to 7 months of age on normal mouse chow were administered either mipomersen (25 mg/kg IP) or control ASO 141923 (25 mg/kg IP) twice weekly for a total of 11 weeks (6 mice in each group, 24 mice total). The treatment was then stopped, and the mice were followed up for an additional 10 weeks. Blood samples were collected at baseline, at 1

<table>
<thead>
<tr>
<th>Lp(a)</th>
<th>NA</th>
<th>0.0002</th>
<th>0.0001</th>
<th>0.06</th>
<th>0.65</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>OxPL/m-apoB</td>
<td>0.46</td>
<td>0.76</td>
<td>0.41</td>
<td>0.06</td>
<td>0.66</td>
<td>NA</td>
</tr>
<tr>
<td>OxPL/apoB</td>
<td>NA</td>
<td>1.41</td>
<td>0.42</td>
<td>NA</td>
<td>0.66</td>
<td>NA</td>
</tr>
<tr>
<td>Apo(a)/m-apoB</td>
<td>NA</td>
<td>0.65</td>
<td>0.66</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA indicates not applicable.
week of treatment, and then at 2-week intervals up to 11 weeks of treatment. After treatment was stopped, blood was also collected at 2, 6, and 10 weeks. Detailed lipoprotein and immunological measurements were then performed as described below.

**Determination of Total Cholesterol, Triglycerides, ApoB-100, Lp(a), and Apo(a) Levels**

Total cholesterol and triglycerides were determined by commercial enzymatic assay using the Roche Cobas Mira Plus Analyzer. Plasma Lp(a) levels [ie, apoB-100 covalently bound to apo(a)] were measured by a validated sandwich ELISA developed in our laboratory in which human apoB-100 from plasma (1:400 dilution) is captured on a plate with monoclonal antibody MB47 (5 μg/mL), and apo(a) was detected with biotinylated murine monoclonal antibody LPA4 (which does not cross-react with plasminogen).13 A standard curve is generated with human plasma of known Lp(a) mass and was used to determine Lp(a) levels. We have previously shown that this method correlates highly (n=500, r=0.96, P<0.0001) with a commercial assay (Diasorin, Inc, Stillwater, Minn).13 This assay does not measure “free” apo(a) levels [ie, apo(a) not bound to apoB-100]. The DiaSorin assay was used to measure total apo(a) [both as Lp(a) and as apo(a) bound to apoB] and was postulated that this mouse model may have excess apo(a) compared with h-apoB-100. However, because the calibrators for this assay are based on Lp(a) standards that are of higher molecular mass than the mini-apo(a) in these mice, the absolute values of apo(a), but not relative changes, are likely to be overestimated, as previously suggested.21 H-apoB-100 levels were determined by a commercial assay (Diasorin, Inc). Mouse apoB levels were determined with a chemiluminescent ELISA with capture murine monoclonal antibody LFS and detection antibody LF3.23 These antibodies recognize different epitopes on mouse apoB-100 (m-apoB-100) but do not recognize h-apoB-100.

We also used an additional methodology to assess whether this Lp(a) mouse model had free circulating apo(a) not bound to h-apoB-100. It was hypothesized that immunoprecipitation with MB47 would pellet all h-apoB-100 particles, including Lp(a), but not free apo(a) or apo(a) noncovalently sticking to other lipoproteins (ie, mouse apoB-100). Thus, persistence of apo(a) in the supernatant would represent apo(a) not bound to h-apoB-100. Plasma was obtained from the 4 Lp(a) mice from study 2 that were treated with mipomersen at baseline and 4-week time points. Increasing doses of MB47 (0 to 20-molar excess MB47:apoB-100 based on a molecular weight of apoB-100 of ~515 kDa and MB47 of ~165 kDa) were added to plasma samples and incubated overnight at 4°C. The samples were then centrifuged at 20 000 rpm to pellet MB47:apoB-100 complexes, and the supernatant was examined for the presence of apoB-100, Lp(a), and apo(a). These assays were performed using a similar ELISA methodology as described above. Briefly, the capture and detection antibodies for apoB-100 were MB47 (5 μg/mL) and a goat anti-human antibody, respectively; for Lp(a), MB47 (5 μg/mL) and LPA4; and for apo(a), LPA4 (5 μg/mL) as the capture antibody and the detection antibody [LPA4 recognizes an epitope present on apo(a) in >1 copy, so it can be used in this manner].

**Determination of OxPL on H-ApoB-100, Mouse ApoB-100, or Apo(a)**

A chemiluminescence ELISA was used to measure the amount of OxPLs present on h-apoB-100 particles (OxPL/h-apoB) using E06 as previously described in detail.14 For this assay, plasma was added to plasma samples and incubated overnight at 4°C. The presence of OxPL was detected with biotinylated E06; and values were expressed as relative light units (RLUs). To determine the amount of h-apoB-100 captured on each well, MB47 was coated on parallel plates; plasma (1:100 dilution) was added; and then biotinylated goat anti–h-apoB-100 was added to determine the amount of apoB-100 (in RLUs) captured. The E06 RLUs were then divided by the apoB RLUs, and the OxPL/apoB ratio was derived.

A similar methodology was used to measure the content of OxPL on apo(a) particles [OxPL/apo(a)], except LPA4 was used as the capture antibody. To determine the amount of apo(a) captured on each well, LPA4 was coated on parallel plates; plasma (1:100 dilution) was added; and then biotinylated LPA4 was added to determine the amount of apo(a) (in RLUs) captured. The E06 RLUs were then divided by the apo(a) RLUs to derive the OxPL/apo(a) ratio.

To determine OxPL on mouse apoB-100 particles (OxPL/m-apoB), we used the same methodology as above except that plates were coated with antibody LF5; plasma (1:100 dilution) was added. To determine the amount of m-apoB captured, parallel plates were coated with LF5; plasma (1:100 dilution) was added; and the amount of m-apoB captured was determined by LF3. The E06 RLUs were then divided by the LF3 RLUs to determine the OxPL/m-apoB ratio.

**Determination of Autoantibodies to Malondialdehyde-LDL**

Mouse IgG and IgM autoantibodies to malondialdehyde-LDL were determined as previously described in detail.23

**RNA and Reverse-Transcriptase Polymerase Chain Reaction Analyses**

Total RNA was extracted from whole-liver tissue and primary hepatocytes isolated (Qiagen RNeasy) as previously described.20 The primers used were as follows: h-apoB-100, 5'-TGCTAAAGGCACATATGGCCT-3' and 5'-CTCAGGTTGCACCTCCATTAG-3' with the fluorescent probe 5'-CTTGGCAGGGATCTACACTGGCCG-3';apo(a), 5'-CCACAGTGCCCCCGGTT-3' and 5'-ACAGGGCTTTCCTAGGGTGTT-3' with the fluorescent probe 5'-CCAAAGCACAGAGCTCTTGAAAG-3'; and m-apoB-100, 5'-CTGTCGCTTCACATTTTGCTT-3' and 5'-ATGTATCTGGTTGGTCTCAAA-3' Expression values were normalized to cyclophilin A. DNA was isolated with the Qiagen DNeasy tissue kit.

**HPLC Analysis of Lipoproteins**

Serum lipoprotein and cholesterol profiling was performed as described.20

**Western Blots for H-ApoB-100, Lp(a), and Apo(a)**

Western blot analyses for h-apoB-100, Lp(a), and apo(a) were performed on mouse plasma subjected to electrophoresis on 4% to 12% Tris-glycine gels overnight at 4°C and transferred to polyvinylidene-fluoride membranes (Invitrogen, Carlsbad, Calif). Nondenaturing gels were used for h-apoB and Lp(a); denaturing gels were used for apo(a). The next day, blots were incubated for 1 hour with a commercially available horseradish peroxidase–conjugated, human-specific apoB antibody (United States Biological, Swampscott, Mass) or LPA4 (1:1000 dilution), followed by a goat anti-mouse IgG horseradish peroxidase–conjugated antibody (1:15 000, BD Biosciences, San Diego, Calif). Protein bands were visualized with the ECL Plus Western blot detection kit (Amersham Biosciences, Inc, Piscataway, NJ). The goat anti-mouse IgG horseradish peroxidase–conjugated antibody also was used to determine the presence of mouse IgG.

Frozen liver sections 10 μm thick were air dried and briefly fixed with 4% paraformaldehyde, rinsed with 60% isopropanol, stained with Oil Red O (3 mg/mL) for 15 minutes, rinsed with isopropanol, and counterstained with Hematoxylin QS (Vector Laboratories, Burlingame, Calif).

**Statistical Analysis**

Analysis of quantitative parameters in mice over the time course of the study was performed with 1-way ANOVA with the posthoc Bonferroni multiple comparison test as appropriate. Differences between the groups were assessed by Students’ t test. Values of P<0.05 were considered significant. The Pearson parametric test was used to calculate correlations between variables.
Table 2. Lipoprotein Variables in Response to Mipomersen or Control ASO

<table>
<thead>
<tr>
<th></th>
<th>Total Cholesterol, mg/dL</th>
<th>Triglycerides, mg/dL</th>
<th>Lp(a), mg/dL</th>
<th>Apo(a), mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mipomersen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>156.3±10.7</td>
<td>121.8±13.8</td>
<td>81.3±24.4</td>
<td>70.1±33.6</td>
</tr>
<tr>
<td>2 wk</td>
<td>105.3±22.6*</td>
<td>82.5±27.10</td>
<td>33.5±9.0‡</td>
<td>92.0±10.2</td>
</tr>
<tr>
<td>4 wk</td>
<td>96.3±27.3†</td>
<td>130.8±55.6</td>
<td>31.8±15.6‡</td>
<td>97.5±8.4</td>
</tr>
<tr>
<td>ISIS 141923</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>205.0±26.1</td>
<td>168.3±34.3</td>
<td>98.3±24.2</td>
<td>92.0±19.6</td>
</tr>
<tr>
<td>2 wk</td>
<td>215.3±34.2</td>
<td>151.0±49.8</td>
<td>95.8±18.6</td>
<td>94.0±10.1</td>
</tr>
<tr>
<td>4 wk</td>
<td>172.0±12.5</td>
<td>160.0±28.6</td>
<td>98.3±24.0</td>
<td>81.0±45.1</td>
</tr>
</tbody>
</table>

*p<0.05, †p<0.01, ‡p<0.001 vs baseline.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Study 1 in H-ApoB-100 and Lp(a) Mice

Baseline Levels of Lipid Variables and OxPLs

The baseline total cholesterol levels (180.6±31.9 versus 148.0±26.1 mg/dL; P=0.021) and h-apoB-100 levels (100.7±18.2 versus 63.4±17.3 mg/dL; P=0.0002) were significantly higher in the Lp(a) mice compared with the h-apoB mice (Table 1), as previously described.

In contrast, apo(a) levels did not change significantly (98.9±35.9 mg/dL at baseline, 104.0±38.72 mg/dL at 3 weeks, 102.1±36.0 mg/dL at 4 weeks; P=0.78 for trend). This was further supported by evaluating hepatic apo(a) mRNA expression in mipomersen versus control ASO (data not shown).

Mipomersen reduced total cholesterol levels over the 4-week treatment period as expected (Table 2). High-performance liquid chromatography analysis documented that this was due in large measure to a reduction in LDL, which was decreased 75% in the 2- and 4-week time points in the mipomersen group (Figure 1). There was no change in high-density lipoprotein (HDL) cholesterol or in the lipoprotein distribution pattern. No significant changes were noted with the control ASO 141923 (Figure 1).

Mipomersen also decreased Lp(a) levels over the 4 weeks (81.3±24.4 mg/dL at baseline, 33.8±9.0 mg/dL at 2 weeks, 31.8±15.6 mg/dL at 4 weeks; P<0.0002 for trend; Table 2). In contrast, apo(a) levels did not change significantly (98.9±35.9 mg/dL at baseline, 104.0±38.72 mg/dL at 3 weeks, 102.1±36.0 mg/dL at 4 weeks; P=0.78 for trend). This was further supported by evaluating hepatic apo(a) mRNA expression in mipomersen versus control ASO 141923 (53.8±37.4% [range, 24% to 108%]; versus 89.2±36.9% [range, 32% to 138%] normalized to cyclophilin A; P=0.29) after 4 weeks of treatment. The 8 Lp(a) mice in this group had variable baseline apo(a) levels (range, 93 to 163 mg/dL), and interestingly, a strong correlation was noted between hepatic apo(a) mRNA expression and the final apo(a) plasma levels (r=0.95, P<0.0001), but a weaker correlation was observed with Lp(a) and apo(a) mRNA levels (r=0.75, P=0.03). Furthermore, there was no difference among groups in mouse apoB mRNA expression (110.1±13.2% versus 98.1±12.9%; P=0.41). As expected, a significant reduction in h-apoB-100 mRNA was noted (26.0±2.6% versus 87.5±7.1%; P=0.0033) in the mipomersen-treated group compared with the control ASO group.

Western blot analysis revealed that h-apoB and Lp(a) levels were significantly reduced, but there was no effect on apo(a) levels (Figure 2). There was no evidence of hepatic steatosis in response to mipomersen, as assessed by Oil Red O staining of hepatic tissue (data not shown).

To further assess the relative excess of apo(a) and Lp(a) in the Lp(a) mice, we immunoprecipitated h-apoB-100 from plasma of Lp(a) mice before treatment with mipomersen with excess MB47 and assayed the content of apoB-100, apo(a), and Lp(a) remaining in the supernatant to provide a measure of the free apo(a). Figure 3 (top) shows changes in these parameters normalized to pre-MB47 values and demonstrates that nearly 90% of both h-apoB-100 and Lp(a) but only 25% of apo(a) was immunoprecipitated [88±4% of h-apoB-100, 92±4% of Lp(a), and 25±22% of apo(a)]. Thus, at baseline in these Lp(a) mice, 75% of apo(a) appears to be free, eg, not bound to h-apoB-100. At the 4-week time point, mipomersen treatment (Figure 3, bottom) resulted in qualitatively similar data, except for the presence of higher levels of free apo(a) after the reduction of h-apoB-100 in response to mipomersen [apoB, 96±1.0%; Lp(a), 95±2.0%; apo(a), 4±22% of baseline levels].

Study 2 in Lp(a) Mice

Effect of Mipomersen on Lipoprotein Levels and Composition and Hepatic ApoB-100 and Apo(a) mRNA Expression

Mipomersen reduced total cholesterol levels over the 4-week treatment period as expected (Table 2). High-performance liquid chromatography analysis documented that this was due in large measure to a reduction in LDL, which was decreased 75% in the 2- and 4-week time points in the mipomersen group (Figure 1). There was no change in high-density lipoprotein (HDL) cholesterol or in the lipoprotein distribution pattern. No significant changes were noted with the control ASO 141923 (Figure 1).

In the Lp(a) mice, concomitant with the decrease in h-apoB-100 levels, there was a significant decline in Lp(a) levels in response to mipomersen (P<0.0001 for trend by ANOVA; Figure 4A). The h-apoB-100 levels reached a nadir by week 3 and remained low until week 11. After mipomersen treatment was discontinued, h-apoB-100 levels returned to baseline levels 10 weeks later. The h-apoB-100 levels in Lp(a) mice also decreased in a similar time-dependent manner when treated with mipomersen (P=0.0001 for trend by ANOVA; Figure 4B). In contrast, there were no significant changes in h-apoB-100 levels in either mouse model with the control ASO. No changes in m-apoB levels were noted with either mipomersen or control ASO (data not shown).

In the Lp(a) mice, concomitant with the decrease in h-apoB-100 levels, there was a significant decline in Lp(a) levels in response to mipomersen (P<0.0001 for trend by ANOVA; Figure 4C). The Lp(a) levels began to decline by 3 weeks and reached a nadir from 5 to 11 weeks. After discontinuation of therapy, the Lp(a) levels returned to baseline after 10 weeks.
To assess the fate of any free apo(a) when Lp(a) levels decreased, m-apoB-100 was captured on microtiter well plates, and the content of apo(a) was assessed with antibody LPA4 [apo(a)/m-apoB]. This analysis revealed a steady increase in the proportion of apo(a)/m-apoB, which peaked at 11 weeks ($P/H110050.014$ for trend by ANOVA; Figure 4D), returning to baseline by 10 weeks after cessation of therapy.

**Effect of Mipomersen on OxPL/H-ApoB and OxPL/M-ApoB**

As noted above, the baseline OxPL/h-apoB levels were $\approx55$-fold higher in the Lp(a) mice than in the h-apoB mice (Figure 4A and 4B). Over the study period, there were no significant changes in OxPL/h-apoB in the h-apoB mice treated with either ASO 301012 or the ASO control (Figure
Antibody E06) in plasma,6,9–15 there was a reduction in the generation of Lp(a) particles in this murine model. Furthermore, because Lp(a) carries OxPL (those detected by antibody LPA4, and the content of OxPL was captured with antibody E06. No changes in OxPL/h-apoB levels over time in the h-apoB mice (Figure 5C) or Lp(a) mice (Figure 5D).

**Effect of Mipomersen on OxPL on Apo(a) Particles and Malondialdehyde-LDL Autoantibodies**

To assess whether the ratio of OxPL on apo(a) particles changed during the treatment in Lp(a) mice, apo(a) particles were captured with antibody LPA4, and the content of OxPL was assessed with the antibody E06. No changes in OxPL/apoB, which reflected a reduction in the mass of Lp(a), rather than a reduced carrying capacity of Lp(a) for OxPL. If apoB-100 synthesis is a rate-limiting step in the generation of human Lp(a) in a similar manner, our findings suggest that mipomersen may provide a novel therapy to reduce plasma Lp(a) levels in patients. In preliminary studies in human subjects treated with mipomersen, Lp(a) levels have been observed to decrease up to 60% (D. Tribble, Isis, Inc, personal communication). Prospective studies in humans to determine the impact of mipomersen on Lp(a) levels seem warranted.

The mechanisms underlying apo(a) synthesis are relatively well known. Apo(a) is synthesized in hepatocytes, and the rate of synthesis is related primarily to apo(a) gene transcription.24 Posttranslational regulation such as formation of the 3 disulfide bonds for each kringle type and N-linked glycation is important for proper apo(a) folding and transport out of the endoplasmic reticulum. This process may take up to 120 minutes and depends on apo(a) size. Once apo(a) exits the endoplasmic reticulum, it travels to the Golgi apparatus, where it undergoes further posttranslational modification and is finally transported to the hepatocyte cell surface. Lp(a) is then thought to be assembled from newly synthesized apoB-100, first by low-affinity, noncovalent interactions between KIV-5 to KIV-8 and then through a disulfide bond between unpaired cysteine 4057 on KIV-9 on apo(a) and cysteine 4326 on the C-terminal region of apoB-100. Because small isoform apo(a) particles are more easily synthesized and secreted, it is postulated that subjects with small apo(a) isoforms have higher Lp(a) levels. Although apo(a) gene transcription contributes significantly to plasma Lp(a) levels,25 some variability in those levels may be attributed to genetic polymorphisms of apo(a).26 It is also quite interesting that a modest inverse correlation is present between triglyceride levels and Lp(a) levels, suggesting that the rate of very-low-density lipoprotein synthesis may affect Lp(a) metabolism.27 In fact, up to 4% of apo(a) is present on very-low-density lipoprotein particles, and these complexes may have a slower catabolism.28,29

In contrast, the clearance of Lp(a) is less well understood. The preponderance of evidence suggests that the LDL receptor does not play a major role in Lp(a) clearance.24 Approximately 10% to 25% of Lp(a) is converted to LDL when apo(a) is cleaved off, and LDL is then cleared by the LDL receptor. There are also some data, although not entirely consistent,30 that apo(a) may not remain covalently attached to a single apoB-100 but may in fact associate with 2 apoB-100 molecules over its metabolic lifetime.27 In patients with renal failure, a small amount (7.5%) of free apo(a), mainly of large isoforms, may be found in the plasma.31 Some Lp(a) is cleaved by metalloproteinases and elastases, which results in apo(a) fragments that are then cleared and/or secreted in urine by the kidney. Recent data in mice also suggest the possibility of an as-yet unidentified apo(a) receptor in hepatocytes of mice because intravenously injected free apo(a) may reduce Lp(a) uptake in hepatocytes.32

ASOs targeting proteins involved in lipoprotein metabolism represent novel therapeutic agents to treat dyslipidemias.18 Proof-of-principle studies initially demonstrated that
ISIS 147764, an ASO targeted to m-apoB-100, reduced mouse apoB mRNA levels in the liver and LDL cholesterol levels in plasma in a dose- and time-dependent manner in C57BL/6, LDLR<sup>−/−</sup> and apoE<sup>−/−</sup> mice. In a phase 2, double-blind, randomized, placebo-controlled, 3-month dose-escalation study, mipomersen reduced apoB-100 (50% at the 200-mg dose) and LDL cholesterol (35% at the 200-mg dose) levels in a dose- and time-dependent manner.<sup>19</sup>

In this study, we hypothesized that reducing hepatic synthesis of apoB-100 would result in a reduction of plasma Lp(a) levels in Lp(a) mice. These mice resemble humans in that they have similar plasma Lp(a) levels and genuine Lp(a) particles. They differ in that the animals have excess apo(a) compared with apoB-100, whereas in most humans, there is an excess of apoB-100 and relatively little free apo(a). After administration of mipomersen, in conjunction with reduced hepatic mRNA expression and plasma apoB-100 levels, a dramatic reduction in plasma Lp(a) levels was evident. This was not due to reduced apo(a) mRNA expression, suggesting that apoB-100 availability is a limiting step in the formation of Lp(a) particles.<sup>17,33</sup> Furthermore, in support of this concept, it was shown that the amount of apo(a) on m-apoB-100, which is known from prior studies to noncovalently associate with m-apoB-100,<sup>17,33</sup> increased in proportion to reduced h-apoB-100 and Lp(a) levels. Overall, these data suggest a novel pathway for reducing Lp(a) levels along with apoB-100 levels. Conversely, it is likely that a reduction in Lp(a) may be mediated by a reduction in apo(a) synthesis using apo(a)-

---

**Figure 3.** Immunoprecipitation experiment using murine monoclonal antibody MB47 to precipitate out h-apoB from plasma of Lp(a) mice (n=4). A 20:1 molar excess MB47:apoB-100 was used. The supernatant was then assayed for the presence of h-apoB-100 (left), Lp(a) (middle), and apo(a) (right). Top, Baseline time point; bottom, the 4-week time point after mipomersen administration.
specific ASOs, although this hypothesis was not tested in this study. This approach will be of interest in future studies once a suitable apo(a) ASO is identified. The fate of free apo(a) also was not evaluated in this study, and understanding its kinetics would be important in future experiments.

The relationship between OxPL and Lp(a) has provided important insights into the potential atherogenicity of Lp(a). In vitro studies have suggested that Lp(a) is the main carrier of OxPL, identified by antibody E06, in human plasma. Additional observations from our laboratory have shown that the OxPL immunoreactivity may be immunoprecipitated along with Lp(a), and detailed density gradient ultracentrifugation experiments showed that nearly all OxPLs recognized by antibody E06 were found in fractions containing apo(a), as opposed to other apolipoproteins. Furthermore, in vitro transfer studies showed that donor OxPLs from oxidized LDL are preferentially donated to Lp(a), as opposed to LDL, in a time- and temperature-dependent manner, even in aqueous buffer. These observations are supported by several clinical studies showing that a strong association occurs between OxPL/apoB and Lp(a) and that OxPL/apoB and Lp(a) show strikingly similar risk for angiographically defined coronary artery disease, the presence and progression of carotid and femoral atherosclerosis, and the prediction of death, myocardial infarction, and stroke in unselected populations. Interestingly, the OxPL/apoB measure tends to be numerically superior in predicting cardiovascular manifestations, and in some but not all studies, the OxPL/apoB ratio was even independent of Lp(a) in predicting coronary artery disease in male subjects <60 years of age.

In this study, it was demonstrated that Lp(a) mice had significantly higher baseline levels of OxPL/h-apoB than h-apoB mice, consistent with prior observations in this model and with the fact that OxPLs measured by E06 are distributed mainly on Lp(a). This was noted in mice on a chow diet and without any other stimulus to generate OxPL. These data, generated even in a weak atherogenic milieu, strongly support the clinical observations noted above that Lp(a) is a preferential carrier of OxPL. Interestingly, the reduction in OxPL/h-apoB appeared to be directly related to the lowering of Lp(a) levels rather than the proportion of OxPL on Lp(a) particles because the OxPL/apo(a) ratio did not change with mipomersen, which lowers apoB-100 levels but is not expected to have direct antioxidant properties. This was supported by the immunoprecipitation experiment showing a reduction in OxPL/h-apoB levels in the supernatant in conjunction with immunoprecipitation of apoB-100 and Lp(a).

We have also previously shown that free apo(a) and recombinant apo(a) peptides bind OxPL. However, the relative proportion bound by Lp(a) versus apo(a) has not been determined yet and is an area of active investigation. Where these OxPL are generated and how they are ultimately

Figure 4. Temporal changes in h-apoB levels in h-apoB mice (A) and Lp(a) mice (B), Lp(a) levels in Lp(a) mice (C), and apo(a) content on mouse apoB-100 particles [apo(a)/m-apoB] (D) in response to mipomersen or control ASO. *P<.001, **P<.01 vs baseline values.
transferred onto Lp(a) in vivo are major questions that merit further exploration. They suggest, however, that the ability of Lp(a) to bind and transport proinflammatory OxPL may be crucial to understanding its atherogenicity and perhaps its potential thrombogenicity.

This study is also the first to document that a reduction in OxPL/apoB levels is achievable with a reduction in Lp(a) levels. This reduction appeared to be dependent on treatment duration; the OxPL/apoB levels returned to baseline when therapy was discontinued. This decrease in OxPL/apoB was directly related to the reduction in Lp(a) levels because the OxPL/apo(a) ratio did not change with therapy. In prior clinical studies with statins and in subjects on low-fat diets, we actually noted an increase in the OxPL/apoB ratio and Lp(a) levels and hypothesized that this increase may reflect an egress of OxPL from the vessel wall, which is then trapped by Lp(a) in the circulation. Those data also could be consistent with upregulation of Lp(a) by statins with concomitant binding of OxPL, as well as other undefined mechanisms. In animal studies of dietary regression, the increase in OxPL/apoB also was noted in rabbits with no Lp(a), along with concomitant removal of OxPL from the vessel wall as assessed by immunostaining before plaque regression, suggesting that these 2 processes were linked mechanistically.

Although further evaluation is needed to understand the relationship between changes in OxPL/apoB and Lp(a), these studies suggest that changes in OxPL are linked to changes in Lp(a). The implications of these observations in clinical risk prediction await outcomes studies.

**Study Limitations**

This study tested the efficacy of mipomersen on 1 specific mini-apo(a) construct under the control of an apoE promoter; therefore, translation to humans studies awaits determination. The role of Lp(a) in atherogenesis in this mouse model, the
effect of mipomersen in cholesterol-fed Lp(a) mice, and whether the reduction in OxPL/apoB will lead to additional antiatherogenic properties will be addressed in additional studies.

Conclusion
Administration of mipomersen resulted in a significant reduction in Lp(a) and OxPL/apoB levels in Lp(a) mice and may have a direct effect of mipomersen in cholesterol-fed Lp(a) mice, and may
reduction in Lp(a) and OxPL/apoB levels in Lp(a) mice and may represent a novel therapeutic agent in reducing LDL-cholesterol, Lp(a), and OxPL/apoB levels in humans.

Sources of Funding
This study was supported by the Fondation Leducq (Drs Witztum and Tsimikas) and an unrestricted gift from Isis Pharmaceuticals to the University of California.

Disclosures
Drs Witztum and Tsimikas are named as inventors in patents and patent applications for the potential commercial use of antibodies to oxidized LDL and have served as consultants to Isis Pharmaceuticals, Inc. M.J. Graham and Drs Mullick and Crooke are employees of ISIS, Inc. The other authors report no conflicts.

References
CLINICAL PERSPECTIVE

Lipoprotein(a) [Lp(a)] is composed of apolipoprotein(a) [apo(a)], which is covalently bound to a low-density lipoprotein by a single disulfide bond on apoB-100. Lp(a) levels are genetically determined and vary widely (0.1 to 250 mg/dL) among individuals. Lp(a) is an independent risk factor for myocardial infarction, stroke, and peripheral arterial disease, particularly in younger patients. However, its pathophysiological role and the underlying mechanisms through which it contributes to cardiovascular disease are unknown. It also has not been determined yet whether lowering Lp(a) levels is clinically beneficial, largely because of the lack of specific agents to lower Lp(a). We have recently discovered that Lp(a) preferentially binds oxidized phospholipids in plasma. In this study, we demonstrate that the antisense oligonucleotide mipomersen, directed to human apoB-100, significantly reduced human apoB-100 levels in Lp(a) transgenic mice [expressing human apoB-100 and apo(a) to make authentic Lp(a) particles], as expected. However, over the 11-week treatment period, compared with baseline, it also reduced Lp(a) levels by ~75% (P<0.0001) in a time-dependent fashion. This was due primarily to limiting the availability of apoB-100 to bind to apo(a). Furthermore, it significantly reduced plasma levels of oxidized phospholipids on apoB and apo(a) particles. This study demonstrates that apoB-100 is a limiting factor in Lp(a) particle synthesis in this Lp(a) transgenic model. If applicable to humans, mipomersen may represent a novel therapeutic approach in not only reducing apoB-100 and low-density lipoprotein cholesterol but also in reducing Lp(a) and associated oxidized phospholipids.
Antisense Oligonucleotide Directed to Human Apolipoprotein B-100 Reduces Lipoprotein(a) Levels and Oxidized Phospholipids on Human Apolipoprotein B-100 Particles in Lipoprotein(a) Transgenic Mice

Esther Merki, Mark J. Graham, Adam E. Mullick, Elizabeth R. Miller, Rosanne M. Crooke, Robert E. Pitas, Joseph L. Witztum and Sotirios Tsimikas

_Circulation_. 2008;118:743-753; originally published online July 28, 2008; doi: 10.1161/CIRCULATIONAHA.108.786822

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/118/7/743

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org/subscriptions/