SUPPLEMENTARY MATERIAL

Molecular mechanisms responsible for the different effects of apoE3 and apoE4 on plasma lipoprotein cholesterol levels

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Preparation of apoE AAV

The apoE3 cDNA in the plasmid pAAVNew.TBGhApoE3 [1] was checked by sequencing with pAAV forward primer and SV40- poly anti primer. This plasmid was then mutated to the apoE4 equivalent using a Quikchange site-directed mutagenesis kit (Stratagene/Agilent) and the C112R sense and reverse primers :

5'-GGA CAT GGA GGA CGT GCG TGG CCG CCT GGT GC-3' and

5'- GCA CCA GGC GGC CAC GCA CGT CCT CCA TGT CC-3' .

The integrity of the new plasmid (intact transgene, promoter, ITRs, poly A) was checked by restriction analysis with PVU11 and Sma1, and sequencing with pAAV forward primer and SV40-poly anti primer. The pAAV plasmids containing transgenes for the various apoE3 and apoE4 deletion mutants used in this study were engineered similarly from the above plasmids.

The plasmids containing the mutant apoE cDNA sequences were then used by the University of Pennsylvania Vector Core to create the apoE AAV. All AAV were manufactured using triple-plasmid transfection of adherent human 293 cells using Ad helper, trans-packaging, and AAV vector plasmids. The AAV were purified from harvested lysates using ion exchange column chromatography. The human apoE AAV, and variants, contain the apoE cDNA insert followed by a SV-40 poly (A) tail and are driven by the liver-specific human thyroglobulin promoter. The AAV were produced with a chimeric packing construct in which the AAV2 rep gene was fused with the cap gene of AAV serotype 8 [1]. The control vector contained the LacZ gene. The concentration (genome copies (gc)/ml) of an AAV8 preparation was measured using quantitative PCR and a set of primers and probes that detected the polyadenylation signals present in the vector genomes. Doses of administered AAV8 are expressed in units of gc.

Mouse procedures

ApoE-null mice (8-10 weeks old, C57/BL6 background; Jackson Laboratories) were fed a chow diet. One day before the treatment with apoE-AAV8, the mice were bled and their plasma cholesterol levels measured as described below. The mice were then placed into groups of five mice so that the average plasma cholesterol level per group was as similar as possible. The mice were injected intraperitoneally with the desired dose of either apoE-AAV8 or LacZ AAV. Blood was drawn from the retro-orbital plexus after 4h of day-time fasting and then spun (10,000 rpm, 7 min, 4°C) to remove cells. Total cholesterol, triglyceride and human apoE levels were measured on a Cobas Mira-L auto- analyzer (Roche Diagnostics Systems). The plasma cholesterol and triglyceride (TG) concentrations were measured using Infinity (Thermo Electron Corporation) and Wako assays, respectively. Human apoE concentrations in plasma were determined by immunoturbidometric assay run on a Cobas Mira-L auto-analyzer using reagents from Kamiya Biomedical Company (Seattle, WA). A pool of 120µl of plasma from five mice was diluted two-fold with FPLC column buffer (1mM EDTA and 154 mM NaCl, pH 8.0). 200µl of diluted sample were run on a Superose 6 column at a flow rate of 0.4ml/min, and 0.5ml fractions were collected. The cholesterol concentration of each fraction was determined using an enzymatic micro-plate reader assay with reagents from Wako.

Liver mRNA levels

Total RNA was isolated from a sample of pooled mouse liver obtained from each test group of mice using an EZ1 RNA mini kit (Qiagen) according to the manufacturer's instructions. 0.1-1.0 µg of total RNA was reverse transcribed using iScript (Bio-Rad). Quantitative real-time PCR analysis was performed in an Applied Biosystems 7500 system using a primer for human apoE (hs00171168_m1) from Applied Biosciences. The relative quantities of mRNA were

determined using the method of comparative changes in threshold cycle and data are expressed as fold change (± SD) relative to 18s rRNA.

ApoE preparation

Human apoE variants were expressed in E. coli as thioredoxin fusion proteins and isolated and purified as described previously [2-3]. The apoE preparations were at least 95% pure as assessed by SDS-PAGE. In all experiments, the apoE sample was freshly dialyzed from a 6M GdnHCl and 10mM DTT solution into a buffer solution before use. ApoE concentrations were determined either by a measurement of the absorbance at 280nm or by the Lowry procedure [4].

LDL receptor binding assay

The abilities of the apoE C-terminal deletion variants to bind to the LDLR were compared using a competitive binding assay with ^{125}I-labeled LDL, as described previously [5]. The recombinant apoE variants were complexed with dimyristoyl phosphatidylcholine (DMPC) and the concentration of apoE/DMPC complex required to give 50% inhibition of the binding of ^{125}I-labeled LDL to human skin fibroblasts at 4° C was determined.

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Supplementary Table I

LDLR binding activities of apoE C-terminal variants

ApoE variant	relative affinity for LDLR (%) (vs. E3/4 (1-191)*
apoE3 (1-191)	100
apoE4 (1-191)	100
apoE3 (1-260)	86
apoE4 (1-260)	86
apoE3 (1-272)	120
apoE4 (1-272)	70
apoE3 (Δ192-260)	32
apoE4 (Δ192-260)	75
apoE3 (Δ261-272)	80
apoE4 (Δ261-272)	29

*Calculated from the concentration (IC_{50}) of apoE in discoidal complexes with DMPC required to displace 50% of the ^{125}I-labeled LDL bound to LDLR on human fibroblasts (see Materials and Methods).The relative affinities for the apoE variants are expressed as the ratio IC_{50} (apoE (1-191)) / IC_{50} (variant); the values are the average of at least two measurements that were similar. The IC_{50} for the wild-type apoE3 (1-191) and apoE4 (1-191) is 6 ± 3 ng / ml (0.2nM).

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure I

Expression of human apoE mRNA in the livers of apoE-null mice as a function of AAV8 dose. Total RNA was extracted from mouse livers two weeks post AAV8 injection and analyzed by real time PCR as described in Methods. A. ApoE3. B. ApoE3 (1-191). C. Relative mRNA levels for apoE3, apoE4 and their (1-272) truncation variants after administration of 3E10 gc of AAV8.

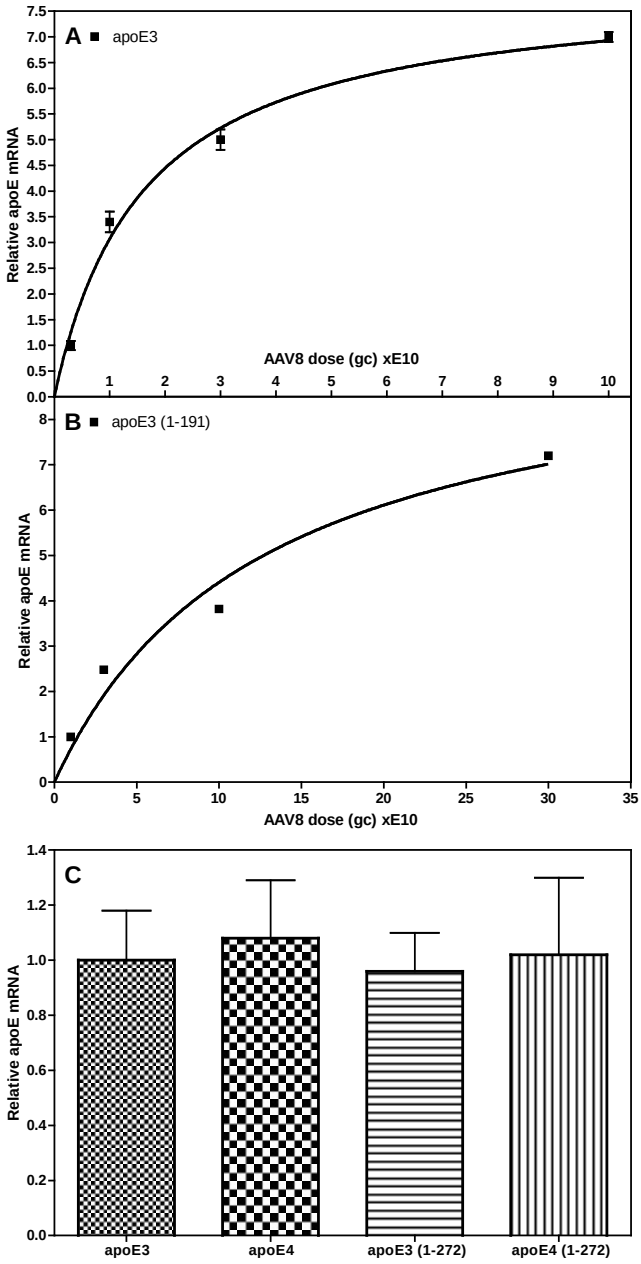
Supplementary Figure II

Influence of apoE3 and apoE4 expression level on plasma VLDL-cholesterol (VLDL-C) and HDL-cholesterol (HDL-C) levels in apoE-null mice. Two weeks after injection of the indicated AAV8 doses, pooled plasma from groups of five mice was analyzed by FPLC to separate the VLDL and HDL fractions as described in Methods and Fig. 2. A. VLDL-C; the relative value of 1.0 for either untreated animals or mice treated with AAV8-null represents an actual VLDL-C level of 167 ± 34 mg/dL (mean ± SEM, n = 4). B. HDL-C, the relative value of 1.0 for untreated animals represents an actual HDL-C level of 26 ± 5 mg/dL (mean ± SEM, n =4). C. The ratio VLDL-C/HDL-C; the value for untreated mice is 6.4 ± 1.8 (mean ± SEM, n=4).

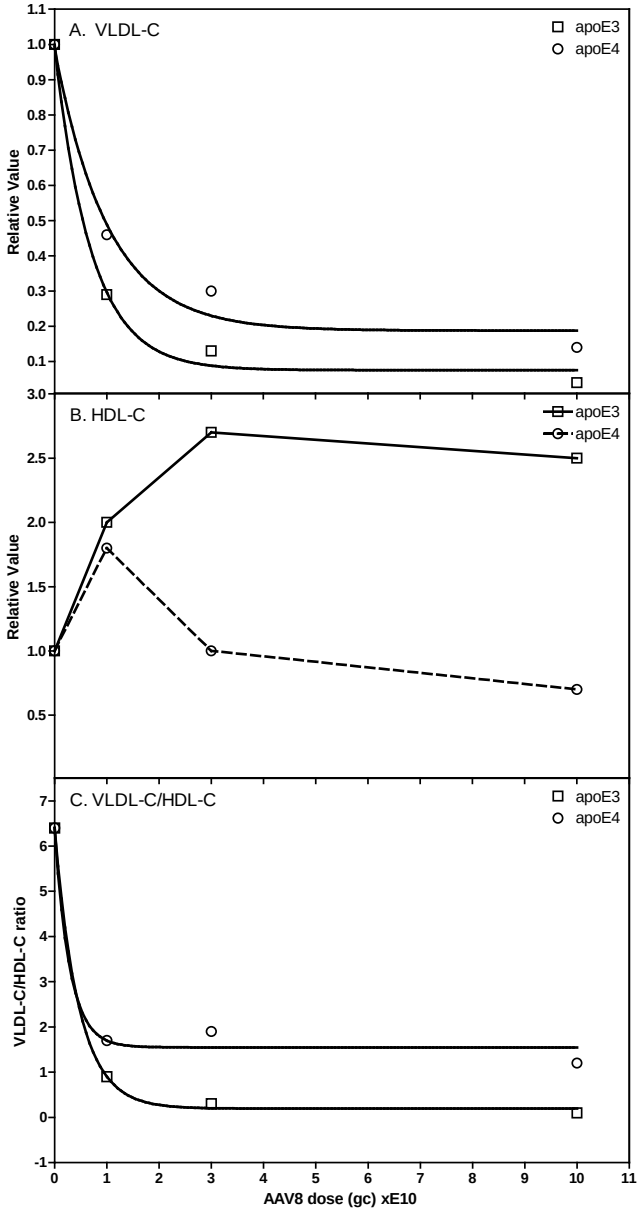
Supplementary Figure III

Influence of removal of the C-terminal domain on the abilities of apoE3 and apoE4 to reduce the plasma cholesterol levels in apoE-null mice. The experiments were performed as described in the legend of Fig. 1. The mice were treated with 1E11 gc of the AAV8 for apoE3(1-299) and apoE4(1-299), and with 1E12 gc of the AAV8 for apoE3 (1-191) and apoE4 (1-191). The reductions in plasma cholesterol level are reported ± SEM, n=4 for apoE3 and apoE4, and n=9 for apoE3 (1-191) and apoE4 (1-191).

Supplementary Figure I



Supplementary Figure II



Supplementary Figure III