Development of Insulin Resistance in the JCR:LA–cp Rat

Role of Triacylglycerols and Effects of MEDICA 16

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The JCR:LA–cp rat develops an extreme obese/insulin-resistant syndrome such that by 12 weeks of age, there is no longer any insulin-mediated glucose turnover. At 4 weeks of age, obese and lean rats have essentially identical basal and insulin-mediated glucose uptake in skeletal muscle. By 8 weeks of age, however, the obese rats no longer exhibit such intake. Plasma insulin concentrations in the normal fed state show only small increases up to 4 weeks, with a rapid rise to a marked hyperinsulinemia thereafter, with an age at half-development of 5.5 weeks. Plasma triacylglycerol concentrations in fed obese rats are elevated at 3 weeks and rise rapidly thereafter. The triacylglycerol content of skeletal muscle is significantly elevated in the obese rats at 4 weeks of age. Histological examination of Oil Red O–stained muscle tissue and transmission electron microscopy shows the presence of intracellular lipid droplets. Treatment with the potent triacylglycerol-lowering agent MEDICA 16 (β,β′-tetramethylhexadecanedioic acid) from 6 weeks of age reduces plasma lipids markedly, but it reduces body weight and insulin resistance only modestly. In contrast, treatment with MEDICA 16 from the time of weaning at 3 weeks of age results in the normalization of food intake and body weight to over 8 weeks of age. The development of hyperinsulinemia is also delayed until 8.5 weeks of age, and insulin levels remain strongly reduced. Plasma triacylglycerol concentrations remain at the same level as in lean rats, and neither an elevated muscle triacylglycerol content nor intracellular lipid droplets are found at 4 weeks of age. The results indicate that insulin resistance develops in the young animals and is not directly due to a genetically determined defect in insulin metabolism. The mechanism of induction instead appears to be related to an exaggerated triacylglycerol metabolism. *Diabetes* 47:770–778, 1998

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Received for publication 8 August 1997 and accepted in revised form 4 February 1998.

2-DG, 2-deoxy-D-glucose; EDL, extensor digitorum longus; KRBH, Krebs-Ringer bicarbonate-HEPES; LPL, lipoprotein lipase; MEDICA 16, β,β′-tetramethylhexadecanedioic acid; NEFA, nonesterified fatty acid; TEM, transmission electron microscopy.

Type 2 diabetes is a reflection of an insulin-resistant metabolic state and is strongly associated with obesity and the metabolic syndrome (1,2). It is a prominent risk factor for both atherosclerosis (3,4) and hypertension (5).

The failure to uptake glucose by target tissues is believed to be the underlying pathophysiology of insulin resistance, and current opinion suggests that muscle may be the first and primary tissue that develops the abnormality (6,7). Skeletal muscle is the major tissue for the postprandial insulin-dependent uptake of glucose. The cellular and molecular mechanisms that link the development of insulin resistance in skeletal muscle and the growth of adipose tissue in obesity remain unknown. Peripheral tissues in fatty Zucker rats (8) and primates (9) have been shown to be insulin resistant earlier than the liver, whereas in high-fat–fed rats, insulin resistance in the liver and adipose tissue precedes its occurrence in skeletal muscle (10). A limited body of evidence suggests that intramuscular triacylglycerol may play a role in the development of insulin resistance (11), but no reports have defined the location within the muscle.

The *cp* gene was first isolated by Koletsky (12), who characterized the fulminant atherosclerosis in the original colony. The JCR:LA–cp strain is one of a number of rat strains with the *cp* gene superimposed on different background genotypes. Rats homozygous for the *cp* gene (*cp/cp*) become obese and insulin resistant, whereas those homozygous normal (+/+) or heterozygous remain lean. Among rodent strains, the JCR:LA–cp rat, if *cp/cp*, appears to be unique in the spontaneous development of vasculopathy, atherosclerosis, and ischemic myocardial lesions (13,14). This development appears to be related to the profound insulin resistance seen in young rats (15,16) and to the extreme hypersecretion of insulin (17).

MEDICA 16 (β,β′-tetramethylhexadecanedioic acid) is a powerful inhibitor of triacylglycerol synthesis at the ATP citrate lyase level (18,19). In the VLDL hyperlipidemic JCR:LA–cp rat, it also significantly decreases the activity of hydroxymethylglutaryl-CoA reductase, probably by reducing the cholesterol requirement for VLDL particle synthesis (19). Long-term treatment of *cp/cp* rats with established insulin resistance caused an 80% decrease in plasma triacylglycerol concentrations, reduced insulin levels, and was both antiatherosclerotic and cardioprotective (20).

The experiments described here were directed at identifying the mechanism underlying the development of the insulin resistance. In addition to characterizing the development of the insulin resistance and abnormal metabolism in young rats, we have modulated these through the reduction of the associated hypertriglyceridemia with MEDICA 16 (19).

RESEARCH DESIGN AND METHODS

Animals. Male rats, either *cp/cp* (obese) or +/+ or +/? (lean), of the JCR:LA–cp strain were bred in the established colony at the University of Alberta, as described previously (20). The rats were maintained in polycarbonate cages on wood-chip bedding and fed a standard low-fat rat diet (Teklad Rodent Diet; Harlan Sprague Dawley, Madison, WI). Rats used for metabolic studies were maintained on a reversed light cycle (lights off at 0600 and on at 1800 h) in an isolated room throughout the protocols. The rats were treated with MEDICA 16 from the time of weaning at 3 weeks of age or from 6 weeks of age. Body weights and food consumption were measured twice weekly. The drug was incorporated into the feed at a concentration of 0.25% (wt/wt), as described previously (20). This gave a dose of 375 mg/kg body weight at 4 weeks of age, decreasing to 250 mg/kg at 12 weeks of age. All metabolic studies and the killing of animals were conducted under halothane anesthesia. During the early portion of the dark (active) phase of the animals' diurnal cycle, blood samples were obtained from the tip of the tail of rats that had been previously conditioned to handling and the procedure. All care and treatment of the rats was in conformity with the guidelines of the Canadian Council on Animal Care and subject to prior institutional review and approval.

Tissue sampling. Animals used for the collection of tissue samples were fasted overnight and lightly anesthetized with halothane; blood was collected by cardiac puncture. The rats were then killed by cervical dislocation, and the soleus and extensor digitorum longus (EDL) muscles were rapidly excised for glucose transport studies. Lateral and medial gastrocnemius and soleus muscles and liver were excised and freeze-clamped in liquid nitrogen for later analysis.

Glucose clearance. These studies were performed in a postabsorptive state with the animals anesthetized with halothane in oxygen using a mask and maintained on a heated table. The rats were given a 10-μCi bolus injection (0.5 ml of a 20-μCi/ml stock solution) of tracer levels of [^{3}H]2-deoxy-D-glucose (2-DG; Du Pont Canada, Mississauga, Ontario, Canada) into the portal vein. Blood (150 μl) was sampled at 2, 5, 10, 20, 30, 40, 50, and 60 min via a femoral artery cannula.

Glucose uptake at basal insulin levels. Rats were given a 10-μCi bolus injection of [^{3}H]2-DG into the portal vein. A cardiac puncture was performed after 10 min, and the animal was exsanguinated. Samples of the liver, diaphragm, heart, brain, and epididymal/oviductal (pelvic), perirenal, and subcutaneous fat were taken from each rat and maintained on ice. Following dissection, 200 mg of each tissue was weighed and solubilized in 1 ml of Solvable (Du Pont Canada). The samples were heated at 60°C until solubilization was complete, and then 15 ml of Atomlight (Du Pont Canada) was added to the solubilized tissue and allowed to stand 48 h before liquid scintillation counting. After initial counting, internal standard (^3H_2O, 5.6×10^4 dpm in 30 μl; Du Pont Canada) was added to the vials and allowed to stand another 48 h before final counting. The results, expressed as dpm/g of tissue, were normalized to the brain and are expressed as relative uptake (i.e., brain = 1.0).

Glucose uptake under hyperinsulinemic conditions. A euglycemic hyperinsulinemic clamp was established under light halothane anesthesia as previously described (16). Once the euglycemic hyperinsulinemic state was stable ((30 min), a bolus injection of tracer levels of [^{3}H]2-DG (10 μCi in 0.5 ml) was injected into the portal vein. After a 10-min period, final blood samples were taken for plasma insulin and radioactivity levels. Exsanguination was performed through cardiac puncture, and tissues were dissected, processed, and counted as described above.

Analytical methods

Plasma samples. Samples were analyzed for plasma glucose by a glucose oxidase technique (Beckman Instruments, Brea, CA) and for insulin by radioimmunoassay (Insulin RIA 100; Kabi Pharmacia, Uppsala, Sweden, with rat insulin standards). Plasma lipids were measured using the total lipid profile technique of Kuksis et al. (21). Nonesterified fatty acids (NEFAs) were determined as described by Novak (22), after extraction with Dole's reagent.

Tissue triacylglycerol determination. Aliquots of frozen skeletal muscle and liver samples were homogenized at 4°C using a Polytron homogenizer (Brinkman, Westbury, NY) in HEPES buffer (10 mmol/l HEPES, 0.25 mol/l sucrose, 1 mmol/l EDTA, and 1 mmol/l dithiothreitol). Triacylglycerols were extracted from the homogenates and from sera by shaking in 20 volumes of chloroform:methanol (2:1 vol/vol) (23) overnight and were quantitated colorimetrically as glycerol using an enzymatic assay kit (Sigma, St. Louis, MO).

Lipoprotein lipase assay. Total tissue lipoprotein lipase (LPL; EC 3.1.1.34) activity was assayed in frozen muscle samples by the measurement of [^{3}H]oleate released from [^{3}H]triolein, as previously described (24).

In vitro determination of glucose transport. Glucose uptake was determined using 2-DG, as previously described (25). Intact soleus and EDL muscles were rapidly suspended, under slight tension, in Krebs-Ringer bicarbonate-HEPES (KRBH) buffer supplemented with 0.1% bovine serum albumin and 2 mmol/l sodium pyruvate, constantly gassed with 95% O_2/5% CO_2, and maintained at 37°C. The ionic composition was 119 mmol/l NaCl, 5 mmol/l HEPES, 2.3 mmol/l $CaCl_2$, 5 mmol/l KCl, 1 mmol/l $MgSO_4$, 1 mmol/l KH_2PO_4, and 25 mmol/l $NaHCO_3$ (pH 7.4). After preincubation for 30 min, the muscles were washed with fresh KRBH buffer and transferred to KRBH buffer supplemented with 0.1% bovine serum albumin and 4 mmol/l 2-DG containing 1 μCi/ml [^{3}H]2-DG in the absence or presence of insulin (100 nmol/l). After a 30-min incubation, the muscles were washed three times in KRBH at 4°C, blotted dry, weighed, and placed in 0.5 ml 0.5 N NaOH overnight. The tissues were homogenized, and radioactive content was determined by counting in a liquid scintillation counter.

Histology. Tissue samples for examination for lipid by light microscopy were frozen and embedded in embedding medium. Frozen sections were cut in a cryostat and stained with Oil Red O and counterstained with hematoxylin.

Electron microscopy. Samples of skeletal muscle and aorta were fixed in cacodylate-buffered glutaraldehyde. After fixation, the samples for visualization of lipid were stained for 1 h with 1% OsO_4 and overnight with 0.1% thymol in 0.34 mmol/l sucrose. After a further 1 h in 1% OsO_4, the samples were stained for 1 h in 4% myrecene in 80% ethanol and a further 1 h in OsO_4 (26,27). All tissues were dehydrated through graded ethanol and embedded in Spurr's resin. Thin sections were mounted on 200-mesh copper grids and stained with uranyl acetate and lead citrate before examination with a JOEL 1200 EX TEM (Japan Electron Optics, Montreal, Quebec).

Statistical analysis. Curve fitting for the clearance of glucose was by nonlinear technique using SigmaPlot (Jandel Scientific, San Rafael, CA) and the two-compartment rate equation, $[2\text{-DG}] = ae^{-k1^t} + be^{-k2^t}$. Statistical analysis was by analysis of variance and unpaired t test, as appropriate, using the statistical package SigmaStat (Jandel Scientific). A value of $P < 0.05$ (two-tailed) was considered to be significant. Logistic curves were fitted using the program ALLFIT (28).

RESULTS

Glucose clearance. The clearance of 2-DG in 3-month-old *cp/cp* and +/+ rats was measured as a function of time under basal conditions. The clearance was exponential and biphasic, and the data were fitted assuming two first-order decays. Rate constants were $k_1 = 0.180 \pm 0.016$ and $k_2 = 0.015 \pm 0.008$ for +/+ rats and $k_1 = 0.161 \pm 0.088$ and $k_2 = 0.0053 \pm 0.0164$ for *cp/cp* rats. Thus, both rate constants were greater for +/+ male rats than for *cp/cp* rats, but the apparent differences between genotypes were not significant ($P < 0.05$).

Tissue uptake of glucose. The relative uptake of 2-DG in various organs in 3-month-old rats at basal insulin levels and under hyperinsulinemic conditions is shown in Fig. 1. At basal insulin levels, *cp/cp* animals showed no significant differences in glucose uptake in the liver, diaphragm, or heart compared with +/+ males. Glucose uptake in the subcutaneous fat depot of *cp/cp* animals was far less than in the same fat depot in +/+ animals ($P < 0.001$). The raw data, expressed as dpm/g of tissue, exhibited the same patterns of results, but with somewhat greater animal-to-animal variance. In the +/+ rats, the elevated insulin levels resulted in no change in the rate of glucose uptake in the brain and similarly had no significant effect on the relative uptake in the liver, heart, or fat depots; however, there was a large increase in uptake in the muscle ($P < 0.001$). The *cp/cp* rats showed no significant change in uptake, in any organ, in response to increased levels of insulin. Steady-state plasma insulin concentrations during the hyperinsulinemic glucose uptake studies were 350 ± 150 for +/+ and 200 ± 70 nmol/l for *cp/cp* rats. These insulin values illustrate that high insulin levels were achieved during the experiment.

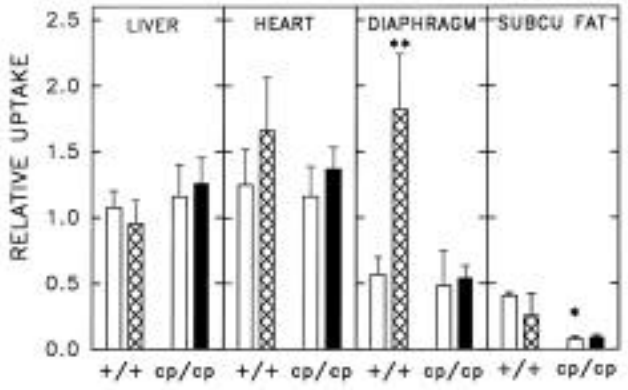

FIG. 1. In vivo uptake of [^{3}H]2-DG at basal levels and under hyperinsulinemic conditions by various tissues normalized to that of the brain. Of each pair of bars, the left (□) represents basal insulin levels, and the right (■, ⊠) represents hyperinsulinemic conditions. Rats were 3 months old; there were 5 rats in each group. Values are means ± SD. *P < 0.05 vs. +/+ basal conditions; **P < 0.001 vs. basal insulin. Absolute uptake in the brain was 222 ± 53 and 183 ± 26 dpm/μg for +/+ and *cp/cp* rats, respectively. These values were not significantly different (P > 0.05).

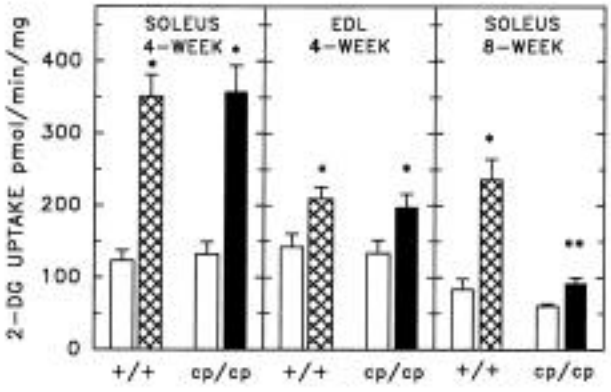

FIG. 2. In vitro uptake of 2-DG by intact soleus and EDL muscles from *cp/cp* and +/+ rats at 4 and 8 weeks of age. □, basal insulin concentrations; ■, uptake under insulin-stimulated conditions. Values are means ± SE. *P < 0.05 vs. basal insulin; **P < 0.01 vs. +/+ insulin-stimulated.

The in vivo glucose uptake studies described above are not possible in very young rats because of the excessive stress that would be imposed on the immature and small animals. Thus, further studies of glucose uptake in 4- and 8-week-old rats were conducted on muscle in vitro using an organ bath technique. Soleus muscle from 4-week-old *cp/cp* or +/+ rats showed a threefold increase in 2-DG uptake in response to insulin (Fig. 2). The EDL muscle of the *cp/cp* rats showed basal and insulin-mediated 2-DG uptake similar to that of the lean animals at 4 weeks of age. In contrast, soleus muscle from 8-week-old *cp/cp* rats showed a reduced uptake of 2-DG and no significant insulin-mediated increase, whereas muscle from +/+ rats retained its threefold response to insulin. The weights of the muscles used from +/+ and *cp/cp* rats, respectively, were: soleus at 1 month, 37.5 ± 1.7 vs. 33.8 ± 3.6 mg, and at 8 weeks, 83.5 ± 3.5 vs. 92.5 ± 2.9 mg (P < 0.05); EDL at 4 weeks, 47.0 ± 1.6 vs. 42.5 ± 3.3 mg. Because the mass/surface area ratio might affect the ability of a tissue to respond to the insulin stimulus, it is possible that the weight differences may have led to a spurious effect. However, the differences were small, and when the data were expressed as uptake per minute per muscle, the insulin-mediated uptake by muscle from *cp/cp* rats remained significantly reduced compared with that from +/+ animals.

Tissue triacylglycerol content. As shown in Fig. 3, the muscle and liver triacylglycerol content of *cp/cp* rats is elevated over that of +/+ rats at 4 weeks of age, before the development of insulin resistance and the increase in fed-state insulin (see below). The marked elevation of tissue triacylglycerols seen in *cp/cp* rats is highly consistent over both age and the two muscles studied, and it increases further up to 12 weeks of age. In *cp/cp* rats treated with MEDICA 16 from 3 weeks of age, the triacylglycerol content of soleus muscle was not elevated at 4 weeks of age (Fig. 4). This effect mirrors the reduction in plasma triacylglycerols (see below) and precedes development of hyperinsulinemia (see below).

Tissue LPL activity. At 12 weeks of age, there was no difference in LPL activity of gastrocnemius muscle between +/+ rats (0.83 ± 0.4 mmol oleate · h^{-1} · mg protein^{-1}) and *cp/cp* rats (0.90 ± 0.06 mmol oleate · h^{-1} · mg^{-1}). In comparison, LPL activity in soleus muscle was lower in *cp/cp* rats (0.18 ± 0.08 mmol oleate · h^{-1} · mg^{-1}) than in +/+ rats (0.41 ± 0.03 mmol oleate · h^{-1} · mg^{-1}; P < 0.05).

Muscle morphology: light microscopy. Oil Red O–stained sections of the lateral gastrocnemius and soleus muscles from *cp/cp* rats at 1, 2, and 3 months of age showed lipid droplets within some muscle bundles and extracellular lipid between the muscle bundles (Fig. 5*A* and *B*). Sections from age-matched +/+ animals contained no visible lipid (Fig. 5*C*). The size and number of lipid droplets appeared to increase with age in the *cp/cp* animals.

Muscle morphology: transmission electron microscopy. As observed by transmission electron microscopy (TEM), lipid droplets were associated with mitochondria in the gastrocnemius muscle from *cp/cp* rats at all ages (Fig. 6*A* and *B*) and were marked in muscle tissue from 1-month-old animals. No such lipid droplets were seen in the muscle tissue from any lean animals examined (Fig. 6*C* and *D*). Membrane remnants within degenerate mitochondria were seen in muscle tissue from animals of both genotypes and at all ages

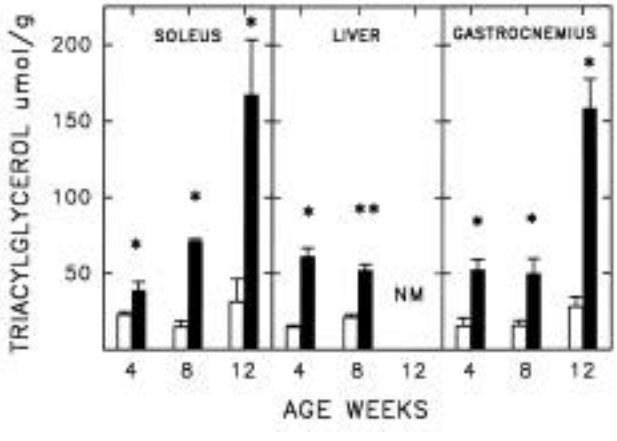

FIG. 3. Triacylglycerol content of muscle and liver of *cp/cp* (□) and +/+ (■) rats showing genotype differences and the changes with age. Values are means ± SE. *P < 0.05; **P < 0.01 vs. +/+.

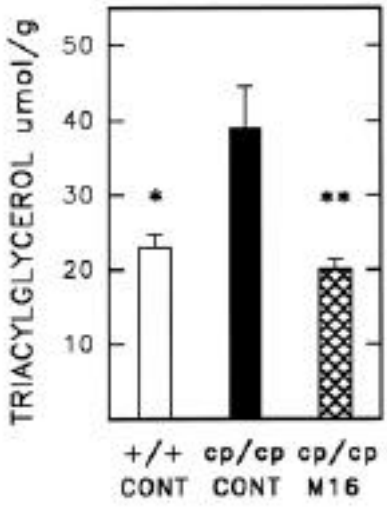

FIG. 4. Effect of MEDICA 16 treatment from 3 weeks of age on triacylglycerol content of soleus muscle at 4 weeks of age. Values are means ± SE. *P < 0.01; ** P < 0.001 vs. *cp/cp* control.

(Fig. 6*A–D*), but they were most evident in tissue from *cp/cp* animals and increased with age. In addition, in *cp/cp* rats, large mitochondrial aggregates with some lipid were present in the peripheral region of the muscle cells (Fig. 7). The distribution of lipid droplets was not uniform within the muscle but was confined to certain bundles. This was evident in the Oil Red O–stained tissues (Fig. 5*A* and *B*) as well as at the TEM level. As observed by TEM, adjacent bundles appeared essentially normal. The lipid visible within the muscle was consistent with the measured triacylglycerol content, above, which was as high as 10% of the muscle mass in the *cp/cp* rats at 12 weeks of age. In muscle tissue from animals treated with MEDICA 16, membrane remnants were present within the mitochondria, but no lipid droplets were seen (Fig. 8). Occasional adipocytes and extracellular lipid were observed in muscles from animals of both genotypes and at all ages.

Plasma triacylglycerol concentrations. At 3 weeks of age, plasma triacylglycerol concentrations of the *cp/cp* rats in the fed state were modestly but significantly elevated compared with those of the +/+ rats (1.63 ± 0.44 vs. 0.42 ± 0.12 mmol/l, P < 0.05; Fig. 9). As the animals matured from 3 to 12 weeks, the +/+ rats showed a slight decrease in triacylglycerol, to 0.33 ± 0.08 mmol/l, whereas in the *cp/cp* rats, triacylglycerol underwent an immediate and apparently linear increase of 0.50 mmol/l per week (Fig. 9). Treatment of male *cp/cp* rats with MEDICA 16 from 6 to 12 weeks of age resulted in a marked reduction in fasting plasma triacylglycerols (2.64 ± 0.95 to 0.61 ± 0.17 mmol/l). Treatment of *cp/cp* rats with MEDICA 16 at 3 weeks of age lowered the plasma triacylglycerol concentration at 4 weeks of age to 0.41 mmol/l, a value identical to that of +/+ rats. At 12 weeks of age, rats treated with MEDICA 16 from weaning at 3 weeks maintained low plasma triacylglycerol levels that were only twofold, and nonsignificantly, greater (P < 0.05) than those of +/+ rats (Fig. 9).

NEFAs. Plasma NEFA was consistently elevated in the *cp/cp* rats (Table 1). Values for *cp/cp* rats increased with age. They were significantly elevated at 8 weeks of age (P < 0.05) and were even more significantly doubled (P < 0.01) at 12 weeks of age.

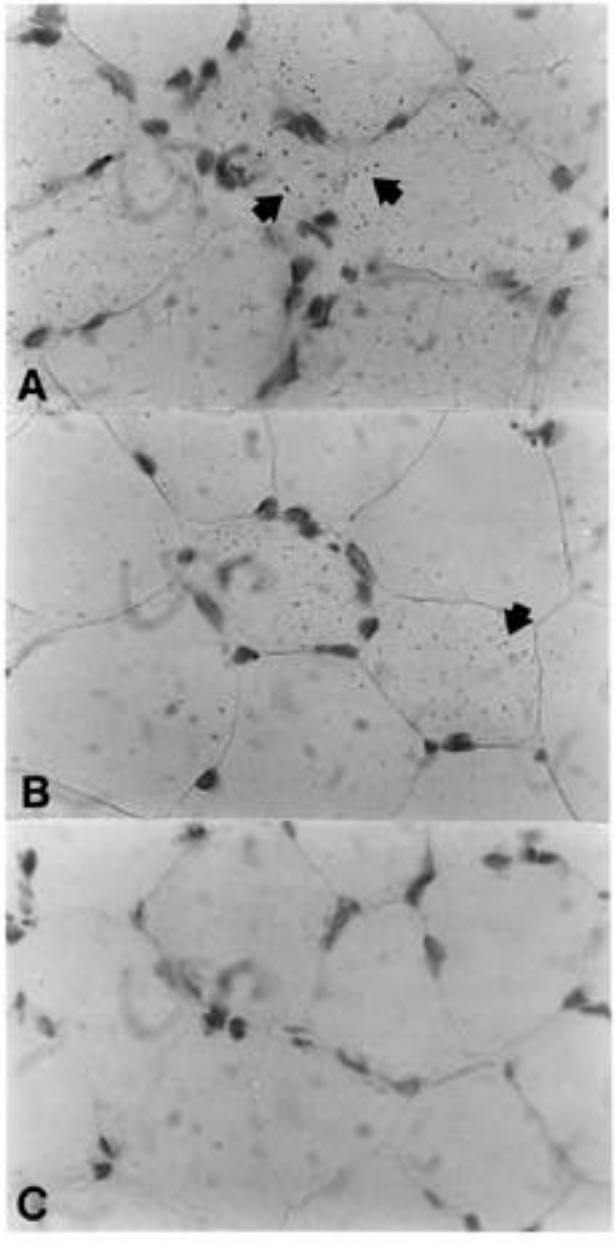

FIG. 5. Photomicrographs of skeletal muscle from JCR:LA–cp rats stained with Oil Red O for neutral lipids and the nuclei counterstained with hematoxylin. *A*: Lateral gastrocnemius muscle from a 12-week-old *cp/cp* rat showing multiple lipid droplets (some indicated by arrows) within the fibers (the lipid appears black). Nuclei appear as larger, stained bodies. *B*: Soleus muscle from a 12-week-old *cp/cp* rat containing visible lipid droplets. *C*: Gastrocnemius muscle from a 12-week-old +/+ rat containing no visible lipid inclusions. Original magnification ×630.

Plasma insulin concentration. Plasma insulin levels of unmanipulated male rats sampled during their dark phase and in a normal fed state are shown in Fig. 10. Whereas +/+ rats showed a modest increase in fed-state insulin as they matured from the time of weaning at 3 weeks to 12 weeks of age, *cp/cp* rats showed an abrupt increase to marked hyperinsulinemia, with an age at half-development of 5.5 weeks. Treatment of *cp/cp* rats with MEDICA 16 from 6 to 12 weeks

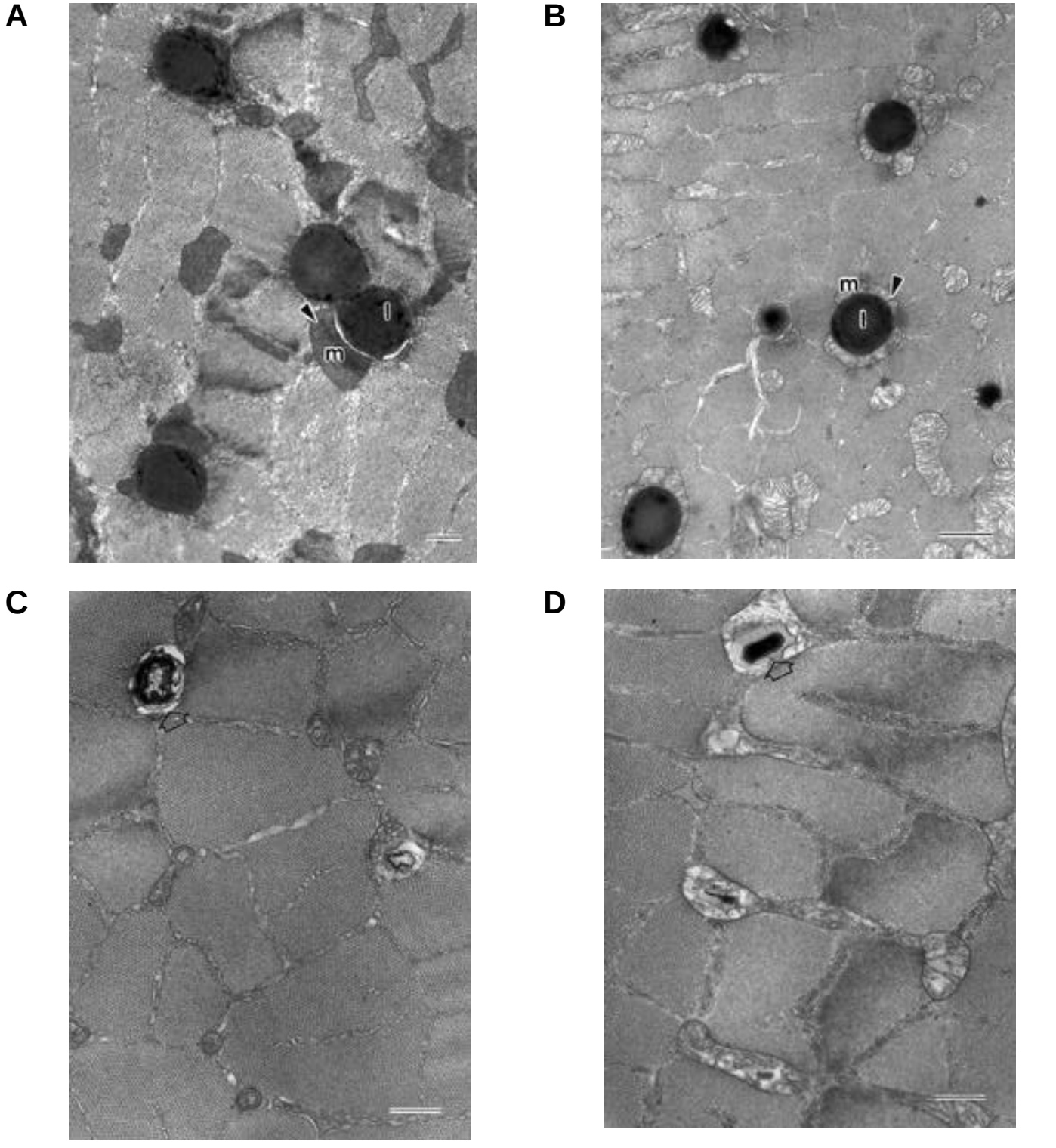

FIG. 6. Transmission electron micrographs of transverse sections of gastrocnemius muscle. The tissue has been treated to retain lipid within the sections. *A*: 1-month-old *cp/cp* rat; *B*: 9-month-old *cp/cp* rat. Lipid droplets (l) are closely associated with mitochondria (m), which contain intact cristae (arrowhead) in the muscle from *cp/cp* rats. *C*: 1-month-old +/+ rat; *D*: 9-month-old +/+ rat. There was no evidence of lipid droplets within muscle from any lean animals. In muscle tissue from all animals, some mitochondria contained distorted membrane-derived forms that appeared to be rich in lipid, and cristae were not evident (open arrow). These degenerate mitochondria were more evident in older animals than in young animals. Bar = 500 nm.

of age did not significantly reduce fasting plasma insulin levels at 12 weeks of age (1,116 ± 394 vs. 1,594 ± 358 pmol/l; $P < 0.05$). Treatment with MEDICA 16 from 3 weeks of age delayed the development of the hyperinsulinemia to the age of 8.5 weeks and markedly reduced the ultimate insulin levels in the fed state (Fig. 10).

Body weight and food consumption. As shown in Fig. 11, *cp/cp* rats do not have significantly greater body weights than

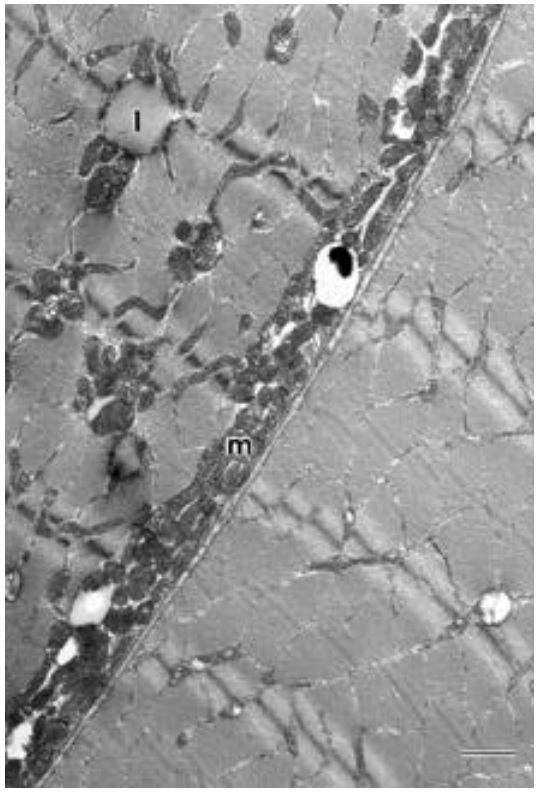

FIG. 7. Transmission electron micrograph from the muscle of a 3-month-old *cp/cp* rat. The peripheral regions of two muscle bundles are shown. In one, there are large lipid droplets (l) and an accumulation of mitochondria. The adjacent bundle shows no obvious abnormalities, including an absence of lipid. Bar = 1 µm.

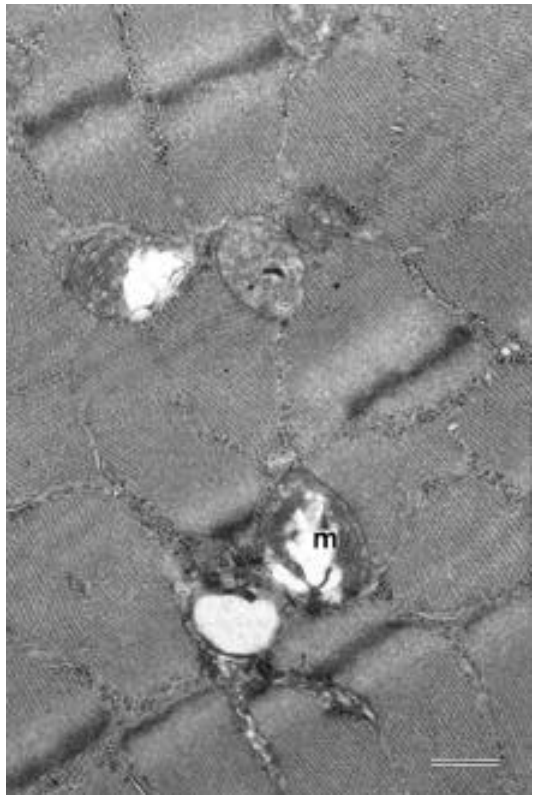

FIG. 8. Transmission electron micrograph from the muscle of a 1-month-old *cp/cp* rat treated with MEDICA 16. There was no evidence of lipid droplets associated with the mitochondria (m), but many of the mitochondria are distorted, and the cristae have degenerated. Bar = 500 nm.

do +/+ rats at the time of weaning (3 weeks of age). Whereas food consumption was significantly greater at or immediately after weaning, this difference was also small. Both food intake and body weights diverged rapidly thereafter, with the *cp/cp* rats becoming markedly obese over the period of a few weeks. Treatment with 0.25% MEDICA 16 from 3 weeks of age normalized the food intake of the *cp/cp* rats to that of the +/+ animals until 6 weeks of age, after which time food intake of the treated rats rose slowly to that of the *cp/cp* control animals. Body weights of the MEDICA 16–treated rats remained identical to those of +/+ controls to beyond 10 weeks of age, before rising slightly (Fig. 11). In contrast, *cp/cp* rats treated with MEDICA 16 from 6 weeks of age, at a time when the insulin resistance and obesity are already well established, showed only a small, nonsignificant decrease in food intake and body weights over the period to 12 weeks of age (Fig. 11).

DISCUSSION

The *cp* gene has recently been identified as a mutation leading to a stop codon in the extracellular domain of the leptin receptor (29). This leads to the absence of any leptin receptor in the plasma membrane of *cp/cp* rats, in contrast to the other obese rat gene, the *fa,* that leads to a reduced binding affinity of leptin and a leptin-resistant state (20). Leptin strongly inhibits pancreatic insulin secretion (30), and the difference in the *fa* and *cp* mutations may explain the more marked hypersecretion of insulin by the *cp/cp* rat (17).

The obesity/insulin-resistant syndrome that is so striking in the mature *cp/cp* rat is only just detectable at the time of weaning at 3 weeks of age. There is, as shown in Fig. 11, no difference in body weights between the genotypes at 3 weeks. Plasma insulin is minimally elevated and does not begin to rise rapidly until the animals are more than 5 weeks of age (Fig. 10). In contrast, the plasma triacylglycerol con-

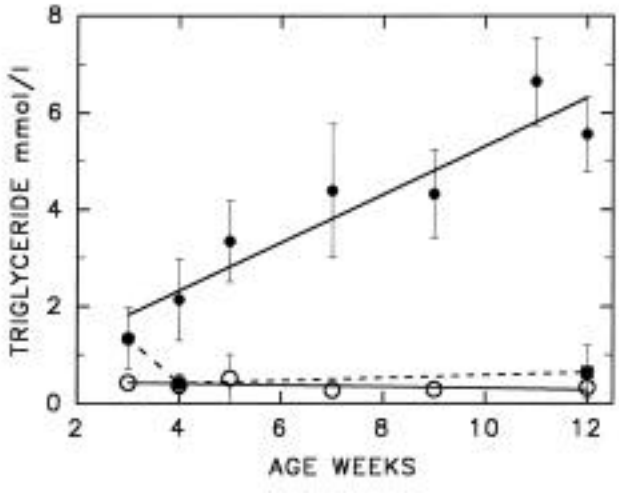

FIG. 9. Plasma triacylglycerol levels as a function of age in +/+ (○) and *cp/cp* (●) rats and *cp/cp* rats treated with MEDICA 16 from 3 weeks of age (■).

TABLE 1
Fasting serum NEFA

	Serum NEFA (g/l)		
	4 weeks old	8 weeks old	12 weeks old
+/+	0.16 ± 0.02 (4)	0.10 ± 0.01 (4)	0.04 ± 0.01 (4)
cp/cp	0.22 ± 0.02 (3)	0.20 ± 0.04* (4)	0.30 ± 0.08**(6)

Data are means ± SE (n). Values represent the fasting state. The values for the *cp/cp* rats are significantly different from those of the +/+ rats at 8 and 12 weeks of age. *P < 0.05; **P < 0.01.

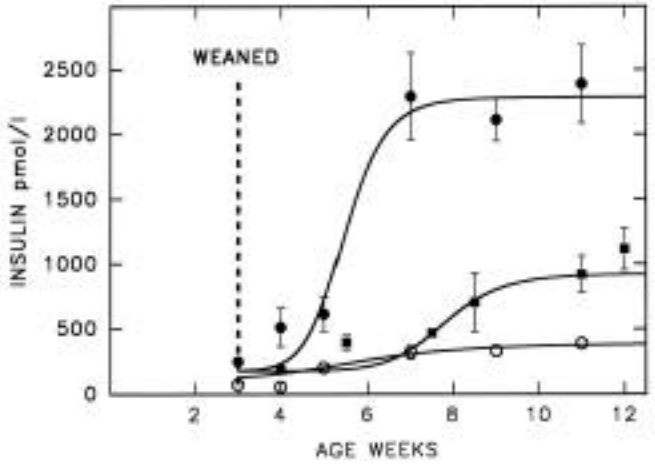

FIG. 10. Plasma insulin levels of freely fed male rats 3 h into the dark phase of their diurnal cycle. ○, +/+; ●, *cp/cp*; ■, *cp/cp* treated with MEDICA 16 from 3 weeks of age. There were no significant differences in the plasma glucose levels between groups. At 11 weeks of age, these were 7.89 ± 1.33, 8.00 ± 0.67, and 8.00 ± 2.11 mmol/l (mean ± SD) for +/+, *cp/cp* control, and *cp/cp* MEDICA-treated rats, respectively.

centration is already very significantly elevated at 3 weeks of age and is rising rapidly. Thus, the significant abnormality of lipid metabolism precedes the insulin resistance and the associated hyperinsulinemia. The insulin resistance clearly is not simply a consequence of a genetic defect directly affecting the insulin/insulin-receptor/glucose-transporter mechanism; rather, it develops as the animal matures. It must thus reflect an induced metabolic dysfunction, one possibly secondary to hyperphagia and exacerbated by the absence of leptin-mediated inhibition of insulin secretion. This is consistent with our findings of essentially normal insulin-binding in the muscle, liver, and brain (D.F.C. Hopkins, G. Williams, J.C.R., unpublished observations).

At 12 weeks of age, *cp/cp* rats under the conditions of this study, both in vivo and in vitro, have no insulin-mediated glucose uptake by skeletal muscle (Fig. 1) (16). The in vitro results in Fig. 2 reveal that this defect is not present at 4 weeks of age, but is evident at 6 weeks of age, and is fully developed by 8 weeks of age. Elevated plasma triacylglycerol levels at 4 weeks of age precede the development of insulin resistance in the *cp/cp* rat (Figs. 9 and 10). MEDICA 16 given from 6 weeks of age does not markedly ameliorate the severity of the established metabolic disturbance, although it does reduce plasma lipids significantly (19), and long-term treatment results in reduced fasting insulin levels and protection against cardiovascular disease (20). However, if the drug is administered from 3 weeks of age, at a time when the insulin resistance is not yet established, the hyperphagia is prevented for >5 weeks and the obesity for >7 weeks (Fig. 11). This results in normalization of plasma triacylglycerol concentrations at 4 weeks; and although the concentrations had increased by a factor of two at 12 weeks of age, they remained dramatically reduced compared with those of the *cp/cp* rats. Eventually, the protection provided by MEDICA 16 is insufficient, and the insulin levels rise. Nonetheless, the ultimate hyperinsulinemia is still greatly reduced. These effects suggest that an abnormality of lipid metabolism underlies the insulin resistance. It appears that MEDICA 16,

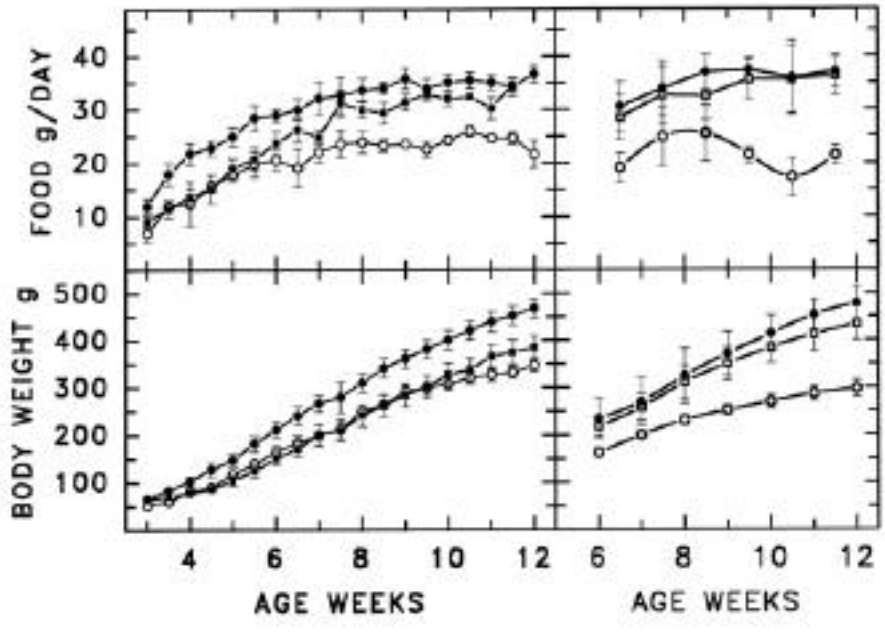

FIG. 11. Food intake and body weights of rats treated with MEDICA 16 from 3 to 12 weeks of age (left panels) and from 6 to 12 weeks of age (right panels). ○, +/+ control; ●, *cp/cp* control; ■, *cp/cp*.

by inhibiting the insulin resistance itself, reduces the need for excessive insulin production and the hyperplasia of the islets of Langerhans, resulting in near-normal morphology of the islets (20).

The muscles studied were of metabolically different fiber types: the soleus is of a red oxidative type, and the EDL is a mixture of oxidative-glycolytic and glycolytic fibers. Thus, the muscles studied may be representative of skeletal muscle in general. The absence of any insulin-mediated glucose uptake in the in vitro diaphragm (Fig. 1), or in the whole animal (16), in the *cp/cp* rats at 12 weeks of age is entirely consistent with the results obtained from the isolated soleus muscle (Fig. 2). It is thus evident that the primary element of the insulin resistance is an inability of the skeletal muscle to respond to insulin with glucose uptake. The hyperinsulinemia appears to be an adaptive response resulting in normoglycemia and is due to islet β-cell hypersecretion (17).

The triacylglycerol content of both soleus and gastrocnemius muscle was significantly elevated in *cp/cp* rats at 4 weeks of age and rose rapidly thereafter. At 4 and 8 weeks of age, the liver of the *cp/cp* rats also showed much greater triacylglycerol content. The results in Fig. 4, showing a reduction in muscle triacylglycerol at 4 weeks of age in MEDICA 16–treated *cp/cp* rats, support the suggested role for intracellular triacylglycerol in the development of insulin resistance. The early rise in plasma triacylglycerol seen in Fig. 9 is also consistent with the hypothesis of a critical role for abnormal triacylglycerol metabolism in the insulin resistance of the *cp/cp* rat. Light microscopy of Oil Red O–stained sections (Fig. 5) indicates that some of the lipid is intramuscular. The TEM images in Fig. 6 confirm that the lipid is within the muscle cells. The lipid is associated with mitochondria, especially those among myofibrils, but also in peripheral mitochondrial aggregates. The 4-week-old *cp/cp* rats had numerous lipid droplets within the muscle, and treatment with MEDICA 16 for 1 week resulted in an absence of detectable intramuscular lipid at 4 weeks of age, consistent with the suggested major role for triacylglycerol in the development of the insulin resistance.

Because the rats had been fed a low-fat, high-starch diet after weaning, the triacylglycerols present in serum, muscle, and liver are mainly the result of de novo fatty acid synthesis. Serum triacylglycerol is almost exclusively present as VLDL, the secretion of which by the liver is excessive (31). Fatty acid synthesis in *cp/cp* rats takes place predominantly in the liver, whereas the high level of serum triacylglycerol suppresses de novo fatty acid synthesis in adipocytes (32). Insulin resistance in older animals decreases glucose uptake by adipose tissue and, thus, the synthesis of free fatty acid from glucose. Therefore, the expansion of adipose tissue seen in the *cp/cp* rats is fueled by high serum triacylglycerol, the uptake of which requires insulin-stimulated LPL activity, and the adipocytes are probably not insulin resistant.

By the time the *cp/cp* rats were 1 month old, triacylglycerol concentrations in serum, muscle, and liver were already fourfold higher than in lean animals, whereas fasting serum insulin levels were within the normal range. Insulin is known to directly promote the synthesis and secretion of triacylglycerol-rich lipoproteins in various liver preparations in vitro and in rats in vivo (33). In confirmation of this, an insulin-responsive element in the promoter region of the fatty acid synthase gene has recently been reported (34). Whether the slight elevation of fasting insulin concentrations was sufficient to cause the observed excessive hepatic triacylglycerol synthesis is debatable, but there is evidence of a defect in the regulation of fatty acid synthesis (35).

In obese *cp/cp* rats, large amounts of triacylglycerol are taken up by adipose tissue and muscle, and the high serum triacylglycerol levels are more a matter of an overloaded system due to increased hepatic VLDL secretion than a problem of clearance (31). Do these high serum or muscle triacylglycerol levels induce skeletal muscle insulin resistance? It can be argued that because mice that are transgenic for apolipoprotein C-III do not become insulin resistant despite extreme hypertriglyceridemia, high serum triacylglycerol alone does not lead to reduced insulin sensitivity (36). However, the elevated serum triacylglycerol levels in these transgenic mice are due to reduced clearance rather than to increased secretion of VLDL, and triacylglycerol levels may be expected to have been low.

Elevated plasma NEFA levels have been implicated in the pathogenesis of insulin resistance (6,7). Enlarged adipose tissue mass may lead to increased lipolysis and NEFA turnover, which would stimulate hepatic production of VLDL and lead, in turn, to elevated serum and muscle triacylglycerol levels. The increased NEFA available to the muscle would compete with glucose as an oxidative substrate, according to Randle's glucose-fatty acid cycle, thus giving rise to insulin resistance. However, the increased plasma NEFA levels of the *cp/cp* rat lag, rather than precede, the development of insulin resistance (Table 1), suggesting that this possible mechanism does not contribute to insulin resistance in the *cp/cp* rat.

By the age of 1 month, skeletal muscle of *cp/cp* rats, in common with that of humans with NIDDM (35) and fat-fed rats (37), contains elevated levels (three- to fourfold) of triacylglycerol (Figs. 3 and 6). Although part of this triacylglycerol may be from adipocytes among the muscle fibers, lipid droplets were visible within the fibers of gastrocnemius and soleus muscle from *cp/cp* rats. The accumulation of triacylglycerol within the fibers cannot be explained by increased muscle LPL activity, which was lower in the muscle of *cp/cp* rats of 1 month of age. However, high fasting serum triacylglycerol may drive an uptake by the muscles on a continual basis, resulting in an increased uptake in the presence of normal or reduced lipase activity. Because insulin has been shown to increase LPL mRNA and LPL synthesis rate, at least in rat adipocytes, and to activate LPL by posttranslational modifications in humans (38), a trend to lower muscle LPL activity may be an early sign of insulin resistance.

Investigation of individual stages of a multifactorial disease such as NIDDM may only be possible in a model like the JCR:LA–cp rat. We are attempting to define the first of the metabolic stages: the onset of insulin resistance during the development of obesity. Based on the present data, we postulate that elevated serum lipids (due to hepatic hypersecretion) promote the uptake and storage of triacylglycerol in muscle. We have reported evidence that stored muscle triacylglycerol may have a direct effect on insulin signal transduction that results in reduced glucose transport (39). The resultant change in insulin secretion would lead to a decrease in glucose tolerance and a compensatory increase in insulin secretion, resulting in a rapid escalation of the hyperinsulinemia. Substantial lowering of serum triacylglycerol would

inhibit this sequence, and, indeed, MEDICA 16 treatment causes such an effect and greatly blunts the development of both the insulin resistance and the obesity. The underlying important question, then, pertains to the origin and mechanism of the lipid-mediated change in signal transduction between the insulin receptor and the glucose transporters. These findings in an extreme animal model of insulin resistance emphasize the role of the development of metabolic abnormalities in human adolescence. They also lead to optimism that drugs such as MEDICA 16 may allow for highly effective treatment of insulin resistance and prevention of its major sequelae.

ACKNOWLEDGMENTS

This work was supported by grants from the Heart and Stroke Foundations of Alberta and the Northwest Territories (J.C.R.), of Ontario (M.R.), and of Nova Scotia (P.J.D.); the Canadian Diabetes Association (G.S., D.C.W.L.); and the Medical Research Council of Canada (D.C.W.L.).

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