Increased hepatic VLDL secretion, lipogenesis, and SREBP-1 expression in the corpulent JCR:LA-cp rat

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Abstract **The corpulent JCR:LA-cp rat (cp/cp) is a useful model for study of the metabolic consequences of obesity and hyperinsulinemia. To assess the effect of hyperinsulinemia on VLDL secretion in this model, we measured rates of secretion of VLDL in perfused livers derived from cp/cp rats and their lean littermates. Livers of cp/cp rats secreted significantly greater amounts of VLDL triglyceride and apolipoprotein, compared with lean littermates. The content of apoB, apoE, and apoCs in both perfusate and plasma VLDL was greater in the cp/cp rat, as was the apolipoprotein (apo)C, apoA-I, and apoA-IV content of plasma HDL. Triglyceride content was also greater in cp/cp livers, as was hepatic lipogenesis and expression of lipogenic enzymes and sterol regulatory element binding protein-1 (SREBP-1). Hepatic mRNAs for apoE, and apoA-I were higher in livers of cp/cp rats. In contrast, the steady state levels of apoC-II, apoC-III, and apoB mRNAs were unchanged. Thus, livers of obese hyperinsulinemic cp/cp JCR:LA-cp rats secrete a greater number of VLDL particles that are enriched in triglyceride, apoE, and apoC. Greater secretion of VLDL in the cp/cp rat in part results from higher endogenous fatty acid synthesis, which in turn may occur in response to increased expression of the lipogenic enzyme regulator SREBP-1c.**—Elam, M. B., H. G. Wilcox, L. M. Cagen, X. Deng, R. Raghow, P. Kumar, M. Heimberg, and J. C. Russell. **Increased hepatic VLDL secretion, lipogenesis, and SREBP-1 expression in the corpulent JCR:LA-cp rat.** ***J. Lipid Res.*** **2001.** 42: **2039–2048.**

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The JCR:LA-corpulent rat, when homozygous for the corpulent (cp) gene (also known as *f*), demonstrates characteristics of hyperphagia, obesity, hyperlipidemia, and hyperinsulinemia with impaired glucose tolerance (1–3). The defect is transmitted as an autosomal recessive trait (4) and has been identified as a Tyr763Stop nonsense mutation in the leptin receptor upstream of the putative transmembrane domain (5). The cp mutation arose in Koletsky's hypertensive rat strain and was crossbred with both LA/N and SHR/N strains (6). Insulin resistance and hyperinsulinemia are more severe in the male JCR:LA-cp rat than in the female, and, unlike the fatty Zucker rat, the male JCR:LA-cp rat develops atherosclerotic vascular lesions (7). Development of cardiovascular disease in the male JCR:LA-cp rat correlates strongly with hyperinsulinemia (7). The JCR:LA-cp rat is therefore a useful animal model for the study of the pathophysiology and metabolic consequences of obesity and hyperinsulinemia.

Hyperlipidemia, in particular higher plasma levels of triglyceride enriched VLDL, is a prominent feature of the corpulent JCR:LA-cp rat (cp/cp) JCR:LA-cp phenotype (3). Although the pathogenesis of hyperlipidemia in the cp/cp JCR:LA-cp rat is not completely delineated, studies using triton WR1339 in intact animals, and with primary hepatocyte cultures from cp/cp rats suggest that overproduction of VLDL may play a significant role (8, 9). Assessment of VLDL secretion in the JCR:LA-cp rat, which exhibits insulin-resistance and hyperinsulinemia, is of interest because previous studies using in vitro hepatocyte cultures demonstrate that prolonged exposure to high levels of insulin increases secretion of both VLDL triglyceride and apoB (10, 11). Recently, it has been demonstrated that induction of the key lipogenic enzymes, fatty acid synthase (FAS), and acetyl-CoA carboxylase (ACC) by insulin is mediated by the transcription factor sterol regulatory element binding protein-1c (SREBP-1c) (12–14), also known as adipocyte differentiation and determination factor (ADD-I) (15). These in vitro observations suggest that up-regulation of endogenous lipid synthesis via regulation of SREBP-1c/ADD-I, FAS, and ACC may play a significant

Abbreviations: ACC-1, acetyl-CoA carboxylase-1; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; apoC-II, apolipoprotein C-II; apoC-III, apolipoprotein C-III; apoE, apolipoprotein E; ADD-I, adipocyte differentiation and determination factor; cp/cp, homozygous corpulent JCR:LA-cp rat; FAS, fatty acid synthase; SREBP-1, sterol regulatory element binding protein-1; +/?, lean JCR:LA-cp rat.

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role in the ability of insulin to increase VLDL secretion via increased availability of fatty acid for triglyceride synthesis. Therefore, we examined rates of secretion of VLDL lipid and apolipoprotein by isolated perfused livers of cp/cp rats in order to assess the effect of hyperinsulinemia on the quantity and composition of secreted VLDL. We also measured the steady-state levels of mRNAs encoding apolipoprotein (apo)B, apoC-II, apoC-III, apoE, apoA-I, FAS, ACC, and ADD-I/SREBP-1 in the livers of cp/cp JCR:LA-cp rats to gain insight into the potential mechanisms by which VLDL secretion is altered in response to hyperinsulinemia.

METHODS

Animals

Male rats were bred at the University of Alberta, Edmonton, Canada, and were shipped to the University of Tennessee at 4–8 weeks of age and maintained on a chow diet (Picolab Rodent Diet 20, Lab Diet 5053, PMI Nutrition International Inc., Brentwood, MO) for several weeks, at which time obese animals attained a weight of 450–500 g, while that of the lean animals was 300–350 g. Obese JCR:LA-cp rats are homozygous for the defective leptin receptor gene (cp/cp), whereas lean animals are heterozygous for the cp gene (cp/+) or homozygous for the functional leptin receptor gene (+/+). Lean animals are phenotypically indistinguishable and are therefore designated as +/? (3).

Liver perfusion

Livers were surgically removed under pentobarbital anesthesia, and liver perfusions were conducted in a humidified perfusion apparatus with recycling perfusion medium consisting of Krebs-Heneseleit-bicarbonate buffer, pH 7.4, with 100 mg/dl glucose, as has been described previously in detail (16). Blood was obtained from the abdominal aorta and placed into tubes containing EDTA prior to removal of the liver. Plasma samples were obtained after a brief centrifugation and processed as described below. Washed outdated human erythrocytes were present in the perfusion medium to provide an initial hematocrit of 25%. The starting perfusion medium contained 0.69 ± 0.03 mM (n = 14) sodium oleate complexed with 3% lipid-free purified BSA. Additional sodium oleate complexed with BSA (6%) was supplied by constant infusion at a rate of 166 μmol/h. The fatty acid concentration achieved at steady state during perfusion did not differ for livers of cp/cp or +/? rats (0.44 ± 0.02 and 0.40 ± 0.03 mM (n = 14) at 1.5 and 3 h, respectively). For experiments in which rates of secretion of newly synthesized apolipoprotein was assessed, trace amounts of [4, 5-^{3}H]L-leucine (25 μCi) were added to the perfusion medium as a pulse and then infused at a rate of 60 μCi/h as described previously (17).

Livers were perfused for up to 3 h. Samples of perfusate were collected at 0, 1, 2, and 3 h. At the termination of the perfusion, the liver was quickly flushed with ice-cold saline. A small piece of liver (0.1 g) was immediately placed in RNAzol for extraction of RNA. After weighing and mincing the remaining portion of the liver, lipids were extracted from 1-g aliquots. Livers that had not been perfused were also analyzed for lipid and mRNA content. Perfusate samples and arterial blood samples were collected in EDTA and perfusate and plasma was obtained after brief centrifugation. All samples were analyzed immediately, as described below, or were stored at −70°.

VLDL uptake studies

Pefused livers have the capacity for re-uptake of secreted nascent VLDL (18). Therefore, greater accumulation of VLDL in perfusate of cp/cp livers could occur as a result of decreased re-uptake rather than greater secretion. Differences in VLDL re-uptake might occur as a result of differing characteristics of cp/cp versus +/? livers, or may be due to differences in composition of the VLDL itself. For this reason, we measured re-uptake of VLDL by perfused livers using a crossover design. Metabolically labeled nascent VLDL was prepared by perfusing normal Sprague-Dawley donor livers with [4, 5-^{3}H]L-leucine (1–3 mCi/liver). Labeled nascent VLDL was introduced into the medium perfusing cp/cp and +/? livers and disappearance of labeled VLDL protein (trichloroacetic acid precipitable counts) was assessed during the first 60 min of perfusion. Conversely, metabolically labeled VLDL produced by +/? and cp/cp donor livers, respectively, was introduced into the perfusate of normal Sprague-Dawley livers. The uptake studies were carried out using oleate-containing medium as described above.

Analytical procedures

Plasma lipoproteins were isolated by sequential ultracentrifugation after density adjustments with sodium bromide at d = 1.006 (VLDL), d = 1.063 (IDL/LDL), and d = 1.21 (HDL). Chemical analyses of all major classes of lipids (triglyceride, phospholipid, cholesterol, free fatty acid, and cholesteryl ester) were conducted after thin layer chromatography of plasma and lipoprotein extracts. Protein analyses were carried out by the method of Lowry as modified by Markwell et al. (19). Apolipoproteins of the various lipoprotein fractions of plasma were separated on Laemmli gradient gels (4–22% polyacrylamide) and stained with Coomassie Brilliant Blue R-250. Densitometric tracing of the gels, using NIH Image 161 software, was used to evaluate the distribution of the apolipoproteins within the particular perfusate or plasma lipoprotein fraction. For experiments in which 4,5-^{3}H-labeled leucine was infused, Coomassie blue-stained apolipoprotein bands from tritium-labeled VLDL perfusion samples were excised for measurement of radioactivity (17).

Measurement of steady state levels of mRNAs encoding various hepatic apolipoproteins, lipogenic enzymes, and SREBP-1

Total RNA was extracted from liver samples using RNA Stat-60 (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions and quantitated by absorbance at 260 nm. Fifteen μg of total RNA was loaded per lane of a formaldehyde/0.8% agarose gel, electrophoresed in 1XMOPS buffer, blotted onto Nytran membranes (Schleicher and Schuell, Keane, NH), and UV cross-linked. Ribosomal RNA bands were visualized by staining with ethidium bromide prior to transfer. Blots were prehybridized for 3 h at 42°C in 50% formamide, 5× SSPE, 5× Denhardt's solution (5Prime-3Prime, Boulder, CO), 7.5% dextran sulfate, 1.5% sodium dodecyl sulfate (SDS), and 100 μg/ml sheared salmon sperm DNA (5Prime-3Prime).

The probe for measurement of rat apoB mRNA was a 518 bp cDNA amplified by RT-PCR from samples of rat hepatocyte total RNA, with forward and reverse primers corresponding to nucleotides +126 to +150 and +609 to +643, respectively. The PCR product is 518 bp within the coding region, but distant from the editing site and free of obvious hairpin loops. Probes were prepared for apoA-I, apoC-II, apoC-III, and apoE using primers based on published sequences of rat liver cDNA for the corresponding protein (20–23). cDNAs were labeled with ^{32}P-dCTP using a random primer labeling kit (Stratagene, La Jolla, CA), and hybridized according to the manufacturer recommendations. mRNA transcripts were visualized by autoradiography using Kodak X-Omat AR film (Rochester, NY). A digital image of the developed film was created and the intensity of bands determined by densitometry (Alpha Innotech, San Leandro, CA).

Plasmids containing cDNAs used for measurement of ACC-1, FAS, and ADD-I/SREBP-1c mRNA levels were generously provided by Dr. Ki-Han Kim (Purdue University, West Lafayette, IN; ACC-1); Dr. Stuart Smith (Children's Hospital Research Institute, Oakland, CA; FAS); and Dr. Bruce M. Spiegelman (Dana Farber Cancer Institute, Boston, MA; ADD-I/SREBP-1c). B-actin mRNA was measured using mouse B-actin DECAprobe (Ambion, Inc., Austin, TX). ACC-1, FAS, ADD-I/SREBP-1c, and B-actin mRNA were detected by overnight hybridization at 42°C with the cDNA probes, ^{32}P-labeled by the random primer method using a commercial kit following the manufacturer's directions (Invitrogen, Carlsbad, CA). Unbound probe was removed by washing with 2× SSC + 0.1% SDS at room temperature and then twice with 0.1× SSC + 0.1% SDS at 65°C for 30 min each. Membranes were exposed to X-Omat AR film (Kodak); a digital image of the developed film was created and bands quantitated by densitometry (Alpha Innotech Corp., San Leandro, CA).

Determination of plasma insulin and glucose levels

Plasma insulin levels were determined using a Micromedic Insulin RIA kit (ICN Pharmaceuticals Inc., Costa Mesa, CA), and glucose was determined using a glucose oxidase kit (Sigma Chemical, St. Louis, MO).

Assessment of hepatic lipogenesis in primary hepatocyte cultures prepared from cp/cp and +/? JCR:LA-cp rats

Hepatocytes were prepared from livers of +/? and cp/cp rats by collagenase perfusion as described previously (10). Cells were suspended in modified Williams E medium (GIBCO/BRL) containing 20% fetal bovine serum (Sigma, St. Louis, MO) and 5.5 mM glucose and were plated on 60 mm culture dishes coated with rat tail collagen (Collaborative Biochemical Products, Bedford, MA) at 3× 10^6 cells per plate. After 4 h, nonadherent cells were removed and the plates washed with PBS before measurement of lipogenesis. Rates of lipogenesis were assessed after incubation of the washed heptocyte monolayer for 3 h in serum-free, 5.5 mM glucose Williams E medium and 1 mM [2-^{14}C]acetate (6 μCi). Lipids were extracted from the cells and medium and separated by TLC (16). Radioactivity in bands corresponding to triglyceride, phospholipids, free fatty acids, and cholesteryl esters was measured by liquid scintillation spectrometry (Beckman Model LS5000).

Statistical analysis

Significance of differences in variables of interest between cp/cp and +/? rats were determined by Student's *t*-test using a microcomputer statistical analysis software package (StatView for Power Macintosh, version 4.51, 1996, Abacus Concepts, Berkeley, CA) on a Macintosh G3 computer.

RESULTS

Characteristics of the obese (cp/cp) and lean (+/?) rats

Both total body weight and liver weight were greater in cp/cp rats than those observed in lean (+/?) controls (**Table 1**). Cp/cp rats also demonstrated higher nonfasting insulin and glucose levels. Total plasma triglyceride, cholesterol, and cholesteryl ester content were also higher in the cp/cp rat. Surprisingly, plasma fatty acid levels in cp/cp rats were comparable to those observed in +/? controls.

Table 2 depicts the apolipoprotein levels observed in plasma VLDL, LDL/IDL, and HDL of cp/cp and +/? rats. VLDL apolipoproteins (B-48, B-100, Cs, and apoA-I) were 4- to 6-fold greater in plasma of cp/cp rats, as compared with +/? rats. Similarly, HDL apolipoproteins' (A-I, A-IV, E, and Cs) levels were approximately 2-fold greater in the plasma of cp/cp rats. In contrast, the apolipoprotein content of the LDL/IDL fraction was comparable in cp/cp and +/? rats. Total plasma apoB-48 levels were higher in cp/cp rats, as were total plasma apoC, E, A-I, and A-IV (Table 2).

TABLE 1. Characteristics of cp/cp and +/? JCR rats

	Lean (+/?) (n = 9)	Corpulent (cp/cp) (n = 9)
Triglyceride (μmol/dl)	49.9 ± 5.1	186.1 ± 25.2[a]
Phospholipid (μmol/dl)	107.7 ± 5.9	185.1 ± 9.9[a]
Cholesterol (μmol/dl)	43.3 ± 2.7	66.1 ± 4.4[a]
Cholesteryl ester (μmol/dl)	92.6 ± 4.8	161.3 ± 11.4[a]
Free fatty acid (μmol/dl)	41.9 ± 3.2	46.7 ± 4.6
Glucose (mg/dl)	210 ± 5 (8)	257 ± 17 (8)[a]
Insulin (μIU)	100 ± 11 (3)	178 ± 13 (3)[a]
Body weight (g)	377 ± 4	538 ± 21[a]
Liver weight (g)	10.7 ± 0.3	17.6 ± 1.0[a]

Results are means ± SEM and the number of individual observations are in parentheses.

[a] $P < 0.05$ compared with +/? rats. The plasma was taken in the fed state at the time of sacrifice.

Hepatic secretion of lipid and lipoproteins in cp/cp and +/? JCR:LA-cp rats

To determine whether greater accumulation of triglyceride-rich lipoproteins in the plasma of cp/cp rats resulted from increased hepatic secretion of VLDL, we assessed lipid and lipoprotein secretion by isolated perfused livers of cp/cp and +/? rats. When livers were perfused with a constant infusion of oleic acid, triglyceride secretion was linear over the 3-h perfusion period and was consistently greater in livers derived from cp/cp rats compared with +/? controls (data not shown). Livers of cp/cp rats secreted 3 times as much triglyceride (VLDL and total perfusate) per gram of liver during the perfusion than did livers of +/? rats (**Table 3**). Secretion of VLDL phospholipid and cholesterol (but not cholesteryl ester) by livers of cp/cp rats was also greater. Because lipid and apolipoprotein secretion rates are presented as amount secreted per gram liver, the absolute magnitude of the difference in lipid and apolipoprotein secretion by livers of +/? and cp/cp rats was even greater, as cp/cp livers were 60% larger than +/? livers. Therefore, whole liver triglyceride secretion rates were up to 5-fold greater in livers of cp/cp rats. Surprisingly, despite increased triglyceride secretion, total fatty acid uptake by livers of cp/cp rats was lower per gram liver than that of +/? livers (Table 3). Because cp/cp livers were larger, however, total fatty acid uptake per liver was comparable to that observed in +/? livers.

Calculation of the core/surface ratio (TG + CE/C + PL) provided an indication of the relative differences in the average size of the VLDL produced by cp/cp versus +/? livers. The core-surface ratios of VLDL produced by cp/cp livers were larger than that of +/? rats (3.53 ± 0.40 versus

TABLE 2. Plasma apolipoproteins in +/? and cp/cp JCR:LA/N rats

Apolipoprotein	ApoB-48	ApoB-100	ApoE	ApoC	ApoA-I	ApoA-IV
VLDL-+/?	0.24 ± 0.02	0.22 ± 0.02	0.11 ± 0.02	0.57 ± 0.18	0.05 ± 0.02	0.11 ± 0.10
VLDL-cp/cp	1.81 ± 0.42[a]	1.07 ± 0.26[a]	0.23 ± 0.10	3.60 ± 0.78[a]	0.26 ± 0.12[a]	0.34 ± 0.21
IDL/LDL-+/?	1.52 ± 0.52	3.27 ± 0.90	1.02 ± 0.21	3.38 ± 1.61	0.20 ± 0.08	0.17 ± 0.14
IDL/LDL-cp/cp	1.36 ± 0.08	3.19 ± 0.51	1.42 ± 0.57	4.42 ± 1.02	0.62 ± 0.25	0.41 ± 0.35
HDL-+/?	ND	ND	2.93 ± 0.69	14.2 ± 2.9	16.8 ± 3.96	3.94 ± 0.92
HDL-cp/cp	ND	ND	6.38 ± 1.30[a]	33.0 ± 3.2[a]	29.5 ± 3.5[a]	8.59 ± 1.11[a]
Total-+/?	1.76 ± 0.53	3.49 ± 0.90	4.06 ± 0.74	18.2 ± 2.3	17.0 ± 3.9	4.19 ± 0.82
Total-cp/cp	3.17 ± 0.43[a]	4.26 ± 0.69	8.02 ± 0.90[a]	41.0 ± 3.8[a]	30.4 ± 3.4[a]	9.33 ± 1.20[a]

Data are mg/dl of plasma apolipoproteins in the VLDL, IDL/LDL, and HDL fractions and the total of the three lipoprotein fractions for +/? (N = 4) and cp/cp (N = 4) JCR:LA-cp rats. Lipoproteins were isolated from the plasma of (non-fasted) +/? and cp/cp JCR:LA-cp rats by sequential ultracentrifugation at d = 1.006 (VLDL), d = 1.006–1.063 (IDL/LDL), and d = 1.063–1.21 (HDL). Apolipoproteins were separated on Laemmli gradient gels (4 to 22% polyacrylamide) and stained with Coomassie Brilliant Blue R-250, and the mass of each apolipoprotein was estimated from densitometric scanning of the stained gels. Separation of apoC species was not sufficient using this methodology to allow separate quantitation of apoC-III and apoC-II. Total apolipoprotein represents the sum of the apolipoprotein in VLDL, IDL/LDL, and HDL fractions. ND = none detectable.

[a] $P < 0.05$ cp/cp versus +/?.

2.44 ± 0.24 for cp/cp versus +/?). This reflects marked enrichment with triglyceride of VLDL secreted by cp/cp livers.

We assessed the rates of secretion of VLDL apolipoproteins by perfused livers of cp/cp and +/? rats both by densitometry on stained SDS-PAGE gels (**Fig. 1A**) and by measuring the secretion of metabolically labeled ([^{3}H]leucine) VLDL apolipoprotein (Fig. 1B). Approximately 85% of secreted apolipoprotein was associated with the VLDL as assessed by both methods (data not shown). Secretion of VLDL associated apolipoproteins was greater in cp/cp rat livers (Fig. 1A and 1B). Although incorporation of [^{3}H]leucine into total hepatic and perfusate protein (TCA precipitable counts) was only somewhat higher in cp/cp livers compared with +/? livers (2.53 μCi/g liver versus 2.11 μCi/g respectively), secretion of labeled apolipoproteins was dramatically increased (Fig. 1B). This indicated a specific increase in incorporation of labeled amino acid into apolipoproteins in the cp/cp livers. Because each VLDL particle has one apoB molecule, the rate of secretion of apoB serves as an index of the number of VLDL particles secreted. Therefore, in addition to increased triglyceride secretion, livers of cp/cp rats also secreted a larger number of VLDL particles. Enrichment of the VLDL fraction with other apolipoproteins (apoE, apoCs) was also observed (Fig. 1). Enrichment of VLDL with triglyceride in the cp/cp rat also resulted in altered distribution of apolipoproteins among the lipoprotein classes. The percentage of apoB-48 associated with VLDL was greater in perfusate of cp/cp livers as compared with that of +/? livers (85% versus 60% for cp/cp versus +/?). Similarly, the percentage of total apoC associated with nascent VLDL secreted by livers of cp/cp rats was greater than that of +/? rats (77% versus 64% for cp/cp versus +/?). In addition to recruitment of apolipoprotein into the VLDL fraction, total secretion of apoB, apoE, apoA-I, and apoCs into all lipoprotein classes was also greater in cp/cp livers (data not shown). This suggests that apolipoprotein synthesis may be greater in livers of cp/cp JCR:LA-cp rats (or, in the case of apoB, intracellular degradation may be lower).

TABLE 3. Secretion of perfusate and VLDL lipids by perfused livers of +/? and cp/cp JCR:LA-cp rats

	+/? (9)	cp/cp (9)
	μmol/g liver/3h	
Triglyceride		
Total	1.11 ± 0.12	3.19 ± 0.35[a]
VLDL	0.85 ± 0.11	2.62 ± 0.31[a]
Phospholipid		
Total	1.62 ± 0.24	1.25 ± 0.18
VLDL	0.27 ± 0.02	0.55 ± 0.06[a]
Cholesterol		
Total	0.24 ± 0.04	0.28 ± 0.05
VLDL	0.10 ± 0.01	0.22 ± 0.03[a]
Cholesteryl ester		
Total	0.14 ± 0.03	0.13 ± 0.04
VLDL	0.06 ± 0.01	0.08 ± 0.02
FFA uptake (μmol/gm/liver/hr)	129 ± 8.8	82 ± 5.2[a]

Results are means ± SEM and the number of individual observations are in parentheses.

[a] $P < 0.05$ compared with +/? rats.

Hepatic expression of apolipoprotein genes in livers of cp/cp versus +/? JCR:LA-cp rats

We determined whether greater rates of secretion of VLDL apolipoprotein by cp/cp livers is accompanied by greater hepatic expression of apolipoprotein mRNA. A modest (50%) but highly significant increase in mRNA content for apoE and apoA-I in livers from cp/cp rats was observed compared with +/? littermates (**Fig. 2**). In contrast, the level of mRNA for apoC-III, apoC-II, and apoB in livers of cp/cp rats and +/? controls was similar (Fig. 2). These findings suggest that greater secretion of apoE and apoA-I in VLDL by cp/cp livers may result from higher levels of mRNA expression. In contrast, increased secretion of apoB and apoCs appears to involve post-transcriptional and/or post-translational mechanisms rather than transcriptional control.

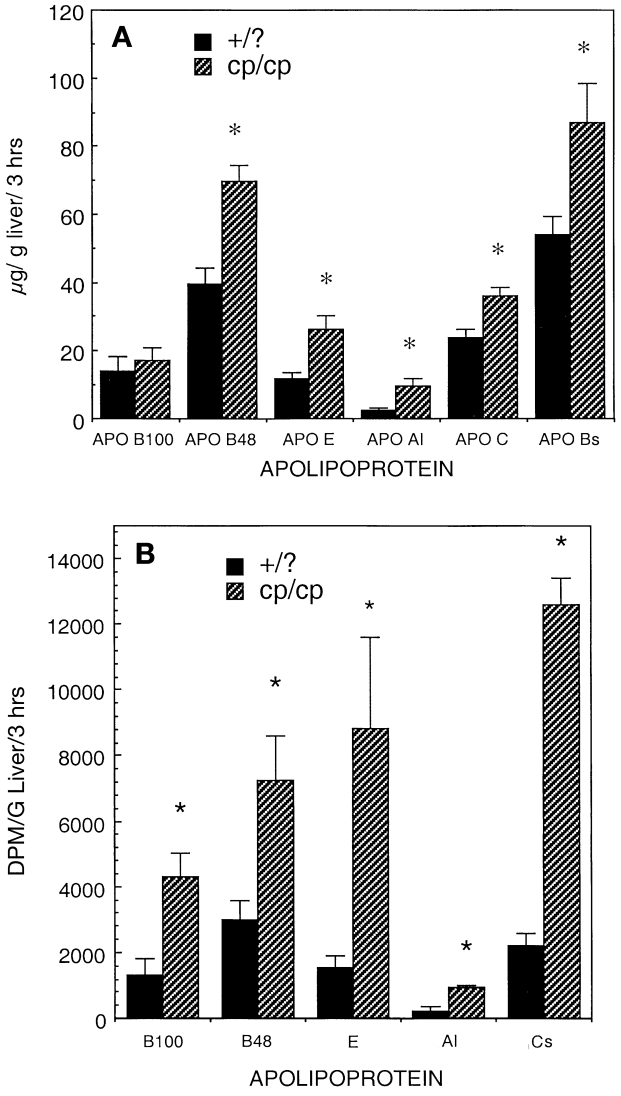
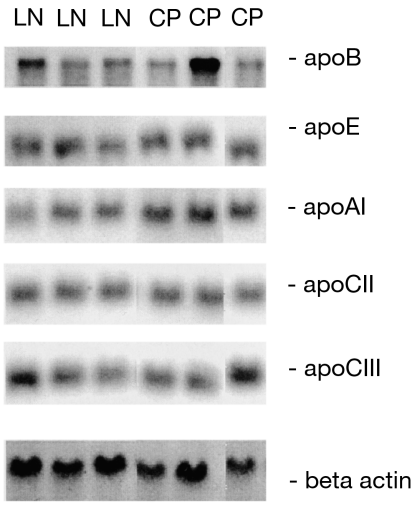


Fig. 1. A: Secretion of VLDL apolipoprotein by perfused livers of +/? and cp/cp JCR:LA-cp rats assessed by stained SDS-PAGE. VLDL was isolated from perfusate by ultracentrifugation at d = 1.006. VLDL apolipoproteins were separated by SDS-PAGE, stained with Comassie Brilliant Blue, and the distribution of apolipoproteins assessed by densitometry. Mass of secreted apolipoprotein was estimated following determination of total VLDL apolipoprotein. Separation of apoC isoforms was insufficient to separately measure their mass, therefore all apoC isoforms are pooled. The data are the mean ± standard error of the mean of nine separate perfusions each for cp/cp and +/? livers. * $P < 0.05$ +/? versus cp/cp by Student's *t*-test. B: Secretion of newly synthesized [^{3}H]leucine labeled apolipoprotein in VLDL by +/? and cp/cp JCR:LA-cp livers. Data are mean ± SEM DPM [^{3}H]leucine incorporated into VLDL apolipoproteins and secreted into the perfusate during the 3 h perfusion period. Apolipoproteins were determined in the VLDL (d < 1.006) fraction following isolation by ultracentrifugation. Radioactivity in each apolipoprotein fraction was determined by liquid scintillation spectroscopy following separation on Laemmli gradient gels stained with Coomassie Brilliant Blue R-250. * $P < 0.05$ +/? (n = 3) versus cp/cp (n = 3) by Student's *t*-test.



	ApoB	ApoCIII	ApoCII	ApoE	ApoA1
+/? (n=5)	163 ± 31	89 ± 9	105 ±12	99 ± 7	98 ± 8
cp/cp (n=5)	218 ± 75	109 ± 9	125 ± 4	141± 4	148 ± 8
P =	.20	.15	.15	.001	.003

Fig. 2. Hepatic expression of mRNA for apolipoproteins in +/? and cp/cp JCR:LA-cp rats. Messenger RNAs encoding for apoB, apoE, apoA-I, apoC-II, apoC-III, and B-Actin were analyzed by hybridization of total RNA from liver samples by Northern blot analysis. A: Representative Northern blots from lean (LN) and corpulent (CP) livers. B: Quantitative data represent the relative intensity of bands normalized to B-actin relative to a +/? reference liver (Reference value = 100). Significance of differences in mRNA levels in +/? versus cp/cp were assessed by Student's *t*-test.

Uptake of VLDL by perfused livers of cp/cp and +/? JCR:LA-cp rats

The perfusion studies described above indicate greater accumulation of VLDL in the perfusate of livers from cp/cp rats; however, because perfused livers also have the capacity to remove VLDL from recirculating perfusate, the observed greater accumulation of VLDL in perfusate of cp/cp livers might have resulted from reduced re-uptake of secreted VLDL. This might occur, either as a result of altered lipid and apolipoprotein composition of VLDL from cp/cp livers, or as a result of intrinsic differences in VLDL re-uptake by cp/cp livers. To determine whether altered VLDL removal resulted in greater accumulation of VLDL in the perfusate of cp/cp livers, we assessed the rate of removal, by perfused livers of Sprague Dawley rats, of metabolically ([^{3}H]leucine) labeled VLDL, derived from livers of cp/cp and +/? JCR:LA-cp rats. As depicted in **Fig. 3A**, rates of disappearance of VLDL from the perfusate of Sprague-Dawley livers were comparable regardless of whether the VLDL was derived from the liver of a cp/cp or +/? JCR:LA-cp rat. Conversely, perfused cp/cp and +/? livers took up VLDL derived from livers of Sprague-Dawley rats at comparable rates (Fig. 3B). These data confirm that increased accumulation of VLDL in perfusate of cp/cp JCR:LA-cp livers is the result of greater secretion rather than reduced re-uptake.

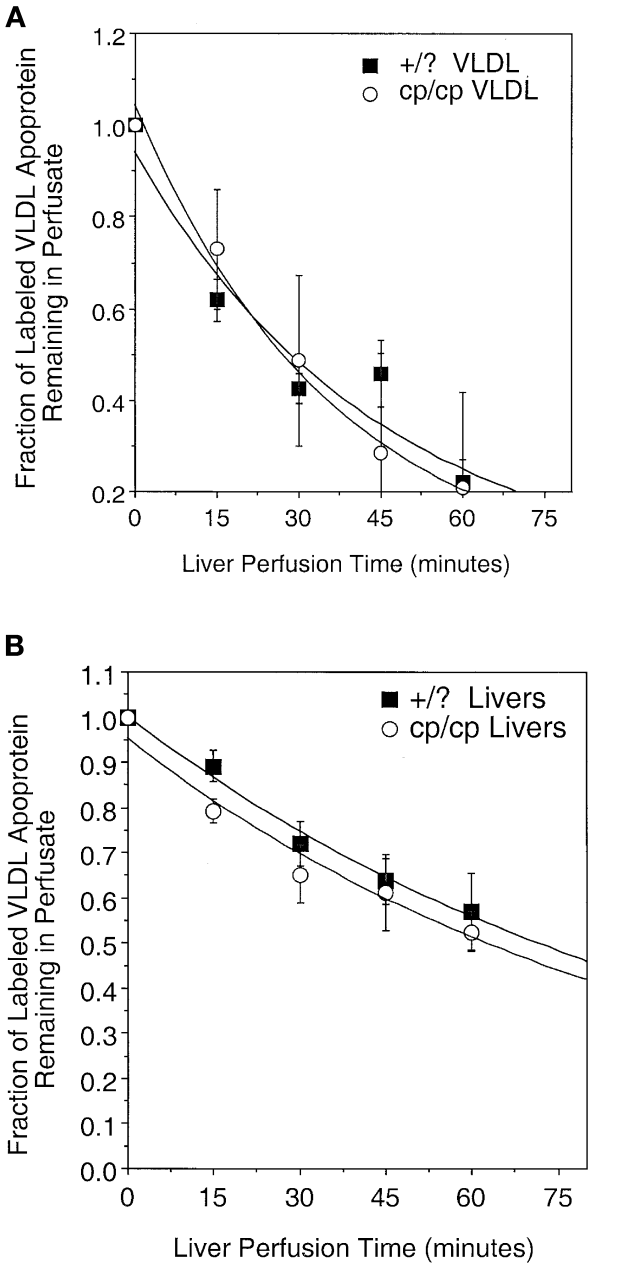


Fig. 3. A: Uptake by perfused livers of control (Sprague-Dawley) rats, of nascent VLDL derived from +/? versus cp/cp JCR:LA-cp rats. To determine whether increased VLDL accumulation in perfusate of cp/cp JCR:LA-cp livers was the result of impaired uptake of cp/cp VLDL by the liver, metabolically labeled VLDL was prepared by infusion of [^{3}H]leucine into perfused livers of cp/cp and +/? JCR:LA-cp rats. ^{3}H-labeled VLDL from cp/cp and +/? donor livers was then introduced into the perfusate of livers derived from Sprague-Dawley rats (N = 2 each for +/? and cp/cp VLDL, respectively) and the disappearance of labeled apolipoprotein was determined. Uptake of [^{3}H]leucine labeled VLDL was similar for VLDL derived from both +/? and cp/cp JCR:LA-cp rats. B: Uptake by perfused livers of +/? and cp/cp JCR-LA/N rats of VLDL derived from control (Sprague-Dawley) rat livers. In order to determine whether increased accumulation of VLDL in perfusate of perfused JCR:LA-cp livers was the result of reduced VLDL uptake rather than increased secretion, VLDL from Sprague-Dawley rats, metabolically labeled with [^{3}H]leucine, was introduced into the perfusate of +/? (N = 3) and cp/cp (N = 3) perfused livers. Disappearance of [^{3}H]leucine labeled VLDL was similar for both +/? and cp/cp livers.

Hepatic lipid content, lipogenesis, and expression of lipogenic enzymes in livers of cp/cp and +/? JCR:LA-cp rats

Availability of triglyceride is an important determinant of VLDL triglyceride and apoB secretion (24–26). We therefore compared hepatic triglyceride content and endogenous lipid synthesis in livers and primary hepatocyte cultures, respectively, from cp/cp and +/? rats. Hepatic triglyceride content was 4-fold greater in livers of cp/cp rats compared with +/? controls, while hepatic content of cholesterol was slightly lower and cholesteryl ester content was unchanged (**Table 4**). To determine whether the higher hepatic triglyceride content in perfused cp/cp livers was the result of perfusion with fatty acid (oleic acid), we also assessed hepatic lipid levels in unperfused livers. The hepatic content of lipids in unperfused cp/cp and +/? livers was comparable to that observed in perfused livers with the exception of reduced free cholesterol and increased cholesteryl ester in perfused livers (Table 4). This likely reflects the utilization of infused fatty acid for cholesteryl ester formation during liver perfusion.

Greater hepatic triglyceride content of cp/cp livers may have resulted from increased dietary intake of fatty acids; however, it may also reflect higher rates of endogenous fatty acid synthesis. To determine whether fatty acid synthesis was increased in livers of cp/cp JCR:LA-cp rats, we assessed rates of synthesis of lipid from [1-^{14}C]acetate in freshly prepared hepatocyte monolayers derived from livers of cp/cp and +/? rats. De novo lipogenesis (assessed by incorporation of labeled acetate into saponified lipid fraction) was increased approximately 2-fold in cp/cp hepatocytes compared with lean (+/?) controls. This was accompanied by increased incorporation of newly synthesized fatty acids into triglyceride (but not phospholipid or cholesteryl ester) in freshly isolated hepatocytes of cp/cp rats (**Fig. 4**).

Expression of the key lipogenic enzymes, FAS, and ACC

TABLE 4. Hepatic lipid content of unperfused and perfused livers

	+/? (9)	Cp/cp (9)
	μmol/g liver	
Triglyceride		
Perfused	4.76 ± 0.31	21.90 ± 2.53[a]
Unperfused	5.07 ± 1.21	24.08 ± 2.71[a]
Phospholipid		
Perfused	23.8 ± 1.0	21.0 ± 1.1
Unperfused	26.9 ± 2.7	23.7± 1.3
Cholesterol		
Perfused	3.78 ± 0.19[b]	3.37 ± 0.14[a]
Unperfused	4.53 ± 0.35	3.56 ± 0.34[a]
Cholesteryl ester		
Perfused	0.51 ± 0.06[b]	0.57 ± 0.07
Unperfused	0.33 ± 0.05	0.44 ± 0.08

Results are means ± SEM for hepatic lipid content of livers of corpulent (cp/cp) and lean (+/?) rats following perfusion for 3 h with oleic acid (VLDL secretion studies) versus that of unperfused livers. The number of individual observations are in parentheses.

[a] $P < 0.05$ compared with +/? rats.

[b] $P < 0.05$ compared with unperfused rats.

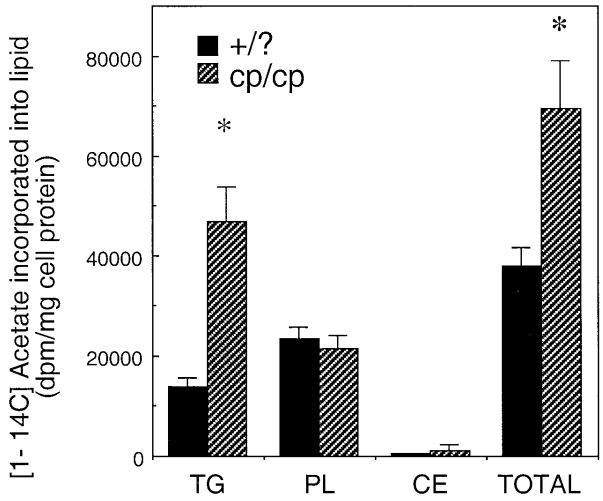
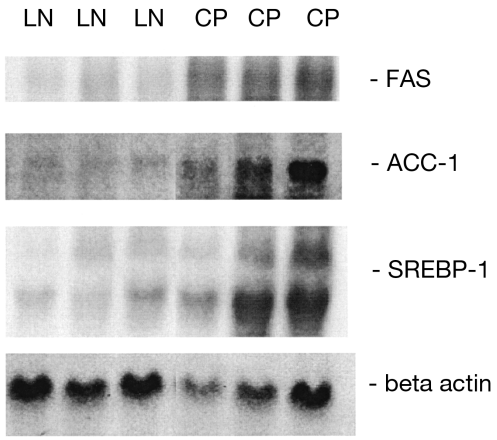


Fig. 4. Incorporation of 1-^{14}C-acetate-labeled fatty acid into lipid products by primary cultures of +/? versus cp/cp JCR:LA-cp rats. Data are the means of three individual hepatocyte cultures (average of three plates per preparation). Hepatocytes from +/? and cp/cp rats were isolated in Williams E medium (20% fetal calf serum, 5.5 mM glucose). After adherence to collagen coated plates, cells were incubated with [1-^{14}C]acetate for 3 h and the amount of label incorporated into cell and medium esterified lipids was determined. "Total" incorporation represents the sum of incorporation of label into all lipid products (triglyceride, phospholipid, cholesteryl ester). Increased total incorporation of label into lipid products in cp/cp hepatocytes was also confirmed by measurement of incorporation of label into total saponified lipids. * $P < 0.05$ compared with +/? hepatocytes (Student's *t*-test.).



Group	FAS	ACC-1	SREBP-1
Lean (n=5)	79 ± 13	113 ± 18	116 ± 15
Corpulent	256 ± 20	401 ± 32	442 ± 77
P =	< .001	<.001	< .003

Fig. 5. Hepatic expression of mRNAs encoding FAS, ACC, SREBP, and B-Actin in cp/cp and +/? JCR:LA-cp rats. Data are mean ± SEM for mRNA levels in lean (LN; +/?, n = 5) versus corpulent (CP; cp/cp, n = 5) livers as determined by Northern blot analysis. A: Depicts representative Northern blots. B: MRNA levels of SREBP-1, FAS, and ACC were quantitated by densitometry and are expressed after correction for B-actin message and relative to mRNA abundance in a (+/?) reference liver (=100). Significance of differences between cp/cp and +/? livers was determined by Student's *t*-test.

is regulated in a glucose-dependent fashion by insulin (14), and the nuclear transcription factor SREBP-1c appears to mediate this effect (12, 13, 27). To determine whether increased hepatic SREBP-1c expression and subsequent increased lipogenic enzyme expression in response to hyperinsulinemia contributes to greater rates of hepatic fatty acid synthesis in the cp/cp JCR:LA-cp rat, we compared levels of ACC, FAS, and SREBP-1 mRNAs in the livers of cp/cp and +/? rats by Northern blot analysis. Consistent with the previously observed in vitro effects of insulin treatment, hepatic expression of mRNA for both ACC-1 and FAS were 3-fold higher in livers of cp/cp JCR LA/N rats (**Fig. 5**). As reported previously (13), SREBP-1 mRNA was represented by two bands of approximately 5 KB in size (Fig. 5). Consistent with its proposed role as a regulator of insulin mediated FAS and ACC-1 transcription, hepatic levels of SREBP mRNA were 4-fold higher in livers of hyperinsulinemic cp/cp JCR:LA-cp rats (Fig. 5).

DISCUSSION

The JCR:LA-cp rat is a good model for the study of the metabolic complications of hyperinsulinemia associated with obesity and insulin resistance. Long-term exposure of cultured hepatocytes to high levels of insulin has been previously shown to stimulate secretion of VLDL triglyceride and apoB (10, 11). To extend these observations to an in vivo model of hyperinsulinemia, we assessed rates of VLDL secretion by perfused livers of cp/cp JCR:LA-cp rats and their lean (+/?) littermates. We determined that livers derived from cp/cp male JCR:LA-cp rats not only secreted greater amounts of VLDL triglyceride, but also greater amounts of the VLDL apolipoproteins apoB, apoCs, and apoE. This was accompanied by greater accumulation in the plasma of VLDL apoB (B-48 and B-100), apoE, and apoC. These findings support the hypothesis that hyperinsulinemia, as well as other metabolic abnormalities associated with insulin resistance, results in increased VLDL secretion in insulin resistant states.

These studies are the first to directly examine VLDL lipid and apolipoprotein secretion by intact perfused livers of cp/cp and +/? JCR:LA-cp rats. They both confirm and extend previous observations of increased rates of accumulation of VLDL triglyceride in plasma following treatment with Triton WR1339 (8) and of increased secretion of VLDL triglyceride and apolipoprotein by primary hepatocyte cultures of cp/cp JCR:LA-cp rats (9). Because reuptake of VLDL by perfused livers of cp/cp and +/? JCR-LA rats was similar, as was that of VLDL derived from cp/cp and +/? livers, the greater accumulation of VLDL in perfusate of cp/cp livers was the result of increased secretion rather than lower re-uptake. As post-heparin lipid clear-

ance is unchanged in cp/cp JCR:LA-cp rats (8), enhanced VLDL secretion appears to play a primary role in the pathogenesis of hyperlipidemia in this model.

The greater rates of VLDL triglyceride and apolipoprotein secretion by livers of cp/cp rats observed in the present study is due to both greater secretion of VLDL per gram of tissue, and to the larger size of cp/cp livers. Similar studies in the fatty Zucker rat have also shown greater rates of hepatic secretion of VLDL lipid and apoB (28, 29). In those studies, increased VLDL triglyceride secretion was attributed to both increased esterification of infused fatty acid into triglyceride and to increased de novo fatty acid synthesis (28, 29). Our studies also show greater rates of fatty acid synthesis and greater incorporation of newly synthesized fatty acid into triglyceride in cultured hepatocytes derived from cp/cp JCR:LA-cp rats.

The finding that the livers of cp/cp rats secrete not only VLDL particles enriched in triglyceride, but also a greater total number of particles, has significant implications for the pathogenesis of hyperlipidemia in insulin resistant states. If increased VLDL particle secretion is not offset by efficient subsequent removal, atherogenic apoB containing remnant particles will accumulate in the plasma. The increased amounts of apoC and apoE associated with the VLDL of cp/cp rats is also significant as both apolipoproteins play important roles in receptor mediated clearance of triglyceride rich lipoproteins (30, 31). A disproportionate increase in apoB-48 compared with apoB-100 in both plasma VLDL and nascent secreted VLDL in cp/cp JCR:LA-cp livers is consistent with the prior observations that insulin increases secretion of apoB-48 in primary hepatocyte cultures via enhanced editing of apoB mRNA (10). This in turn is due to increased expression of the catalytic subunit of the apoB mRNA editing complex, apobec-1 (32, 33).

In the present study, increased secretion of VLDL by perfused livers of cp/cp rats reflects a response to long-term in vivo hyperinsulinemia in the setting of insulin resistance. It is important to clearly distinguish between short-term and long-term effects of insulin on VLDL secretion. Although acute insulin treatment inhibits VLDL secretion both in vivo in humans (34, 35) and in vitro in rat hepatocytes (36, 37), long-term hyperinsulinemia in humans (38, 39), Zucker rats (29), and hepatocyte cultures (10, 11) is associated with increased VLDL secretion. The effect of long-term hyperinsulinemia to increase hepatic VLDL secretion may result both from attenuation of the suppressive effect of insulin on VLDL secretion (40–42) and an unabated lipogenic response to insulin as a result of differential development of insulin resistance (43). Recent studies also indicate that the insulin response of both perfused liver and hepatocyte cultures is influenced by the prior nutritional status of the donor animal (44, 45).

Greater hepatic expression of mRNA for apoE and greater secretion of apoE by livers of cp/cp rats is of particular interest in view of recent observations of a stimulatory effect of apoE on VLDL production in transgenic mice (46) and rabbits (47). On the other hand, increased hepatic apoB mRNA levels did not accompany greater secretion of apoB by livers of cp/cp rats. Thus transcriptional regulation of apoB in the cp/cp JCR:LA-cp rat is unlikely. Transcriptional regulation of apoC-III or apoC-II also appears unlikely in this animal model, as steady state levels of mRNA for these apolipoproteins were not increased in the cp/cp rat. On the other hand, the finding that apoC-III mRNA was not reduced in the cp/cp rat was somewhat unexpected in view of previous findings of transcriptional down-regulation of the apoC-III gene following insulin treatment of diabetic mice and HepG2 cells transfected with an apoC-III luciferase reporter construct (48). The higher levels of apoA-I mRNA in livers of cp/cp rats is consistent with earlier findings of increased expression of endogenous apoA-I mRNA and enhanced activity of a rat apoA-I promoter in transfected Hep G2 cells in response to insulin (49). Greater apoA-I mRNA expression in cp/cp livers was accompanied by greater secretion of apoA-I by perfused livers and increased plasma content of HDL apoA-I and cholesterol (data not shown).

The availability of triglyceride is an important determinant of VLDL secretion. Triglyceride is synthesized in the liver from both dietary fatty acid and as a result of de novo lipogenesis. In these experiments, perfused livers were continuously infused with fatty acid. The rate of fatty acid uptake by perfused livers of cp/cp rats was not greater than that of +/? livers, and there was no significant depletion of the storage pool of triglyceride in perfused livers of cp/cp rats. We also demonstrate that de novo lipogenesis is increased in cp/cp livers. Therefore, greater secretion of triglyceride by cp/cp livers was likely the result of both preferential utilization of exogenous fatty acid for triglyceride synthesis and increased endogenous lipogenesis. Furthermore, higher rates of de novo fatty acid synthesis in livers of cp/cp rats was in part due to increased hepatic expression of mRNA for the key lipogenic enzymes ACC-1 and FAS. These data are consistent with prior observations of induction of lipogenic enzymes with insulin in vitro (14) and suggest that, in addition to increased dietary intake of fatty acid and carbohydrates, increased de novo lipogenesis is an important underlying cause of VLDL hypersecretion in the cp/cp rat.

Other factors (e.g., glucose and fatty acid) can also increase hepatic VLDL secretion and lipogenesis (14, 24, 50, 51). However, in the present experiments, non-fasting plasma glucose was only marginally higher in cp/cp rats and plasma free fatty acid was not increased. Furthermore, cp/cp livers secreted more VLDL despite the provision of equal amounts of fatty acid in the perfusate. A role of elevated free fatty acids in the pathogenesis of insulin resistance and hyperlipidemia in the cp/cp rat cannot be completely excluded, however, because these animals have an exaggerated FFA response to stressful stimuli (52). Higher rates of VLDL secretion in the cp/cp rat may also occur in response to increased dietary carbohydrate intake, with subsequent conversion into triglyceride by the liver (53). However, the findings of increased lipogenesis in vitro and increased mRNA for ACC and FAS clearly indicate that altered dietary carbohydrate intake is accompanied by an adaptive increment in the capacity for endogenous lipid synthesis in the cp/cp liver.

The observation of elevated levels of mRNA for the transcription factor SREBP-1 (also known as ADD-I) in the livers of hyperinsulinemic cp/cp rats is both novel and important in view of the proposed role of SREBP-1c in mediating the effect of insulin to regulate FAS and ACC (12, 13, 25). SREBPs were initially identified as transcription factors mediating cholesterol homeostasis [as reviewed in ref. (54)] and adipocyte differentiation (15). Two genes coding for SREBPs have been identified, SREBP-1 and 2. SREBP-2 seems to be involved primarily in regulating genes associated with cholesterol synthesis or turnover, whereas SREBP-1 regulates genes required for fatty acid synthesis. The gene for SREBP-1 has alternative promoters leading to expression of SREBP-1a or SREBP-1c, differing in their first exon. Although the probe used in the current studies does not specifically distinguish between the two forms of SREBP-1, SREBP-1c is the predominant product derived from this gene in liver and has stronger effects on transcription of lipogenic enzymes than on those involved in cholesterol metabolism (55). SREBP-1c mRNA is also elevated in other models of insulin resistance and hyperinsulinemia despite down-regulation of IRS-2, an essential component of the insulin-signaling pathway in liver (43). IRS-2 down-regulation occurs primarily as a result of transcriptional down-regulation by insulin (56). The persistence of elevated SREBP-1c in these animals and in the obese JCR rat reflects the continued ability of insulin to stimulate fatty acid synthesis despite IRS-2 down-regulation, and is in direct contrast to the loss of the ability of insulin to suppress both hepatic glucose synthesis and adipocyte lipolysis in insulin resistant states. This differential development of insulin resistance results in simultaneous overproduction of glucose and fatty acids by the liver, as well as increased flux of fatty acid to the liver from adipose tissue stores, which serves as a further stimulus for hepatic VLDL production as described by McGarry (57).

In summary, secretion of VLDL apolipoprotein and lipid by perfused livers of obese, hyperinsulinemic cp/cp JCR:LA-cp rats was greater than that observed in their lean littermates. De novo fatty acid synthesis, as well as expression of mRNA for the lipogenic enzymes FAS, ACC, and of the lipogenic regulatory factor SREBP-1c was also greater in the cp/cp rat. On the other hand, hepatic expression of apolipoprotein mRNA is either unchanged (apoB, apoC-III, apoC-II) or is only modestly increased (apoE, apoA-I). These data indicate that secretion of a greater number of VLDL particles that are enriched with triglyceride, apoE, and apoCs underlies the development of hyperlipidemia in the cp/cp JCR:LA-cp rat. Furthermore, up-regulation of hepatic lipogenesis and of the nuclear transcription factor SREBP-1c by insulin may be an important factor in the pathogenesis of VLDL hypersecretion in the cp/cp JCR-LA rat.

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