An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis

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Abstract **High levels of plasma apolipoprotein B-100 (apoB-100), the principal apolipoprotein of LDL, are associated with cardiovascular disease. We hypothesized that suppression of apoB-100 mRNA by an antisense oligonucleotide (ASO) would reduce LDL cholesterol (LDL-C). Because most of the plasma apoB is made in the liver, and antisense drugs distribute to that organ, we tested the effects of a mouse-specific apoB-100 ASO in several mouse models of hyperlipidemia, including C57BL/6 mice fed a high-fat diet, *Apoe*-deficient mice, and *Ldlr*-deficient mice. The lead apoB-100 antisense compound, ISIS 147764, reduced apoB-100 mRNA levels in the liver and serum apoB-100 levels in a dose- and time-dependent manner. Consistent with those findings, total cholesterol and LDL-C decreased by 25–55% and 40–88%, respectively. Unlike small-molecule inhibitors of microsomal triglyceride transfer protein, ISIS 147764 did not produce hepatic or intestinal steatosis and did not affect dietary fat absorption or elevate plasma transaminase levels. These findings, as well as those derived from interim phase I data with a human apoB-100 antisense drug, suggest that antisense inhibition of this target may be a safe and effective approach for the treatment of humans with hyperlipidemia.**—Crooke, R. M., M. J. Graham, K. M. Lemonidis, C. P. Whipple, S. Koo, and R. J. Perera. **An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. *J. Lipid Res.* 2005.** 46: **872–884.**

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Coronary heart disease (CHD) has been the leading cause of death in the United States for over a century, and complications from atherosclerosis are the most common cause of death in Western societies (1–3). LDL has been causally implicated in atherogenesis, and elevated levels of plasma LDL cholesterol (LDL-C) are a major risk factor for cardiovascular disease (1, 4, 5).

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In humans, apolipoprotein B (apoB)—the principal apolipoprotein of LDL and its metabolic precursor, VLDL (6–8)—is the main structural protein for VLDL assembly in the liver. In the intestine, apoB plays a vital role in the assembly of chylomicrons and in the uptake of dietary fats. The human apoB gene yields two proteins, apoB-100 and apoB-48 (6, 8–10). ApoB-48 contains the N-terminal portion 2,152 amino acids of apoB-100 and is formed as a result of apoB mRNA editing, which changes apoB codon 2153 to a stop codon. Assembly of apoB-containing lipoproteins occurs in the endoplasmic reticulum and requires microsomal triglyceride transfer protein (MTP) (10–13).

Elevated apoB and LDL-C levels are associated with premature atherosclerosis in several inherited diseases, including familial hypercholesterolemia, familial defective apoB-100, and familial combined hypercholesterolemia (14–19). Abnormalities in apoB-100 metabolism that increase the risk for CHD are also observed in diabetes mellitus and obesity (20–22). Conversely, mutations that interfere with apoB synthesis lower plasma levels of apoB and LDL-C and appear to be associated with reduced levels of atherosclerosis (23–26). These genetic observations have prompted interest in pharmacologic inhibition of apoB synthesis. Regrettably, the potential benefits of directly reducing apoB synthesis via traditional therapeutics have never been explored. However, the effects of blocking lipoprotein secretion have been assessed with small-molecule inhibitors of MTP (27, 28), with genetic interventions in mice that inhibit MTP (27, 29, 30) and apoB synthesis (31, 32). Enhancement of hepatic apoB-100 mRNA editing by providing *apobec-1* to transgenic mice and rabbits

Abbreviations: AMPK, AMP-activated protein kinase; apoB, apolipoprotein B; ASO, antisense oligonucleotide; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HF, high fat; LDL-C, LDL cholesterol; 2′MOE, 2′-*O*-methoxyethyl; MTP, microsomal triglyceride transfer protein; SCD, stearoyl CoA desaturase; SREBP, sterol-responsive element binding protein; SSC, standard sodium citrate; TG, triglyceride.

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reduced plasma apoB-100 (33) but resulted in hepatocellular dysplasia and carcinoma.

Antisense oligonucleotides (ASOs) are novel therapeutic agents that safely and selectively reduce levels of a specific mRNA (34). The pharmacokinetics of antisense drugs has been extensively characterized in multiple animal models and in clinical settings (35–39). After parenteral administration, first- and second-generation ASOs are widely distributed in tissues; the highest concentrations are found in kidney and liver (35–39), and the drug levels in these organs correlate directly with pharmacological activity in vivo (37, 38).

Because antisense drugs are distributed to the liver and because there is a strong therapeutic rationale for blocking hepatic apoB secretion, we designed an antisense molecule specific for murine apoB-100 and tested its efficacy in several mouse models of hyperlipidemia. Our findings suggest that direct inhibition of apoB-100 may be a safe and novel approach to lowering LDL-C levels in various hyperlipidemic states.

MATERIALS AND METHODS

Oligonucleotides/atorvastatin

A series of uniform chimeric 20-mer phosphorothioate oligonucleotides containing 2′-*O*-methoxyethyl (2′MOE) groups at positions 1–5 and 15–20 targeted to murine apoB-100 were synthesized and purified as described (40) with an automated DNA synthesizer (380B; Perkin-Elmer-Applied Biosystems; Foster City, CA). The two most potent ASOs complementary to murine apoB-100 (GenBank accession number M351861) were ISIS 147764: 5′-GTCCCTGAAGATGTCAATGC-3′, position 541 of the coding region, and ISIS 147483: 5′-ATGTCAATGCCACATGTCCA-3′, position 531 of the coding region. To demonstrate the specificity of inhibition, we used control ASOs: ISIS 29837, 5′-TCGATCTCCTTTTATGCCCG-3′; ISIS 113529, 5′-CTCTTACTGTGCTGTGGACA-3′; ISIS 141923, 5′-CCTTCCCTGAAGGTTCCTCC-3′; and ISIS 144479 (an antisense inhibitor of MTP), 5′-CACCTGCCACTTGCTTCCCG-3′.

Atorvastatin (Lipitor), an inhibitor of HMG-CoA reductase in humans and rodents, was used as a positive, lipid-lowering control. It was administered orally once daily at 20 mg/kg.

Cell culture and oligonucleotide treatment

Murine apoB-100 ASOs were evaluated for their ability to reduce apoB-100 mRNA expression in primary murine hepatocytes isolated by in situ liver perfusion as described (41). The cells were seeded in 60 cm^2 6-well plates at 3×10^5 cells/well in high-glucose DMEM (Invitrogen; Carlsbad, CA) containing 10% FBS and allowed to adhere overnight. The medium was then replaced with serum-free DMEM containing 9.0 μg/ml lipofectin (Invitrogen) and various amounts of murine apoB-100 or control ASOs. After incubation for 4 h, the treatment medium was replaced with 10% DMEM, and the cells were incubated overnight. The hepatocytes were then lysed in RNA lysis buffer (Qiagen), and total RNA was prepared for RT-PCR analysis.

RT-PCR analysis

Total RNA was extracted from whole liver tissue and primary hepatocytes with Qiagen RNeasy isolation kits, as described (42), and 50 ng of each sample was subjected to RT-PCR analysis with a Prism 7700 Sequence Detector (Applied Biosystems); all reagents were from Invitrogen. The mouse-specific primer probes for apoB-100 quantification were: forward primer 5′-CGTGGGCTCCAGCATTCTA-3′, probe 5′-CCAATGGTCGGGCACTGCTCAA-3′, and reverse primer 5′-AGTCATTTCTGCCTTTGCGTC-3′. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) and/or Ribogreen levels, assessed with forward primer 5′-GGCAAATTCAACGGCACAGT-3′, probe 5′-AAGGCCGAGAATGGGAAGCTTGTCATC-3′, and reverse primer 5′-CGCTCCTGGAAGATGGTGAT-3′. In both cases, the probes were labeled with 5′ FAM (a 6-carboxyfluorescein reporter) and 3′ TAMRA [a 5(6)-carboxytetra-methyl-rhodamine quencher]. After 40 amplification cycles, absolute values were obtained with SDS analysis software (Applied Biosystems).

Northern analysis

Total RNA was extracted from whole-liver tissue and primary hepatocytes as described previously (42). After RNA isolation, 15 μg of total RNA was separated on a 1% 1× MOPS agarose gel containing ethidium bromide and 20 mM formaldehyde. The RNA was transferred to a Hybond TM-N+ membrane (Amersham; Les Ulis, France) by overnight capillary transfer with 20× saline sodium citrate (SSC). Stained membranes were examined with UV light to confirm the quality of transfer and to mark the migration of the 28S and 18S RNAs. After UV fixation, prehybridization was performed at 65°C for 2 h in Rapid-Hyb solution (Amersham Biosciences; Piscataway, NJ). The mouse-specific cDNA probes were produced by amplification of a 500 bp region of apoB-100 with forward (5′-CGGATGAAGGCATACATTCGT-3′) and reverse (5′-CAGCTTGCTTGTTGGGAC-3′) primers. The sequence identity of the product was confirmed, asymmetric PCR was performed, and the probe was ^{32}P-dCTP labeled with a GeneAmp DNA labeling kit (Applied Biosystems). After hybridization at 65°C for 2 h, the membranes were washed in 0.1× SSC/0.1% SDS twice at room temperature for 30 min and once at 65°C for 30 min. To further demonstrate transfer equivalency, blots were rehybridized with a mouse G3PDH cDNA probe (BD Biosciences Clontech; Palo Alto, CA), and the RNA bands were quantified using ImageQuant analysis software (Molecular Dynamics).

ApoB Western blots

Cells or tissues were harvested in lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% NP40, 1% Triton, 0.25% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail 1:100, 0.2 mM sodium orthovanadate), and protein concentrations were measured with the BioRad DC kit (BioRad; Hercules, CA). Cellular lysates, mouse plasma, and liver and intestinal homogenates were subjected to electrophoresis on 4–12% Tris-glycine gels and transferred to polyvinylidene-fluoride membranes (Invitrogen). ApoB-100 Western blot analyses were performed overnight at 4°C with a primary anti-mouse apoB-100 antibody (1:1,000, kindly provided by Dr. Stephen G. Young, UCLA Department of Medicine/Division of Cardiology). The next day, blots were incubated for 1 h with a goat anti-mouse IgG secondary antibody conjugated with peroxidase (1:15,000; BD Biosciences; San Diego, CA). Protein bands were visualized using the ECL Plus Western blot detection kit (Amersham Biosciences). ApoB-48 Western blots were performed in a similar manner using a primary anti-rabbit polyclonal antibody that recognizes both apoB-100 and apoB-48 (1:2,000, also provided by Dr. Young) and a goat anti-rabbit IgG secondary antibody conjugated with peroxidase (1:15,000; BD Biosciences). Protein bands were quantified using ImageQuant analysis software (Molecular Dynamics).

HPLC analysis of lipoproteins

Serum lipoprotein and cholesterol profiling was performed as described (43) with a Beckman System Gold 126 HPLC system,

507e refrigerated autosampler, 126 photodiode array detector (Beckman Instruments; Fullerton, CA), and a Superose 6 HR 10/30 column (Pfizer; Chicago, IL). HDL, LDL, and VLDL fractions were measured at a wavelength of 505 nm and validated with a cholesterol calibration kit (Sigma). For each experiment, a three-point standard curve was performed in triplicate to determine the absolute concentration of each lipoprotein fraction.

NMR analysis of lipoproteins

Serum lipoprotein subclass particle number and size were determined at Liposcience® using a NMR spectral analysis process as described (44).

Metabolic measurements

Serum concentrations of glucose, transaminases, ketones, triglycerides (TGs), and total cholesterol were determined with an Olympus AU400e automated clinical chemistry analyzer (Melville, NY). Total cholesterol, LDL, and HDL were measured directly with the autoanalyzer, and the data were corroborated by the HPLC method described above.

Murine pharmacology experiments

All animal experiments were conducted according to the Institutional American Association for the Accreditation of Laboratory Animal Care guidelines. Wild-type C57BL/6, *Apoe*-deficient mice (ApoE^{tm1unc}), and *Ldlr*-deficient mice (C57BL/6J-*Ldlr*^{tm1Her}) were obtained from The Jackson Laboratory (http://www.jax.org). A majority of the mice were male, and studies began with animals 4–5 weeks of age. The mice were maintained on a 12-h light/12-h dark cycle and fed ad libitum. C57BL/6 mice were fed a Western diet consisting of 60% lard (Research Diets; New Brunswick, NJ) or regular rodent chow. *Apoe*-deficient mice were fed a Western diet containing 0.15% cholesterol, and *Ldlr*-deficient mice were fed regular chow.

Oligonucleotides were administered to mice based upon body weight by intraperitoneal injection, with the dosing solution formulated at 10 mg/ml. At study termination, mice were anesthetized, whole blood was obtained through cardiac puncture, and liver, spleen, and epididymal fat pads were removed and weighed. Liver was processed and RNA, protein, metabolic parameters, total cholesterol, and lipoproteins were analyzed as described above.

Liver and intestinal morphology was evaluated using hematoxylin and eosin–stained paraffin-embedded tissue.

DNA array analysis

Liver samples derived from apoB-100 ASO–treated mice were used for DNA array transcriptional profiling or microarray analysis. Mouse liver total RNA (10 μg) was used as the starting material for cDNA synthesis. For the first-and second-strand cDNA synthesis, SuperScript reverse transcriptase (Invitrogen) and oligo-dT primer containing T7 RNA polymerase promoter sites were used. The in vitro transcription reaction was produced using a high-yield transcription labeling kit from Enzo (Enzo Bioarray/Affymetrix). The cDNA (15 μg) was fragmented and hybridized to an Affymetrix Murine genome 430A chip using standard methods described by the manufacturer (Affymetrix). Each chip contained ~22,000 genes. Statistical analysis for the microarray analysis was performed using multi-sample inference with ANOVA. Raw *P* values were adjusted using the Benjamin-Hochberg procedure. All statistical computations were performed using SAS® and S-Plus®.

Liver studies

Hepatic TG levels were assayed as described (45). Neutral lipids were detected in frozen liver sections by Oil Red O staining (46). The concentrations of ISIS 147764 and metabolites were measured by capillary gel electrophoresis (37).

Dietary fat absorption studies

Dietary fat absorption was evaluated in high-fat (HF)-fed C57BL/6 mice treated with twice weekly injections of ISIS 147764 for 8 and 20 weeks (50 mg/kg and 25 mg/kg, respectively) using modifications of fecal isotope protocols described previously (47, 48). At those times, [^{14}C]oleic acid and [5,6-^{3}H]sitostanol, a nonabsorbable phytosterol standard formulated in olive oil, were administered by gavage to control- and apoB-100 ASO–treated mice, and feces were collected for 72 h. Dietary fat absorption was calculated based upon the ratio of [^{14}C] to [^{3}H] in the fecal extracts.

Statistical analysis

In general, pharmacology experiments were performed at least two to three times in groups of five to eight mice. Total cholesterol, LDL-C, HDL, and plasma and liver TGs are reported as means ± SD. Statistical analysis was performed using one-way ANOVA, with comparisons made between saline- and drug-treated mice. Differences were considered significant at $P < 0.05$.

RESULTS

In vitro characterization of murine apoB-100 antisense oligonucleotides

Multiple 2′MOE chimeric ASOs targeting various regions of the apoB-100 gene were evaluated for their ability to reduce mouse apoB-100 mRNA in primary murine hepatocytes. Approximately 25% of these compounds were active. The two most potent compounds—ISIS 147764 and ISIS 147483—were selected for additional evaluation. Both reduced apoB-100 mRNA levels in a dose-dependent fashion; inhibition was maximal (90%) at 150 nM. A control ASO, ISIS 113529, had no effect on apoB-100 expression. Doses of any ASO up to 300 nM did not affect cell viability. ISIS 147764 was chosen for further study in mouse hyperlipidemia models (data not shown).

In vivo reduction of murine hepatic apoB-100 and apoB-48 in HF-fed C57BL/6 mice

ISIS 147764 (5, 25, and 50 mg/kg) was administered intraperitoneally to HF-fed C57BL/6 mice twice weekly for 6 weeks, and liver apoB-100 mRNA levels were measured by RT-PCR and Northern blotting. ApoB-100 mRNA levels were reduced in a dose-dependent manner (**Fig. 1A**), with the greatest inhibition (87%) at a dose of 50 mg/kg. Neither the control ASO (an antisense inhibitor of MTP) nor atorvastatin affected mRNA levels. These results were confirmed in at least five experiments with a variety of scrambled and mismatched ASO controls, including a six-base mismatch of ISIS 147764 that was pharmacologically inactive. ISIS 147764 did not affect mRNA expression of nontargeted genes, including ACAT, MTP, and HMG-CoA reductase (not shown), indicating that inhibition of apoB-100 expression was both target- and sequence-dependent.

ApoB-100 and apoB-48 protein levels in liver and serum were analyzed by Western blotting. After 6 weeks of treatment with the apoB-100 ASO, dose-dependent reductions in liver apoB-100 protein were observed relative to saline-treated controls (Fig. 1B). At the highest dose (50 mg/kg), protein levels were almost undetectable. Neither control

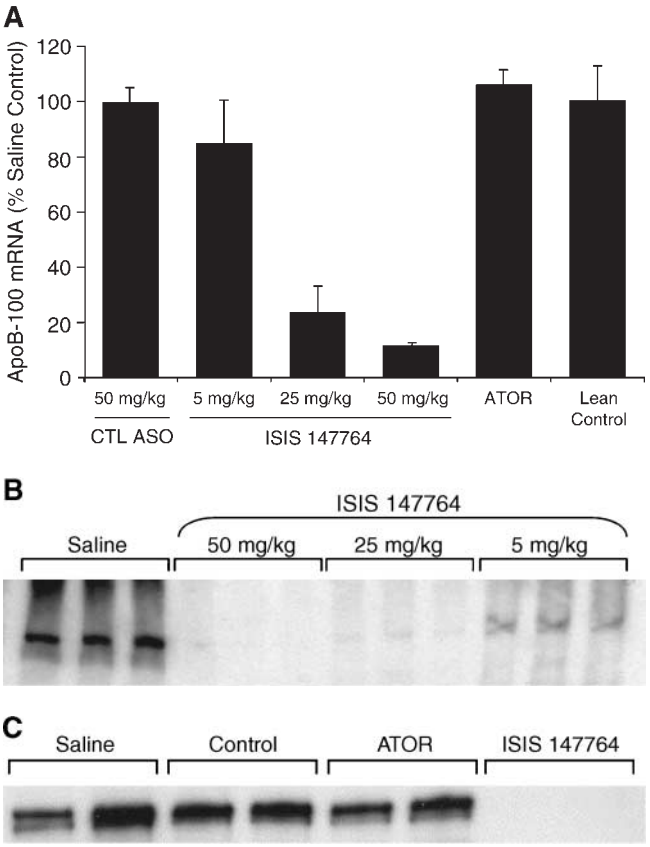

Fig. 1. ISIS 147764 specifically reduces apolipoprotein B-100 (apoB-100) mRNA and protein levels in livers of high-fat (HF)-fed C57BL/6 mice. In this representative experiment, mice received ISIS 147764 at the indicated doses or a control antisense oligonucleotide (ASO) twice weekly for 6 weeks and orally administered atorvastatin (20 mg/kg daily). A: Total mRNA was prepared from liver, and apoB-100 mRNA expression was analyzed by RT-PCR (n = 6 per group). The results were also confirmed by Northern analysis (not shown). Data are expressed as the mean percentage of mRNA levels in saline-treated, HF-fed C57BL/6 mice. The apoB-100 signal was normalized against the signal for glyceraldehyde-3-phosphate dehydrogenase (G3PDH). CTL, control ASO; Lean Control, chow-fed C57BL/6 mice; ATOR, atorvastatin. B: Dose-dependent reduction of apoB-100 protein expression in liver. Western analysis of proteins in liver lysates derived from individual mice treated for 6 weeks with drug doses as described above. Each lane contains 50 μg of protein. C: Reduction of serum apoB-100. A control ASO and atorvastatin had no effect on the protein. Each lane represents the serum apoB-100 of an individual animal treated with saline, atorvastatin, or 100 mg/kg ASO weekly.

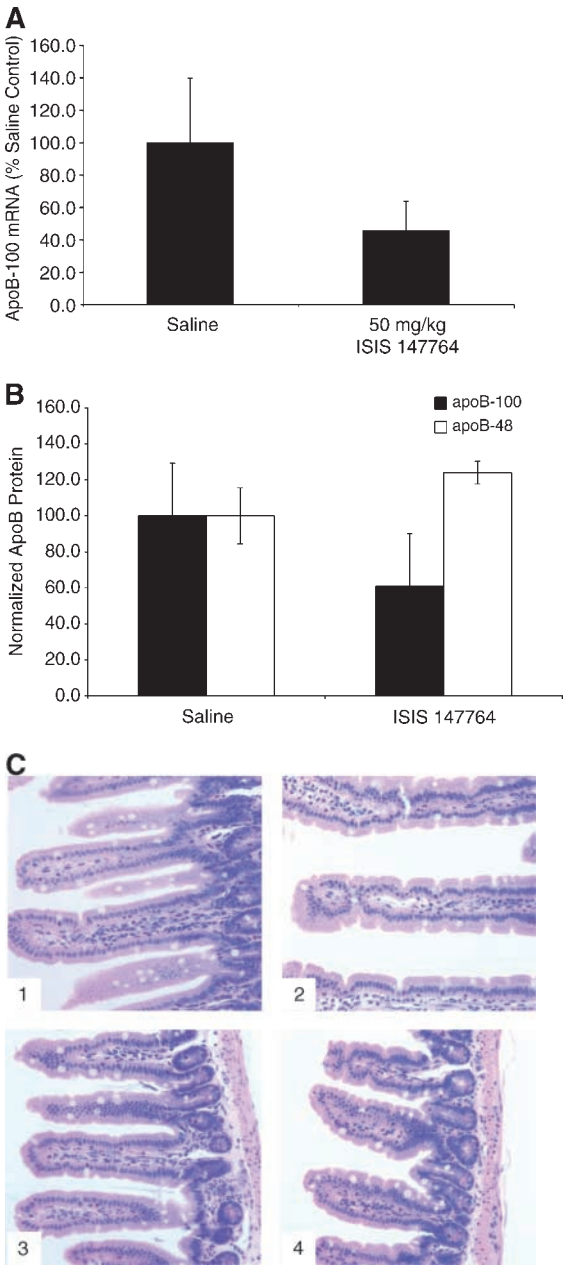

Fig. 2. Effects of ISIS 147764 on intestinal apoB in HF-fed C57BL/6 mice. In this representative experiment, mice were administered 50 mg/kg ISIS 147764 twice weekly for 8 weeks. A: Total mRNA was prepared from intestine and apoB-100 mRNA expression analyzed by RT-PCR (n = 4 per group). Data are expressed as the mean percentage of mRNA levels in saline-treated, HF-fed C57BL/6 mice. The apoB-100 signal was normalized against Ribogreen. B: Differential effects of ASO treatment on apoB-100 and apoB-48 proteins. ApoB-100 protein levels were suppressed, whereas apoB-48 protein levels were not reduced by systemically administered drug. Protein levels in intestinal lysates from mice treated with saline alone or ISIS 147764 were analyzed by Western blotting. Each lane contains 50 μg of protein. C: Representative hematoxylin and eosin-stained histologic sections of the small intestine of HF-fed mice after 6 weeks of treatment with ISIS 147764 (25 mg/kg twice weekly). Panel 1, duodenum of saline-treated mice; Panel 2, duodenum of apoB-100 ASO–treated mice; Panel 3, jejunum of saline-treated mice; Panel 4, jejunum of apoB-100 ASO–treated mice.

ASOs nor atorvastatin (data not shown) had any effect. Plasma apoB-100 protein levels were suppressed as well (Fig. 1C). ApoB-48 protein levels in liver and serum, like those of apoB-100, were also significantly reduced (90%; data not shown).

Effect of apoB-100 ASO treatment on intestinal apoB in HF-fed C57BL/6 mice

Intestinal apoB-100 mRNA and protein and apoB-48 protein levels were also measured after systemic administration of the ASO (50 mg/kg twice weekly). After 8 weeks of treatment with ISIS 147764, intestinal apoB-100 mRNA

levels in HF-fed mice were reduced (54%) compared with saline-treated animals (**Fig. 2A**). The extent of mRNA suppression was less than that observed previously in livers of ASO-treated mice (Fig. 1A). Interestingly, although the intestinal apoB-100 protein levels were reduced (40%), apoB-48 protein levels were not statistically different from those of saline-treated controls (Fig. 2B). Histological evaluation of the small intestine from several experiments has demonstrated that there were no differences between saline- and apoB-100 ASO–treated mice (Fig. 2C).

Effect of apoB-100 suppression on lipid and lipoprotein levels

We hypothesized that ASO treatment would also reduce total cholesterol and LDL-C levels. ApoB-100 mRNA suppression correlated tightly with reduction in total cholesterol levels in HF-fed C57BL/6 mice (**Fig. 3A**). Treatment with 50 mg/kg ISIS 147764 lowered apoB-100 mRNA levels by 88% and those of total cholesterol by 55%, and effectively normalized cholesterol to levels reported in the literature and observed routinely in our chow-fed, saline-treated control mice (82 ± 15 mg/dl). As predicted, reduction of apoB-100 mRNA lowered circulating LDL-C levels (Fig. 3B), reducing LDL-C levels by 66% at 25 mg/kg and 88% at 50 mg/kg. The absolute particle numbers of all apoB-100-containing lipoproteins, as assessed by NMR spectroscopy, were significantly suppressed as well. However, chylomicrons were not reduced (data not shown). Serum TG levels were also reduced (25% inhibition) in these mice in all experiments. Administration of 50 mg/kg ASO decreased TG levels from 117.5 ± 19.0 mg/dl to 87.3 ± 16.3 mg/dl after 6 weeks of treatment ($P < 0.05$).

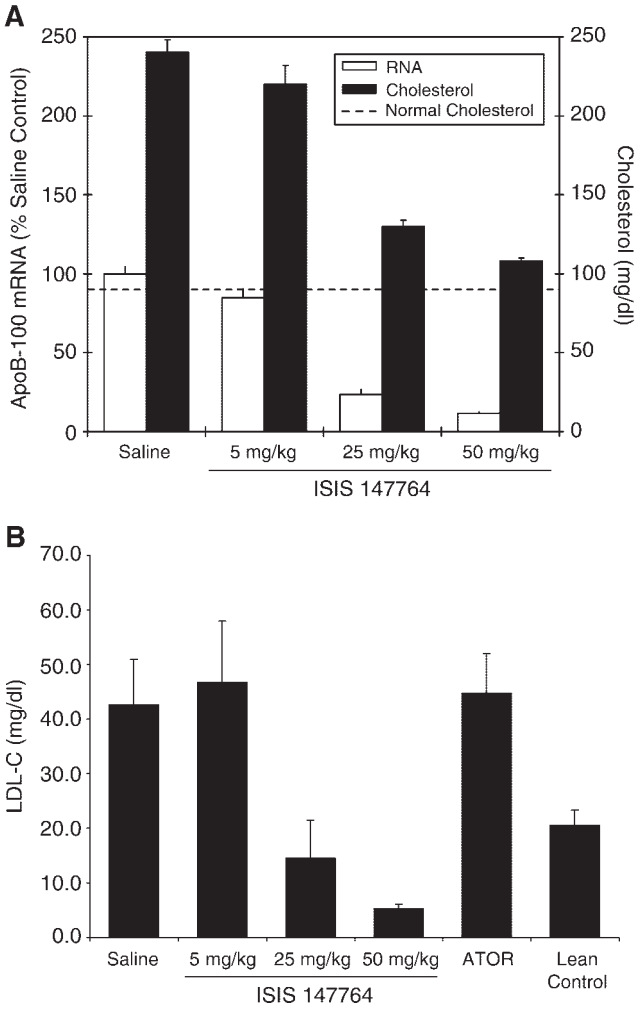

Fig. 3. Total cholesterol and mRNA (A), and LDL cholesterol (LDL-C) (B) levels in HF-fed C57BL/6 mice after 6 weeks of treatment with ISIS 147764, atorvastatin (ATOR), or both. Lean Control, chow-fed C57BL/6 mice. Dose-dependent reductions of cholesterol correlated with reductions of mRNA in liver. RNA was prepared from liver, and apoB-100 mRNA expression was analyzed by RT-PCR (n = 6 per group). Data are expressed as the mean percentage of mRNA levels in saline-treated, HF-fed C57BL/6 mice. The apoB-100 signal was normalized against the signal for G3PDH.

Onset of action

To determine the kinetics of apoB-100 ASO inhibition, we treated HF-fed C57BL/6 mice with ISIS 147764 (50 mg/kg twice weekly) for up to 6 weeks. Hepatic concentrations of intact drug, liver apoB-100 mRNA and protein levels, plasma apoB-100, total cholesterol, and LDL-C levels were assessed 48 h after the first dose and at 1, 2, 4, and 6 weeks. ApoB-100 mRNA, total cholesterol, and LDL-C (**Table 1**) were suppressed in a time-dependent manner; hepatic mRNA and liver protein (**Fig. 4**) were reduced by 50% as early as 48 h after the first dose. Significant amounts of full-length ISIS 147764 were detected in the liver by 48 h (85.0 μg/g) and increased steadily, reaching a maximum (513.0 μg/g) at 6 weeks. Total cholesterol was reduced by 65% at 4 weeks, and LDL-C by 40% ($P < 0.05$) after 1 week. This reduction in LDL-C correlated with suppression of liver apoB-100 mRNA and protein levels (Table 1 and Fig. 4) and serum apoB-100 (not shown). Reductions in mRNA, protein, total cholesterol, and LDL-C levels were maximal 4–6 weeks after treatment.

Duration of effect

To determine the duration of the lipid-lowering effects, we treated HF-fed C57BL/6 mice with ISIS 147764 (25 mg/kg twice weekly) for 6 weeks and examined apoB-100 liver drug concentrations, liver apoB-100 mRNA and protein (not shown), and serum levels of total cholesterol, LDL-C (**Table 2**), and apoB-100 protein (**Fig. 5**) for 6–8 weeks after termination of dosing. All experimental parameters gradually returned to control levels after cessation of treatment; however, the lipid-lowering effects of ISIS 147764 were prolonged, consistent with the extended half-life of 2′MOE ASOs in the liver (34, 38, 39). Six weeks after cessation of dosing, the liver ISIS 147764 concentrations were reduced from 335.0 μg/g to 70.0 μg/g. Hepatic apoB-100 mRNA levels were reduced by 37% (compared with 84% at baseline), total cholesterol by 38%, and LDL-C by 36% ($P < 0.01$). The pharmacologic effects of the drug were insignificant after 8 weeks (Table 2), coincident with decreased hepatic ASO levels.

TABLE 1. Hepatic levels of ISIS 147764 and apoB-100 mRNA, total cholesterol, and LDL-C levels in HF-fed C57BL/6 mice during 6 weeks of treatment (50 mg/kg twice weekly)

Time/Treatment	Hepatic ASO Level	RNA Reduction	Total Cholesterol	Total Cholesterol Reduction	LDL-C	LDL-C Reduction
	μg/g	%	*mg/dl*	%	*mg/dl*	%
48 H						
Saline	0		177.5 ± 23.0		ND	ND
ISIS 147764	85.0	50	159.8 ± 22.0	10	ND	ND
1 Week						
Saline	0		165.8 ± 14.8		31.5 ± 5.5	
ISIS 147764	121.0	57	146.4 ± 15.7	12[a]	19.3 ± 5.1	40[a]
2 Weeks						
Saline	0		162.0 ± 23.3		27.6 ± 7.9	
ISIS 147764	195.0	73	121.4 ± 8.6	25[b]	14.3 ± 3.3	50[b]
4 Weeks						
Saline	0		200.0 ± 25.5		49.7 ± 9.5	
ISIS 147764	350.0	82	70.4 ± 13.1	65[b]	6.9 ± 1.5	86[b]
6 Weeks						
Saline	0		173.1 ± 21.3		45.6 ± 17.4	
ISIS 147764	513.0	88	56.6 ± 6.4	66[b]	6.6 ± 1.0	87[b]

ApoB, apolipoprotein B; ASO, antisense oligonucleotide; HF, high fat; LDL-C, LDL cholesterol; ND, not determined.
[a] $P < 0.05$.
[b] $P < 0.01$.

Comparison of ISIS 147764 in multiple mouse hyperlipidemia models

Mouse models are unreliable in predicting the efficacy of hypolipidemic drugs in humans (30, 49). In addition, mice edit apoB-100 to apoB-48 in the liver, and their apoB-100 levels vary (30, 49, 50). Therefore, we compared the effects of ISIS 147764, 50 mg/kg twice weekly for 6 weeks, in HF-fed C57BL/6, *Apoe*-, and *Ldlr*-deficient mice. ISIS 147764 was also administered to chow-fed C57BL/6 mice to determine whether apoB-100 reduction could also lower apoB-100 mRNA, cholesterol, and LDL-C in these lean animals. Hepatic apoB-100 mRNA expression was reduced by 73–87% in all treated mice; the inhibition was greatest in HF-fed mice (**Table 3**). The reductions in total cholesterol and LDL-C varied, depending on the model; the greatest efficacy was observed, as before, in HF-fed mice. *Apoe*-deficient mice, which had the highest levels of apoB-48 and the lowest levels of apoB-100 (26), were least sensitive to the lipid-lowering effects (25% reduction in cholesterol, 40% reduction in LDL-C). In *Ldlr*-deficient mice, which have elevated amounts of apoB-100 (30), LDL-C levels were reduced by 62%.

HDL levels were reduced by 34% in chow-fed C57BL/6 mice, by 48% in HF-fed C57BL/6 mice, and by 14% in *Ldlr*-deficient mice. Atorvastatin also reduced HDL levels (not shown). Lieu et al. (30) reported similar findings in Reversa mice and suggested that mature HDL particles require components from apoB-containing lipoproteins. Therefore, a significant loss of VLDL and LDL should produce similar reductions in HDL.

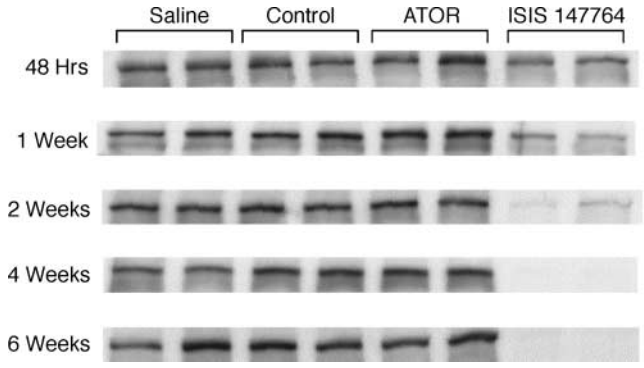

Fig. 4. Time-dependent reduction of liver apoB-100 protein in HF-fed C57BL/6 mice. Mice (n = 6) received 50 mg/kg of ISIS 147764 and a control ASO twice weekly for 6 weeks and orally administered atorvastatin (ATOR, 20 mg/kg daily) for various times. Protein levels in liver lysates from mice treated with the drug for 48 h or 1, 2, 4, or 6 weeks were analyzed by Western blotting. Each lane contains 50 μg of protein isolated from an individual animal.

Safety profile after apoB-100 reduction

To monitor hepatotoxicity, we routinely measured transaminase levels. The levels of aspartate aminotransferase and alanine aminotransferase in ASO-treated HF-fed mice were similar to those observed in saline- and ASO control–treated mice even after 20 weeks of treatment (**Fig. 6A**). Comparable effects on transaminases and other metabolic parameters (glucose, ketones, and liver, body, and spleen weights) were also observed in chow-fed C57BL/6, *Apoe*-deficient, and *Ldlr*-deficient mice after 6 weeks of treatment, suggesting that this compound is safe in mice.

Pharmacologic inhibition of apoB-100, like that of MTP, should block the secretion of TG from hepatocytes, potentially causing steatosis (27, 29). To address this concern, we evaluated the effects of ISIS 147764 on murine liver in all of our experiments by gross histopathology, by Oil Red O staining for lipids (mainly TG), and by measuring hepatic TG levels. Results from cumulative studies in all models described above over several years have failed to demonstrate hepatic steatosis beyond that of the phenotype of the hyperlipidemia model, nor were the effects dif-

TABLE 2. Hepatic levels of ISIS 147764 and reductions in apoB-100 mRNA, total cholesterol, and LDL-C levels after cessation of treatment in HF-fed C57BL/6 mice

Time/Treatment	Hepatic ASO Level	RNA Reduction	Total Cholesterol	Total Cholesterol Reduction	LDL-C	LDL-C Reduction
	μg/g	%	*mg/dl*	%	*mg/dl*	%
Baseline[a]						
Saline	0		187.0 ± 35.8		21.7 ± 4.8	
ISIS 147764	335.0	84	87.6 ± 19.9	53[b]	5.3 ± 2.0	76[b]
2 Weeks						
Saline	0		143.9 ± 26.7		23.9 ± 2.6	
ISIS 147764	163.0	78	67.1 ± 5.6	53[b]	13.8 ± 3.8	42[b]
4 Weeks						
Saline	0		194.5 ± 18.2		34.4 ± 4.2	
ISIS 147764	124.0	49	110.0 ± 9.9	43[b]	17.8 ± 2.3	48[b]
6 Weeks						
Saline	0		221.4 ± 30.3		36.9 ± 6.4	
ISIS 147764	70.0	37	138.0 ± 17.5	38[b]	23.6 ± 2.6	36[b]
8 Weeks						
Saline	0		159.9 ± 35.6		22.3 ± 4.1	
ISIS 147764	43.0	19	134.9 ± 28.2	16	20.8 ± 4.4	NC

NC, no change ($P > 0.05$).
[a] After administration of ISIS 147764 (25 mg/kg) twice weekly for 6 weeks.
[b] $P < 0.01$.

ferent from those in multiple mice treated with a control ASO or atorvastatin. Moreover, in chow-fed C67BL/6 mice, ISIS 147764 (100 mg/kg weekly for 6 weeks) did not produce hepatic steatosis (not shown).

To define the long-term consequences of apoB-100 inhibition, we administered ISIS 147764 to HF-fed mice over 20 weeks. Mice were sacrificed at 6, 12, and 20 weeks. ApoB-100 mRNA, total cholesterol, and LDL-C were significantly and consistently reduced to levels observed after 6 weeks of dosing in the experiments described previously (not shown). As described above, transaminase levels were unaffected (Fig. 6A). Liver TG stores did not change significantly after 6 weeks of treatment (Fig. 6B). This trend has been uniformly observed in all HF-fed C57BL/6 pharmacology experiments (not shown). However, by 20 weeks, hepatic TG stores were significantly lower than in controls (Fig. 6B). In fact, as shown by Oil Red O staining, the hepatic steatosis expected with long-term HF feeding was not observed in mice treated with the apoB-100 ASO (Fig. 6C).

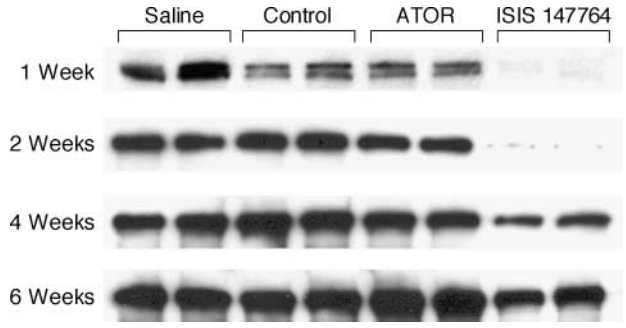

Fig. 5. Duration of apoB-100 ASO activity. HF-fed C57BL/6 mice (n = 6) were treated with saline, a control ASO (25 mg/kg twice weekly), atorvastatin (ATOR, 20 mg/kg daily), or ISIS 147764 (25 mg/kg twice weekly) for 6 weeks, and serum apoB-100 protein levels were analyzed by Western blotting at 1, 2, 4, and 6 weeks after cessation of treatment. Each lane contains 50 μg of protein isolated from an individual animal.

As described earlier, apoB-48, which is produced by the posttranscriptional editing of apoB-100 mRNA in murine hepatocytes and enterocytes (6, 12), is the essential apoB lipoprotein in chylomicrons and, hence, uniquely involved in dietary fat absorption. To evaluate the effect of apoB-100 suppression on dietary fat absorption, HF-fed C67BL/6 mice were treated with ISIS 147764 for 8 and 20 weeks (50 mg/kg and 25 mg/kg twice weekly, respectively), and [^{14}C]oleic acid absorption was determined using modifications of fecal isotope protocols described previously (47, 48). Consistent with our observations that intestinal apoB-48 protein levels were unaltered, (Fig. 2B) ASO treatment had no effect on [^{14}C]oleic acid absorption (Fig. 6D).

A number of experiments performed using transcriptional profiling (microarray analysis) of liver mRNA derived from apoB-100 ASO–treated mice as a function of both time and dose provided possible insight into why antisense inhibition of apoB-100 did not produce hepatic steatosis. These analyses suggested that a number of key genes involved in cholesterol and fatty acid biosynthesis, as well as transport, were downregulated as a result of the reduction in apoB-100. Examples of the affected genes include stearoyl CoA desaturase 1 (SCD1) (**Fig. 7A**), hepatic lipase, fatty acid binding protein 2, and the transcriptional activator sterol-responsive element binding protein-1 (SREBP-1) (Fig. 7B). Concomitant with these effects on SREBP-1c, fatty acid synthase protein levels were reduced as early as 1 week after ASO administration (Fig. 7C). These effects were specific to apoB-100 reduction and were not observed with control ASOs or atorvastatin in HF-fed mice (unpublished results).

Because so many genes involved in fundamental metabolic pathways were affected by apoB-100 ASO treatment, we considered AMP-activated protein kinase (AMPK) as a possible master regulatory factor. AMPK is a well-characterized enzyme thought to be a key sensor of cellular en-

TABLE 3. ApoB-100 mRNA and lipid levels after 6 weeks of ISIS 147764 treatment (50 mg/kg twice weekly) in mouse models of hyperlipidemia

Mouse Model/Treatment	RNA Reduction	Total Cholesterol	Total Cholesterol Reduction	LDL-C	LDL-C Reduction	HDL-C	HDL-C Reduction
	%	*mg/dl*	%	*mg/dl*	%	*mg/dl*	%
Chow-fed C57BL/6							
Saline		93.3 ± 14.0		17.2 ± 0.7		67.3 ± 15.1	
ISIS 147764	73	53.5 ± 7.5	43[a]	6.2 ± 1.5	64[a]	44.6 ± 4.3	34[a]
HF-fed C57BL/6							
Saline		240.0 ± 22.3		42.6 ± 8.3		192.3 ± 15.0	
ISIS 147764	87	109.0 ± 1.4	55[a]	5.3 ± 0.7	88[a]	99.2 ± 1.4	48[a]
Apoe-deficient							
Saline		1,767.0 ± 147.0		526.0 ± 87.2		51.4 ± 9.6	
ISIS 147764	75	1,494.7 ± 200.5	25[a]	399.9 ± 57.5	40[a]	61.1 ± 15.0	NC
Ldlr-deficient							
Saline		279.2 ± 17.0		134.7 ± 13.0		125.4 ± 8.0	
ISIS 147764	74	166.0 ± 12.0	40[a]	52.2 ± 7.0	62[a]	107.0 ± 8.0	14[a]

NC, no change ($P > 0.05$).
[a] $P < 0.01$.

ergy levels that affects multiple catabolic and anabolic metabolic pathways (51, 52). Upregulation of AMPK is thought to decrease cholesterol and fatty acid biosynthesis and increase fatty oxidation through several mechanisms (51). As shown in Fig. 7D, AMPK mRNA levels were doubled at the highest dose level after 6 weeks of treatment with the apoB-100 ASO. Consistent with these alterations in lipogenesis/energy balance, although apoB-100 ASO–treated mice do gain weight, they appear to be protected over time from the incremental increases in body mass typically seen in HF-fed control animals (Fig. 7E).

DISCUSSION

In this in vivo characterization of the mouse-specific apoB-100 ASO ISIS 147764 in several mouse models of hyperlipidemia, we demonstrated a dose- and time-dependent correlation between reduction of apoB-100 mRNA and protein in the liver and serum apoB-100, total cholesterol, and LDL-C levels. These findings were consistent with our hypothesis that inhibiting apoB-100 would have a beneficial effect on circulating lipid levels. This inhibition was highly specific and selective, as shown by the lack of effect on other proteins (ACAT and MTP) that participate in the assembly of apoB-containing particles. Nor did we observe effects on the HMG-CoA reductase gene, demonstrating a mechanism of cholesterol lowering that is clearly different from that produced by statins. Because 2′MOE ASOs do not interact with the cytochrome P450 system (53), a human apoB-100 antisense inhibitor could be ideal for use with statins and other second-tier lipid-lowering agents and with the many other drugs often used in hyperlipidemic diabetic patients.

Because both forms of apoB (apoB-100 and apoB-48) are products of a single gene and because mice edit apoB-100 to apoB-48 in both liver and intestine (6, 8, 12), we expected to see some effects on apoB-48 protein levels in our apoB-100 ASO–treated mice. Although reductions in apoB-100 mRNA and protein were observed in the intestine, apoB-48 protein levels were unchanged, suggesting, perhaps, that intestinal apoB-48 protein was translated more efficiently than that of apoB-100 or that apoB-48 protein was more stable than that of apoB-100 within enterocytes. The increased stability of apoB-48 in intestine has been observed by others in *apobec-1*-deficient mice (54). The mechanisms of these differential effects are under investigation in our laboratory. Nonetheless, these data were consistent with the lack of effects on absolute chylomicron particle number and dietary fat absorption in mice treated for varying amounts of time with ISIS 147764. Additionally, histological analysis of the small intestines from mice in several experiments revealed that the enterocytes of ASO-treated mice resembled those of saline-treated animals. The phenotype of apoB-100-treated mice was in sharp contrast to that of genetically altered mice that were unable to produce apoB in the intestine (55). Those animals could not form chylomicrons, were unable to absorb cholesterol or dietary fat from the intestine, and exhibited marked fat accumulation in the enterocytes of their duodenum and jejunum.

The 2′MOE ASOs, such as ISIS 147764, are more potent and nuclease-resistant and possess a longer target organ half-life than do first-generation phosphorothioate ASOs (34, 38, 39). Moreover, the pharmacokinetic and pharmacodynamic properties of these compounds have been extensively studied in our laboratories. For example, after subcutaneous injection in mice and other species, liver concentrations of 2′MOE ASOs reach a maximum after 48 h. The elimination half-life is 11–19 days, depending on the dose and organ. The rapid onset of action and prolonged duration of effect of ISIS 147764 are consistent with published data and correlate with hepatic levels of the antisense drug.

Treatment with ISIS 147764 caused no changes in liver transaminases or other metabolic parameters, indicating that the drug can be safely administered to mice. Although mice displayed modest, but not statistically significant, increases in liver TG stores up to 6 weeks after administration of the drug, similar results were seen in atorvastatin-

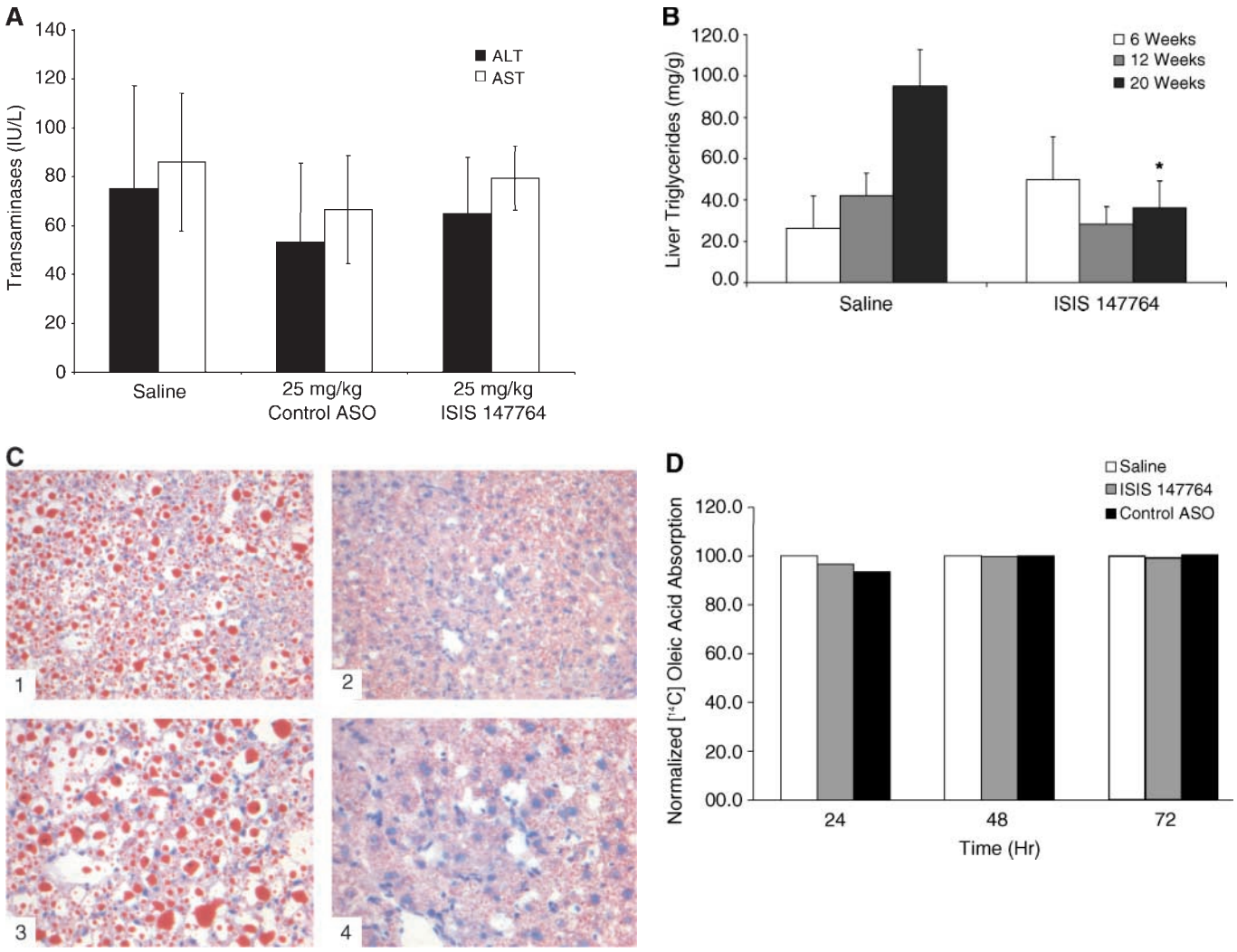

Fig. 6. Safety profile after apoB-100 reduction. A: Effects of apoB-100 ASO treatment on markers of hepatotoxicity in mice. Representative experiment (n = 6 per group) showing aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in HF-fed C57BL/6 mice after 20 weeks of treatment with a control ASO (25 mg/kg twice weekly) and ISIS 147764 (25 mg/kg twice weekly). B: Effect of ISIS 147764 on liver triglyceride stores in HF-fed C57BL/6 mice administered drug for 20 weeks (25 mg/kg twice weekly). Statistically significant differences were observed only at 20 weeks ($*P < 0.01$). C: Oil Red O–stained liver sections from saline control HF-fed mice (panels 1 and 3) and HF-fed mice administered ISIS 147764 (panels 2 and 4) for 20 weeks (25 mg/kg twice weekly). Magnification: panels 1 and 2, 10×; panels 3 and 4, 20×. D: Representative experiment in HF-fed C57BL/6 mice (3–6 per group; 50 mg/kg twice weekly) demonstrating that ISIS 147764 had no effect on dietary fat absorption. After 8 weeks of treatment, [^{14}C]oleic acid and [5,6-^{3}H]sitostanol, a nonabsorbable phytosterol standard, were administered by gavage to control and apoB-100 ASO–treated mice, and feces were collected for 72 h. [^{14}C]oleic acid absorption was determined by the fecal ratio methods previously described (47, 48). Similar results were also observed in HF-fed C57BL/6 mice administered drug (25 mg/kg twice weekly) for 20 weeks. Error bars represent SD.

treated mice, suggesting that the effects were not unique to ISIS 147764 or to inhibition of apoB-100. In mice treated with ISIS 147764 for 20 weeks, liver TG stores were lower than those of control mice. On the basis of our data, pharmacological inhibition of apoB-100 is clearly different from that of MTP, inasmuch as interference with that protein using small-molecule inhibitors usually resulted in increased hepatic and intestinal TG levels in multiple species in vivo (56).

Microarray analysis of liver mRNA suggested that the early, slight accumulation of liver TGs triggered compensatory mechanisms in HF-fed mice treated with the apoB-100 ASO. These effects included downregulation of lipid and fatty acid synthetic and transport proteins, including fatty acid binding protein 2, hepatic lipase, SREBP-1, SCD, and fatty acid synthase, the rate-limiting enzyme responsible for the biosynthesis of saturated long-chain fatty acids. This downregulation of the fatty acid synthetic pathway is consistent with metabolic adaptations previously described in mice with a targeted apoB-38.9 mutation (57). Additionally, we observed the upregulation of AMPK, a key regulator of cellular energy levels (51, 52). Activation of this enzyme is thought to decrease cholesterol and fatty acid biosynthesis and increase fatty oxidation through several

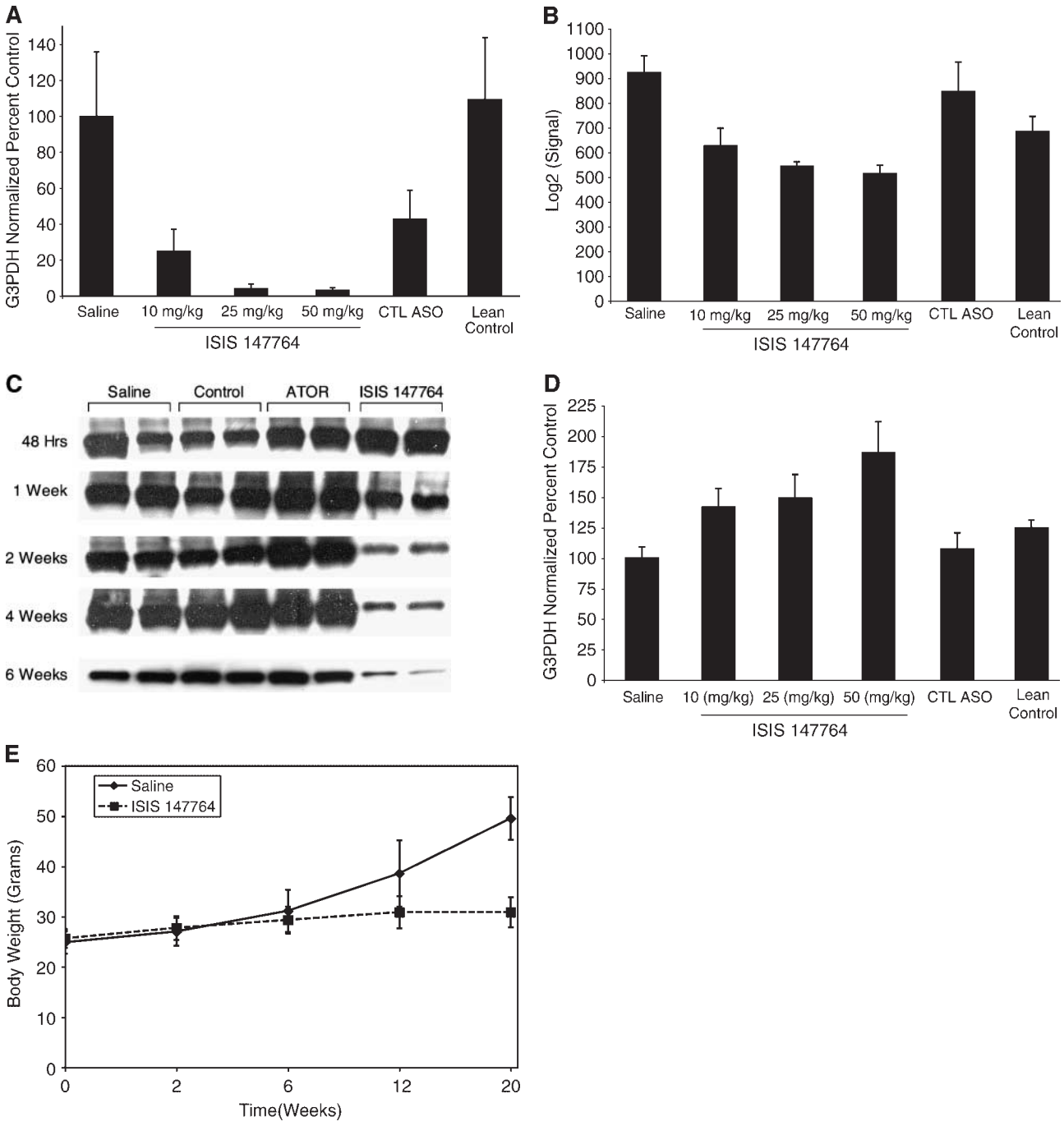

Fig. 7. Compensatory effects in HF-fed C57BL/6 mice after apoB-100 ASO treatment. A: Dose-responsive reduction in SCD-1 mRNA levels after 6 weeks ASO treatment (twice weekly administration) as determined by RT-PCR analysis. Data are reported as means ± SEM for each treatment group. B: Dose-responsive reduction in SREBP-1c mRNA levels after 6 weeks ASO treatment (twice weekly administration) as determined by microarray analysis. Data are reported as means ± SEM for each treatment group. C: Representative experiment in HF-fed C57BL/6 mice (n = 8 per group) demonstrating the time-dependent reduction in fatty acid synthase. Proteins from liver lysates from mice treated with 50 mg/kg drug for 48 h and twice weekly for 1, 2, 4, and 6 weeks were analyzed by Western blotting. Each lane contains 50 μg of protein isolated from an individual animal. D: Dose-responsive induction of AMPK-α2 mRNA levels after 6 weeks ASO treatment (twice weekly administration) as determined by RT-PCR analysis. Data are reported as means ± SEM for each treatment group. E: Body weights of apoB-100 ASO–treated mice as a function of time. HF-fed C57BL/6 mice were administered ISIS 147764 for 20 weeks (25 mg/kg twice weekly).

mechanisms, including inhibition of SREBP-1, acety-CoA carboxylase, and malonyl-CoA (51, 52). The resolution of hepatic steatosis via the upregulation of AMPK observed with ISIS 147764 treatment is consistent with metabolic effects (including stabilization of weight gain without fat malabsorption) observed in *ob/ob* mice treated with metformin, a drug used for the treatment of type 2 diabetes (58, 59).

Although the pharmacological activity of ISIS 147764 looks promising in mice, murine cholesterol homeostasis varies greatly from that of humans. Therefore, the true test of the antisense therapeutic approach to apoB-100 inhibition lies in results obtained from human clinical trials. Indeed, an antisense inhibitor to human apoB-100, ISIS 301012, is being evaluated in phase I clinical trials. Interim analysis of data from this study indicated that ISIS 301012 safely reduced total cholesterol, VLDL, LDL-C, and serum apoB-100 in a dose-responsive fashion in healthy human volunteers (60), with no evidence of gastrointestinal side effects (steatorrhea, abdominal pain, bloating) commonly observed with MTP inhibitors (56).

Human apoB-100 is an excellent target for pharmacological intervention with antisense therapeutics. Humans with heterozygous familial hypobetalipoproteinemia are hypolipidemic and, most importantly, have a low incidence of coronary artery disease (14, 23–26). Most heterozygous subjects do not have clinical symptoms, and the disorder is usually detected during routine cholesterol screening programs (14, 23–26). Typically, the plasma apoB and LDL-C levels are 25–50% of normal, and apoB secretion rates are approximately 30% of normal. In some cases, depending upon the disease subclass, TG levels are also significantly reduced, suggesting that reducing apoB-100 with an antisense drug could lower TGs as well as LDL-C.

The most recent data from the National Cholesterol Education Program Adult Treatment Panel III studies (61) and others (62) suggest that target LDL-C levels are frequently not attained and that cardiovascular mortality rates are still high, especially in high-risk patients. More-stringent LDL-C-lowering goals, coupled with a clearer understanding of the molecular basis of various types of dyslipidemias, especially those involving the atherogenic apoB-containing lipoproteins, underscore the need to develop new therapeutics. Our data suggest that an antisense inhibitor of apoB-100 could be such an agent.

In summary, pharmacologic inhibition of apoB-100 with ISIS 147764 directly and specifically reduced apoB-100 in multiple mouse models of hyperlipidemia without adverse side effects. Because of the selectivity, specificity, lack of interaction with the cytochrome P450 system, and novel mechanism of action, an antisense inhibitor of human apoB-100 could be a useful single or combination therapeutic agent. Such an agent could be broadly applicable for several clinical groups, including statin-intolerant patients (63, 64) and other high-risk patients (e.g., those with diabetes or metabolic syndrome) whose lipid levels are not adequately controlled by statins and existing combination agents.

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