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Mouse and Other Rodent Models of C to U RNA Editing

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Abstract

Substitutional RNA editing represents an important posttranscriptional enzymatic pathway for increasing genetic plasticity by permitting production of different translation products from a single genomically encoded template. One of the best-characterized examples in mammals is C to U deamination of the nuclear apolipoprotein B (apoB) mRNA. ApoB mRNA undergoes a single, site-specific cytidine deamination event yielding an edited transcript that results in tissue-specific translation of two distinct isoforms, referred to as apoB100 and apoB48. Tissue- and site-specific cytidine deamination of apoB mRNA is mediated by an incompletely characterized holoenzyme containing a minimal core complex consisting of an RNA-specific cytidine deaminase, Apobec-1 and a requisite cofactor, apobec-1 complementation factor (ACF). The underlying biochemical and genetic mechanisms regulating tissue-specific apoB mRNA editing have been accelerated through development and characterization of physiological rodent models as well as knockout and transgenic animal strains.

Keywords

Lipid metabolism; RNA editing; Apobec-1; Hepatocytes; Hormonal regulation; Diet; Primer extension; Subcellular distribution

1. Introduction

Substitutional cytidine to uridine (C to U) RNA editing is an enzymatic process that alters a genomically encoded sequence through site-specific deamination of cytidine residues in nuclear RNA transcripts. C to U RNA editing of the apolipoprotein B (apoB) mRNA has been extensively characterized and involves enzymatic deamination of a C to U base in the nuclear apoB mRNA that converts a glutamine codon (CAA) into a stop codon (UAA), leading to a premature interruption of translation and synthesis of a truncated form of apolipoprotein B, defined as apoB48. The unedited version of apoB mRNA encodes a full-length protein, apoB100 (1, 2).

Posttranscriptional C to U deamination of apoB mRNA is regulated in a species and tissue-specific manner. ApoB mRNA editing, and consequently apoB48 synthesis, occurs in enterocytes of the small intestine of all mammals. For yet incompletely understood reasons some species, such as mice and rats, also exhibit apoB RNA editing in the liver (2) and secrete both apoB100 and apoB48. Human liver, by contrast, synthesizes and secretes only apoB100 (3).

Introduction of a translational stop codon in the edited apoB transcript leads to translation of a carboxy-terminal truncated protein (apoB48) that lacks key domains present in the full-length (apoB100) protein. ApoB100-containing lipoproteins are recognized by a

ubiquitously expressed low-density lipoprotein receptor (LDLR) through interactions with a domain localized in the C terminus of apoB100. Because they lack the LDLR binding domain present in apoB100, apoB48-containing lipoproteins are cleared through a distinctive receptor expressed predominantly in the liver (4). Thus, intestinal apoB48-containing lipoproteins represent an evolutionary adaptation to efficiently deliver dietary triglycerides and fat-soluble vitamins to the liver. The fine regulation of plasma cholesterol homeostasis by contrast is regulated via LDLR-mediated uptake of apoB100-containing lipoproteins.

The enzymatic deamination of apoB mRNA is mediated by a holoenzyme whose minimal core is composed of an RNA-specific cytidine deaminase (Apobec-1) (5) and an essential RNA-binding protein cofactor, apobec-1 complementation factor (ACF) (6, 7). Apobec-1 expression is limited to the stomach, small intestine, and colon in humans, but is not expressed in human liver (3). By contrast, ACF shows a broad expression pattern both in rodents and humans, with greatest abundance in liver, small intestine, and kidney (6, 8).

Both apobec-1 and ACF mRNA and protein expression undergo developmentally regulated increase in the fetal and neonatal small intestine, presumably to accommodate some of the developmental changes taking place in lipid metabolism in which the relative production of either apoB48 or apoB100 would confer a metabolic advantage (9, 10). This prediction is consistent with the numerous studies demonstrating tissue-specific regulation of C to U RNA editing in the setting of metabolic adaptations involved in lipid metabolism. This review addresses the approaches and methods to studying developmental, nutritional, hormonal, and genetic modulation of C to U RNA editing in murine and other rodent models.

2. Materials

2.1. Animals

2.1.1. Congenitally Hypothyroid Mice (Pax8$^{-/-}$)—*Pax8*$^{-/-}$ mice are generated by homologous recombination (11). *Pax8*$^{-/-}$ mice manifest a phenotype of early growth delay and death after weaning. These animals are characterized by the absence of thyroxine-producing follicular cells in the thyroid, which results in the inability to autonomously produce thyroid hormone at weaning (12). *Pax8*$^{-/-}$ mice exhibit decreased hepatic apoB RNA editing associated with reduced mRNA and protein levels of ACF (13).

2.1.2. Zucker Rats—Adult fatty Zucker rats (*fa/fa*) obtained from Harlan Industries (Indianapolis, IN) carry a missense (A to C) mutation in the leptin receptor (OB-receptor) (14). *fa/fa* rats are characterized by obesity, hypercholesterolemia, hyperlipidemia, hyperglycemia, and hyperinsulinemia. This rat model of acquired insulin resistance recapitulates the observations reported in isolated rat hepatocytes incubated with insulin (15). Specifically, Zucker rats demonstrate elevated hepatic apoB mRNA editing in conjunction with increased Apobec-1 mRNA abundance (16). Insulin may itself modulate ACF expression since incubation of rat primary hepatocytes with 10 nM insulin produced a 1.5-fold increase of apoB editing activity that correlated with increased ACF nuclear localization (17). Further studies demonstrated that insulin-dependent ACF nuclear import is related to an increase in ACF phosphorylation (18). These findings suggest a new regulatory mechanism controlling apoB RNA editing.

2.1.3. Ob/Ob Mice—Mutant *ob/ob* mice in a C57BL/6J background are obtained from Jackson Laboratory (Bar Harbor, ME). These mice gain weight rapidly, exhibit hyperphagia, hyperglycemia, glucose intolerance, and insulin resistance with elevated plasma insulin

levels. *In vivo* studies report an increased ratio of apoB100/apoB48 without changes in apobec-1 expression (19).

2.1.4. db/db Mice—The *db* gene encodes for the leptin receptor, Ob-R. The spontaneous homozygous *db* mutation results in the truncation of the Ob-Rb spliced form leading to loss of signaling activity (20). The *db/db* mice in a C57BL6/J background are obtained from Jackson Laboratory (Bar Harbor, ME). The mutant animals exhibit severe obesity and hypoglycemia coupled with hyperinsulinemia (21). *db/db* mice, like *ob/ob* mice, exhibit increased serum apoB100/apoB48 ratios whose mechanism is incompletely understood (22).

2.1.5. Apobec-1$^{-/-}$ Mice—Three independent lines of *apobec-1*$^{-/-}$ lines were generated by homologous recombination (23, 24). Homozygous mice appear healthy and fertile with no alterations in serum cholesterol or triglyceride concentration. *Apobec-1*$^{-/-}$ mice lack apobec-1 protein, fail to mediate C-to-U RNA editing in any tissue, and consequently synthesize and secrete exclusively apoB100 (23, 24).

2.1.6. Liver-Specific Apobec-1 Transgenic Mice—A full-length rabbit apobec-1 cDNA was cloned downstream of a liver-specific promoter (pLiv11) (25). Four lines of apobec-1 transgenic animal were generated ranging from 3 to 17 copies of the gene. Transgenic overexpression of rabbit apobec-1 results in loss of specificity for the canonical cytidine 6666 and C to U editing at multiple other cytidines in apoB RNA, a phenomenon known as hyperediting (26). In addition other RNAs undergo C to U RNA editing including the tumor suppressor gene NAT1 which undergoes multiple C to U modifications resulting in reduced NAT1 expression. This loss-of-function of NAT1 leads to hepatic dysplasia and hepatocellular carcinoma (25).

2.1.7. Tetracycline-Dependent Liver-Specific Conditional Apobec-1 Transgenic Mice—Rabbit apobec-1 was cloned downstream of a tetracycline-mediated transactivator (tTA) response element and a line of transgenic mice generated and crossed into a liver-activating protein (LAP)-tTA transgenic mice which express tTA specifically in hepatocytes (27). Thus, rabbit apobec-1 can then be turned on and off by removing from or adding tetracycline to the drinking water (28) (see Note 1).

2.2. Hormonal Regulation of C to U RNA Editing

2.2.1. Thyroid Hormone—3,5,3′-triiodo-L-thyronine (Sigma) (T3) stock solutions are prepared in saline containing 10 mg/mL bovine serum albumin adjusted to pH 11. Aliquots (20 μL) are kept frozen at −20°C.

2.2.2. Insulin—Bovine insulin (Sigma, St Louis, MO) is added to the feeding medium of primary hepatocytes in a concentration range of 0–67 nM (400 ng/mL).

2.2.3. Estrogen—17α-Ethinyl estradiol and 17β-estradiol stock solutions (Sigma, St Louis, MO) are prepared in propylene glycol (1 mg/mL).

Rodent show diet is from ICN Biochemicals, Cleveland, OH.

[1]Two-to-four-month-old, tetracycline-dependent, liver-specific conditional apobec-1 transgenic mice are administered 10 mg/mL doxycycline hydrochloride in the drinking water. The expression of rabbit apobec-1 is suppressed within 2 days and followed by suppression of apoB RNA editing activity and apoB48 synthesis within 4 days of treatment.

2.3. Dietary Modulation of C to U RNA Editing

2.3.1. Diet-Induced Hypothyroidism—Propylthiouracil (2-thio-4-hydroxy-6-*n*-propylpyrimidine) (Sigma, St Louis, MO) is dissolved in H_2O at a concentration of 1 mg/mL and aliquots are kept at −20°C.

2.3.2 Ethanol—Control liquid diet: 180 Kcal/L protein; 350 Kcal/L fat; 470 Kcal/L carbohydrate.

Ethanol liquid diet: 180 Kcal/L protein; 350 Kcal/L fat; 115 Kcal/L carbohydrate; 355 Kcal/L ethanol (6.7% v/v) (Bio-Serv Inc., Frenchtown, NJ).

2.3.3. High Carbohydrate—A high carbohydrate (58.45% sucrose) fat-free diet (ICN Nutritional Biochemicals) is used to augment hepatic lipogenesis and to stimulate the accumulation of fat in the liver.

2.3.4. Fasting–Refeeding Protocol—Rats (Sprague–Dawley, Charles River Wilmongton, MA).

Purina rodent chow diet (Purina Mills).

2.4. Buffers and Media

2.4.1. Primary Hepatocyte Isolation Culture Medium

1. Male Sprague–Dawley rats or C57BL/6 mice (8–12 weeks old).
2. Anesthesia: 87 mg/kg ketamine HCl (Fortdodge Animal Health), 13.4 mg/kg xylazine (Lloyd Laboratories) (intraperitoneal administration).
3. “All purpose sponge” (2 × 2 in.) (Kendall).
4. Sterile catheter (22G × 1 in.) (Terumo).
5. [$4^{1/2''}$ straight] Iris scissors (Miltex Instruments).
6. 10× liver perfusion buffer: 1.2 M NaCl; 60 mM KCl; 22 mM NaH_2PO_4; 40 mM Na_2HPO_4; 5.5 M glucose; 25 mM $MgSO_4$. Working perfusion solution is prepared by diluting 10× perfusion buffer with $NaHCO_3$ (1.7% w/v final concentration) and 0.2 mM EGTA; pH 7.4. Filter the solution through 0.22-μm filter (Techno Plastic Product) and add 1 mL of 100× penicillin/streptomycin (Mediatech Cellgro).
7. Liver Digest Medium supplemented with Collagenase-Dispase (Invitrogen, Gibco).
8. Pump settings (Minipuls 2 peristaltic pump, Gilson): 3–4 mL/min. Wash tubing with 70% ethanol for 5 min followed by 5 min of 1× PBS and 5 min of perfusion working solution.
9. 100-μm filter (BD Falcon).
10. Collagen I-coated dish (35 mm) (BD Biosciences).
11. Wash medium: William’s medium E (Invitrogen) supplemented with 0.64 mM L-ornithine, 38 mM sodium bicarbonate, 10 mM HEPES, 10 mM dextrose, 100 mg/mL streptomycin and 100 IU/mL penicillin G, 50 nM triiodothyronine, 1 mg/mL bovine serum albumin, 5 mg/mL linoleic acid, 0.1 mM $CuSO_4$, 3 nM $NaSeO_3$, 50 pM $ZnSO_4$.
12. L15 Medium (Invitrogen).

13. Culture medium: wash medium supplemented with 5% FBS and 1× penicillin/streptomycin.

2.4.2. Protein Extraction

1. Tissue lysis buffer: 20 mM Tris pH 8.9; 1 mM sodium vanadate; 150 mM NaCl; 2 mM EDTA; 100 mM sodium fluoride; 5% glycerol; 50 mM β-glycerophosphate and protease inhibitors (Roche Maxi or Mini (for 50 and 10 mL lysis buffer, respectively)).
2. 10× detergent buffer: 10% Triton X-100; 1% SDS in lysis buffer.
3. Dignam A: 10 mM HEPES pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl.
4. Dignam B: 300 mM HEPES pH 7.9; 1.4 M KCl; 30 mM $MgCl_2$.
5. Dignam D: 20 mM HEPES pH 7.9; 0.1 M KCl; 20% glycerol.
6. Protein concentration is assessed using Bio-Rad Protein assay (BIO-RAD).

2.4.3. RNA Analysis

1. RNA extraction: TRIzol (Invitrogen) following manufacturer's protocol.
2. DNAse treament: DNA-free kit (Ambion).
3. First strand synthesis: High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The reactions are performed in a 2720 Thermo Cycler (Applied Biosystems).
4. Quantitative PCR: SyBR GreenER qPCR SuperMix (Invitrogen). Amplifications are performed with an ABI Prism 7000 instrument (Applied Biosystems).

2.4.4. Poisoned Primer Extension

1. apoB amplification: Taq DNA polymerase (Invitrogen).
2. PCR purification: QIAquick PCR purification kit (QIAGEN).
3. Primer extension: 10× annealing buffer: 400 mM Tris–HCl pH 7.5.
4. 200 mM $MgCl_2$; 500 mM NaCl.
5. Extension buffer: 10 mM DTT; 25 mM d(ATP/dCTP/dTTP); 0.8 mM ddGTP; 1.5U T7 DNA polymerase (USB).
6. PhosphorImager/ImageQuant (GE Healthcare).

2.4.5. 10× In Vitro Conversion Buffer—100 mM HEPES, pH 7.9, 1 M KCl, 1 mM EDTA, 2.5 mM DTT, 10% glycerol, 250 ngtRNA and 20 units RNAsin Rinonuclease Inhibitor.

2.4.6. Cellular Fractionation Buffers

1. Hypotonic buffer A: 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and protease inhibitors.
2. Buffer B: Buffer A adjusted to 300 mM HEPES, 1.4 mM KCl, 30 mM $MgCl_2$.
3. Buffer C: 20 mM HEPES, 500 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and protease inhibitors.

4. Dialysis buffer: 20 mM HEPES (PH 7.9), 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 20% glycerol, 0.5 mM benzamidine, 0.5 mM PMSF.

2.4.7. Immunohistochemistry Solutions

1. Fixative solution: 10% formalin (Sigma).
2. Rehydration solution: citrate buffer (0.01 M pH 6).
3. Blocking solution: 5% normal goat serum/3% bovine serum albumin.

Biotinylated goat anti-rabbit IgG (Vectastain Elite, Vector Labs, Burlingame, CA).

Avidin-conjugated horseradish peroxidase (Vector Labs).

2.4.8. Fast Protein Liquid *Chromatography*—Chromatography buffer: 1 mM EDTA, 150 mM NaCl, and 10 mM sodium phosphate, pH 7.4.

2.4.9. Analysis of Serum Lipids and Lipoproteins Content—L-type TG H, L type TG-M-ColorA and L type TG-M – ColorB kits (Wako Diagnostics).

Cholesterol E kit (Wako Diagnostics).

2.5. Primers

2.5.1. Quantitative PCR Primers—Murine apobec-1: Forward 5′-ACCACACGGATCAGCGGAAA-3′ Reverse 5′-TCATGATCTGGATAGTCACACCG-3′; murine apoB: Forward 5′-CACTGCCGTGGCCAAAA-3′ Reverse 5′-GCTAGAGAGTTGGTCTGAAAAATCCT-3′; murine ACF: Forward 5′-GATGAAAAAAGTCACAGAAGGAGTTG-3’ Reverse 5′-CAAATCCCCGGTTTTTGGT-3′; murine GAPDH Forward 5′-TGTGTCCGTCGTGGATCTGA-3′; Reverse 5′-CCTGCTTCACCACCTTCTTGA-3′.

2.5.2. Murine apoB mRNA *Amplification*—Forward: 5′-ATCTGACTGGGAGAGACAAGTAGC-3′; Reverse: 5′-ACGGATATGATACTGTTCATCAAGAA-3′.

2.5.3. Poisoned Primer Extension (Antisense Primer)—5′-CCTGTGCATCATAATTATCTCTAATATACTGATCA-3′.

3. Methods

3.1. Hormonal Regulation of C to U RNA Editing

3.1.1. Thyroid Hormone—Chemically induced hypothyroid animals (mice or rats) are injected intraperitoneally with 3,5,3′-triiodo-L-thyronine (T3) at a physiological dose (0.5 mg/100 g of body weight) to normalize thyroid hormone levels or at pharmacological doses (50 mg/100 g of body weight) for 7 days to induce hyperthyroidism (29). For particular studies, when a time course of apoB mRNA editing is required, animals may undergo a single intravenous bolus of T3 at 100 mg/100 g of body weight a minimum of 3 h before determining apoB100 synthesis (29).

Congenitally hypothyroid (*Pax8*$^{-/-}$) mice (2-week-old) are injected intraperitoneally with a combination of T4 (2.5 mg/kg) and T3 (0.25 mg/kg) in 100 μL PBS daily for 2–4 days (12). This combination of TH has been demonstrated to promote hyperthyroidism in mice (30).

3.1.2. Insulin—Bovine insulin is added to the feeding medium of primary hepatocytes in a concentration range of 0–67 nM (400 ng/mL). The culture medium, with or without insulin, is replaced daily for a period of time ranging from 6 h to 5 days.

3.1.3. Estrogen—17α-Ethinyl estradiol solutions are administered subcutaneously (3–5 mg/kg/day) to 200–280 g Sprague–Dawley male rats for 5 consecutive days between 8:30 and 10:30 a.m. The animals have access to standard rodent chow diet. Twelve hours before sacrifice, the animals are fasted to reduce fluctuations of plasma cholesterol. Male mice are administered 17β-estradiol at a dose of 3 mg/kg body weight/day for 5 consecutive days. The animals have access to standard rodent chow. The animals are fasted overnight and sacrificed on the seventh day (31).

3.2. Dietary Modulation of C to U RNA Editing

3.2.1. Diet-Induced Hypothyroidy—The animals (mice or rats) are fed with chow diet supplemented with 0.1% (w/w) propylthiouracil (2-thio-4-hydroxy-6-*n*-propylpyrimidine) for 21–28 days in order to induce hypothyroidism followed by assessment of plasma thyroid hormone levels (T4, T3). Induction of hypothyroidism results in abnormal lipid metabolism and also changes C to U RNA editing in the liver of experimental animals.

3.2.2. Ethanol—Male Wistar rats (200–250 g) are fed regular rat chow for a minimum of 10 days before starting the diet regimen and are then acclimated for another 7 days to receiving a liquid diet as their unique feeding source. The amount of ethanol is progressively increased as followed: first 3 days: 1/3 ethanol 2/3 control diet; fourth to seventh day: 2/3 ethanol 1/3 control diet; eighth day on: pure ethanol diet. The diet is provided for a period of 15, 20, 30 up to 40 days. Alcohol feeding stimulates hepatic lipogenesis and results in hepatic fat accumulation (32).

3.2.3. High Carbohydrate—A high carbohydrate (58.45% sucrose) fat free diet is used to augment hepatic lipogenesis and to stimulate the accumulation of fat in the liver. This diet is given ad libitum for a minimum of 7 days prior to study.

3.2.4. Fasting–Refeeding Protocol—Rats are acclimated to a 12 h light/12 h dark cycle and fed a Purina rodent chow diet for 10 days and then fasted for 48 h. Selected groups are studied at the end of the 48 h fasting period. Other groups of rats are refed a high sucrose-fat free diet (high carbohydrate diet described above) for 24 or 48 h. This regimen induces dramatic hepatic lipid accumulation in the refed animals and results in alterations in C to U RNA editing of apoB RNA (33). All animals are allowed free access to water.

3.3. Isolation of Mouse Primary Hepatocytes

Upon anesthesia, a midline incision is made, the small intestine is gently deflected to the left side of the mouse, and kept in position with a saline soaked “all purpose sponge” exposing the portal vein. A single use sterile catheter connected to the perfusion solution prewarmed at 37°C is introduced into the vena cava and the portal vein is cut to allow the perfusate to flow out of the liver. During the entire procedure, the perfusion is performed at a flow rate of 3–4 mL/min and all perfusion solutions are maintained at 37°C. The liver is perfused with 35 mL perfusion working solution and massaged with Q-Tips dipped in saline to promote buffer flow until liver becomes pale (1–3 min). The liver is then perfused with Liver Digest Medium for 10 min followed by 3 min of perfusion working solution to wash out digest medium (see Note 2). Before removing the liver, the gallbladder is removed by cutting the fibrous band connecting the gallbladder to the diaphragm and the liver using a $4^{1/2''}$ straight Iris scissors. The gallbladder is then disconnected from the bile duct by incision of the junction between the gallbladder and the bile duct, allowing the gallbladder to be removed

intact. The digested liver is then gently teased apart with a cell scraper in 20 mL of L15 Medium containing 5% FBS and antibiotics. The homogenate is filtered through a 100 μm filter into a 50-mL centrifuge tube. Cells are collected by centrifugation at 50 × *g* for 5 min at 4°C. The pelleted cells are washed twice with 40 mL hepatocyte wash medium and counted using a hematocytometer. Viability should be ~80%. Hepatocytes are then resuspended in culture medium and seeded at 6×10^5 cells/mL in a 35 mm collagen I coated dish. Hepatocytes are incubated at 37°C in a humidified atmosphere of 5% CO_2 for a period up to 5 days.

3.4. Protein and RNA Extraction

All liver tissues are minced and flash frozen in liquid nitrogen and stored at −80°C. Either whole small intestine or separate fractions (proximal, middle, and distal intestine) are minced and flash frozen similarly to the liver tissues. However, in some cases, it may be advantageous to scrape the mucosa in order to enrich for enterocytes rather than smooth muscle and supporting submucosal tissues. In that case, the content of the small intestine is flushed with 5–10 mL of ice-cold saline and the intestine is opened along the antimesenteric border to expose the mucosa that is then scraped on glass microscope slides. The scraped mucosa is then flash frozen in liquid nitrogen and stored at −80°C. Proteins are extracted from 100 mg tissue in 1 mL of lysis buffer. The homogenates are adjusted with 0.11 volume of 10× detergent buffer and incubated on ice for 10 min (see Note 3). Extracts are cleared from cellular debris by centrifugation at 16,000 × *g* for 10 min. Protein concentrations in tissue homogenates are evaluated before being resolved on SDS-PAGE. RNAs are extracted by homogenization in TRIzol following the manufacturer's instructions and kept at −80°C (see Note 4).

3.5. Analysis of ACF and Apobec-1 mRNA Expression

Extracted RNAs (10 μg) are treated with two units of DNAse for 30 min at 37°C in 40 μL final volume to remove genomic DNA. One microgram of DNAse-treated RNA is added to 20 μL mixture containing Reverse Transcription buffer, 4 mM dNTP, 1× random primer and MultiScribe Reverse transcriptase, all provided in the High Capacity cDNA Reverse transcription kit. The reverse transcription reaction is performed as follows: step 1: 10 min at 25°C; step 2: 120 min at 37°C; step 3: 5 s at 85°C. The cDNAs are then kept at −80°C or used immediately for PCR amplification. Apobec-1 and ACF mRNA abundance is determined by quantitative PCR. Real-time quantitative PCRs are performed using SYBR Green Master mix following manufacturer's instructions. RNA abundance is determined by normalization against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level in each sample.

3.6. Evaluation of apoB C-to-U RNA Editing

3.6.1. In Vivo apoB RNA Editing by Poisoned Primer Extension—After RNA extraction and reverse transcription as described above, a 275-bp sequence of the murine apoB RNA (nt 6,512–6,786) flanking the editing site is amplified. PCR products are purified from the oligonucleotides using PCR purification kit and 20 ng of purified PCR product is annealed to 100 pg of 5′end-labeled antisense primer located 39 nucleotides downstream of the edited site (see Note 5). The annealing reaction is performed at 70°C for 10 min. Primer

[2]Time of digestion is critical for the quality of the hepatocyte preparation Consequently, it is recommended to adjust the duration of digestion to the size of the liver. Small livers will be sufficiently digested in 6–7 min. Check the texture of the liver: the surface should evolve from a smooth and organized liver to a liver with a dissociated appearance in which cellular components can be distinguished.
[3]The detergent is added to the crude protein extract after homogenization to avoid too much foaming. Add detergent and vortex before the incubation on ice.
[4]Use 1 mL of TRIzol per 100 mg of tissue.

extension is initiated by addition of 1.5 units of T7 RNA polymerase. The reaction is performed at 42°C for 3 min (3). The products are precipitated and resolved on an 8% polyacrylamide-7M urea gel (40 cm long) (see Note 6). The proportion of apoB48 and apoB100 is determined using Phosphor Imager/Image Quant).

3.6.2. Determination of apoB RNA Editing in S-100 Extracts—Tissues from liver, small intestine, and kidney are minced, homogenized sequentially in a Dounce homogenizer using a type A followed by a type B pestle, in Dignam A buffer, and centrifuged at 750 × *g* in an SS34 rotor (Sorvall). The supernatants are adjusted with 0.11 volume of Dignam B buffer and centrifuged at 100,000 × *g* in an SW55 Ti rotor (Beckman). After overnight dialysis at 4°C against 1,000–2,000 volumes of Dignam D buffer, the S100 extracts are stored at −80°C (3).

Exactly 10 fmol of *in vitro* transcribed 361 nt apoB RNA (nucleotides 6,512–6,872) is incubated with determined amounts of S100 extract (5–10 mg) for 3 h at 30°C in 1× *in vitro* conversion buffer supplemented with 10% glycerol, 250 ng tRNA, and 20 units RNAsin in a final volume of 20 mL. The reaction is stopped upon incubation with 200 ng/mL proteinase K. The RNA is extracted and subjected to RT-PCR followed by poisoned primer extension as described above. The proportion of edited and unedited apoB RNA is determined using PhosphorImager/ImageQuant.

3.7. Cellular Distribution of apobec-1 and ACF

3.7.1. Cellular Fractionation—Hepatic tissues (~100 mg) are minced in hypotonic buffer A, homogenized in Buffer B with a Dounce type B pestle.

The homogenate is centrifuged at 1,000 × *g* for 5 min in an SS34 rotor. The supernatant is centrifuged further at 100,000 × *g* yielding cytoplasmic extracts. The cytoplasmic extracts are dialyzed overnight at 4°C against Dignam D (1 or 2 L) and stored at −80°C. Pellets from the low-speed centrifugation are homogenized in Buffer C with a Dounce pestle B and centrifuged at 26,000 × *g* for 10 min in an SS34 rotor resulting in nuclear extracts, kept at −80°C (34).

3.7.2. Western Blot Analysis of Apobec-1 and ACF Cellular Distribution—Cytoplasmic and nuclear extracts (~100 mg) are resolved on a 10% polyacrylamide-SDS gel and transferred to PVDF membranes. The blots are probed with rabbit polyclonal anti-apobec-1 antibody (35) or with rabbit polyclonal anti-ACF antibody (36). The blots are subsequently probed with cytoplasmic and nuclear-specific markers to confirm no cross contamination from one compartment to the other during subcellular fractionation (see Note 7).

3.7.3. Immunohistochemistry—Pieces (1–2 mm) of tissues (liver or small intestine) are fixed and paraffin-embedded. Immunohistochemistry is performed on 5-μm sections. The slides are rehydrated and then microwaved for 15 min in citrate buffer (0.01 M pH 6) before incubation with rabbit polyclonal ACF antibody (1:500 dilution) (36). For detection of apobec-1, slides are blocked for 15 min in 5% normal goat serum/3% bovine serum albumin, followed by exposure to rabbit anti-peptide apobec-1 antibody (40 mg/mL dilution) (35).

[5]5′ end-labeled primer is purified from the unincorporated nucleotide using QIAquick Nucleotide Removal kit (QIAGEN).
[6]Resuspend the poisoned primer extension products in 3 mL of loading dye containing 95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 18 mM EDTA, 0.025% SDSs and incubate at 95°C for 4 min. Run the gel at 70 W and stop the electrophoresis when the Bromophenol blue reaches the two-thirds of the gel.
[7]Primary antibody solutions can be kept at 4°C for future experiments.

Following incubation with a biotinylated goat anti-rabbit IgG diluted in 0.3% Triton/1% BSA, the slides are treated for 15 min with 1% hydrogen peroxide in methanol to block endogenous peroxidases. Finally, the slides are treated with avidin-conjugated horseradish peroxidase prior to color development.

3.8. Serum apoB Analysis

Blood samples are drawn by exsanguination via direct cardiac puncture or aortic cannulation or by retro-orbital bleed, and serum is obtained by centrifugation at 4,000 × *g* for 20 min at 4°C. Two microliters of serum is resolved on a 4–12% polyacrylamide gradient-SDS gel. After transfer, membranes are incubated with rabbit polyclonal anti-mouse apoB antibody (37) and visualized by enhanced chemoluminescence.

3.9. Analysis of Serum Lipids and Lipoproteins Content

Serum triglycerides are analyzed using L-type TG H kit (Wako Diagnostics, L type TG-M-ColorA, L type TG-M-ColorB). Serum cholesterol is assessed using Cholesterol E kit. Lipoproteins in 200 μL of plasma are fractionated by Superose 6 chromatography. Specifically, two 25-cm Superose 6 columns are connected in tandem and 500 μL fractions are collected. Samples are either stored at 4°C for up to 2 weeks or kept at −20°C until analysis. Cholesterol and triglycerides content of each individual fraction can be assessed using enzymatic detection kits described above.

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