

Pharmacogenetic Analysis of Rosiglitazone-Induced Hepatosteatorosis in New Mouse Models of Type 2 Diabetes

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Although thiazolidinediones suppress hyperglycemia in diabetic (NON × NZO)F1 males, these mice exhibit unusual sensitivity to drug-induced exacerbation of an underlying hepatosteatorosis only rarely experienced in human patients. To establish the pharmacogenetic basis for this sensitivity, a panel of recombinant congenic strains (RCSs) with varying degrees of obesity and diabetes was generated by fixing selected NZO HILt alleles on the diabetes- and hepatosteatorosis-resistant NON/Lt background. Four new strains in this panel were exposed to chronic rosiglitazone treatment. Only one, NONcNZO8 (designated RCS8), exhibited an F1-like hepatosteatorotic response. In both the F1 and RCS8 males, this adverse effect correlated with rosiglitazone suppression of already impaired hepatic phosphatidylcholine biosynthetic enzymes in both arms of the biosynthetic pathway, the phosphatidylethanolamine methyltransferase pathway, and the CDP-choline pathway, including choline kinase and CTP-cholinephosphate cytidyltransferase. This adverse response was not reproduced by CL316,243, a β₃-adrenergic receptor agonist with potent antihyperlipemic effects. Genome comparison showed that RCS8 differed from the other strains in carrying NZO-derived genome on virtually all of chromosome 16 and in smaller segments on chromosomes 6, 14, and 17. Thus, these RCSs present a panel of new mouse models exhibiting differential levels of obesity and diabetes as well as different drug responses. This panel can be used to screen for treatments for type 2 diabetes and its complications. *Diabetes* 54: 1854–1862, 2005

The most common forms of human type 2 diabetes are polygenic in origin with contributions from both sides of the pedigree. In mice, obesity-associated diabetes (diabesity) also has a complex polygenic basis. Admixing genomes of unrelated strains of inbred mice provides insight as to how a complex disease such as type 2 diabetes can become more common as genetic heterogeneity increases in an outbreeding human population. As strains of laboratory mice become increasingly more inbred, genomes are selected that are capable of sustaining reproductive fitness in the face of increased mutational load. As mutations occur that affect the expression or function of multiple quantitative trait loci (QTLs), continued reproductive fitness requires co-adaptation within the overall genetic architecture for sets of QTLs that must interact to maintain metabolic homeostasis. Variation among inbred strains in physiologic parameters associated with glucose homeostasis (e.g., nonfasting plasma glucose or insulin concentrations) reflects strain-dependent systemic adaptations to strain-unique genetic polymorphisms at multiple QTLs. To model how a genetic outcross can destabilize such metabolic adaptations, we previously crossed two unrelated inbred strains, nonobese nondiabetic (NON) and New Zealand obese (NZO). The NON/Lt strain carries QTLs conferring latent type 2 diabetes susceptibility, whereas NZO/HILt mice carry numerous QTLs contributing to male diabesity in a threshold fashion (1). Whereas diabesity spontaneously developed in 0% of NON/Lt males and in ~50% of NZO/HILt males, the two sets of parental QTLs synergized in a way that 90–100% of F1 males exhibited the diabesity trait (1). Genetic analysis subsequently identified seven QTLs contributed by the NZO parent and two QTLs contributed by the NON parent (1,2).

The hyperglycemia, hyperinsulinemia, and hyperlipidemia that developed in diabetic F1 males were effectively suppressed by chronic treatment with the thiazolidinedione (TZD) compound, rosiglitazone, incorporated into the diet. However, these antidiabetic effects were accompanied by marked exacerbation of an underlying hepatosteatorosis (3). Because choline deficiency is known to produce hepatosteatorosis in mice (4,5), we tested the lipid composition of milk from untreated F1 lactating dams; this analysis showed a marked deficiency in all classes of phosphatidylcholine (S. Watkins, Lipomics, Sacramento, CA, personal communication). This result suggested to us

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PEMT, phosphatidylethanolamine methyltransferase; PPAR γ , peroxisome proliferator-activated receptor- γ ; QTL, quantitative trait locus; RCS, recombinant congenic strain; TZD, thiazolidinedione.

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that the genetic milieu of the F1 mice combined QTLs that adversely affect the two phosphatidylcholine biosynthetic pathways in liver and that this effect is influenced by treatment with TZDs.

In mouse liver, the phosphatidylethanolamine methyltransferase (PEMT) pathway contributes ~30% and the CDP-choline pathway, including choline kinase and CTP-cholinephosphate cytidyltransferase, contributes ~70% of the total hepatic phosphatidylcholine. Male mice with a targeted mutation in the gene encoding PEMT are sensitized to high-fat/high-cholesterol-induced hepatosteatosis, with hepatocytes showing a marked defect in export of VLDLs containing triglycerides and apolipoprotein B100 (6–8). Similarly, genetic disruption of the gene encoding cholinephosphate cytidyltransferase- α decreased secretion of both HDLs and VLDLs (9). Hence, polygene combinations producing impaired hepatic phosphatidylcholine biosynthesis would be predicted to predispose to hepatosteatosis risk in response to pharmacologic agents that can increase triglyceride transport into the liver. The activity of the PEMT reaction seems to be governed largely by the supply of the substrates phosphatidylethanolamine and *S*-adenosylmethionine (10). Because the phosphatidylethanolamine is derived from diacylglycerol via the CDP-ethanolamine pathway, the amount of diacylglycerol could influence the activity of PEMT. The regulation of phosphatidylcholine biosynthesis via the CDP-choline pathway in liver and other tissues and cells focuses on the cholinephosphate cytidyltransferase reaction, which is considered to be the rate-limiting step (10). The active form of cholinephosphate cytidyltransferase is found associated with membranes, and the inactive form is in a soluble form largely located in the nucleus. There is a rapid movement of the enzyme between these two locations that is regulated by the level of phosphatidylcholine and diacylglycerol in the membranes. If the level of phosphatidylcholine is high, there is release of active cholinephosphate cytidyltransferase from the membrane into the inactive reservoir. If phosphatidylcholine levels are low and/or diacylglycerol levels are high in membranes, cholinephosphate cytidyltransferase is translocated to membranes where it is activated. The activity of cholinephosphate cytidyltransferase is also regulated at the level of transcription best documented in studies on the cell cycle (11). The activity of choline kinase is generally not considered to regulate the biosynthesis of phosphatidylcholine (10).

Our objective is to use mouse models of diabetes to understand how natural allelic variation at multiple genetic loci can affect responsiveness to drug-mediated therapy. The polygenic obesity/diabetes syndromes in mice are particularly relevant because the “metabolic syndrome” associated with most human diabetogenic obesities also has a complex genetic basis. To elucidate the pharmacogenetic basis for the adverse responses of the F1 liver to TZD exposure, we compared the hepatic activities of the phosphatidylcholine biosynthetic enzymes in both parental strains with the F1 and in a panel of genetically characterized recombinant congenic strains (RCSs). The RCSs were produced by backcrossing the F1 for two cycles onto the parental NON/Lt background, with selection for different subsets of diabetes QTLs sorting into

each RCS (12). For example, RCS10 contains the greatest number of diabetes contributions from both parental backgrounds (on chromosomes 1, 4, 5, 11, 12, and 18) and develops the highest (F1-like) diabetes frequency (90–100%) of any of the RCSs. In contrast, RCS1, selected to contain the diabetogenic QTLs mainly on chromosomes 1 and 15, showed only 50% diabetes frequency. RCS2, a strain lacking most of the NZO-derived diabetes contribution on chromosome 15 while otherwise carrying the same genetic composition as RCS1, showed an even lower (25%) diabetes frequency (12). In the present study, we show that hybridizing the two parental genomes produced an unusual lowering of hepatic phosphatidylcholine biosynthetic enzyme activities in control F1 males and that rosiglitazone treatment further reduced these enzymatic activities. Screening a selected panel of RCSs showed that only one, NONcNZO8, developing a diabetes frequency of ~75%, exhibited the extreme hepatosteatotic response to rosiglitazone when compared with F1 males. The results demonstrate how susceptibility genes from both parental genomes contribute additively or codominantly to a complex disease and further demonstrate the utility of the RCS panel for identifying genetic architectures that might predict positive versus adverse responses to drug therapy.

RESEARCH DESIGN AND METHODS

(NON \times NZO)F1, NON/Lt, and four previously reported RCSs (12) (NONcNZO1 [designated RCS1], NONcNZO2 [RCS2], NONcNZO8 [RCS8], and NONcNZO10 [RCS10]) were bred in our research vivarium. They were housed two to four per pen in double-pen Plexiglas boxes on shaved pine bedding and given free access to food and acidified water. All mice shared the same mouse room with controlled temperature and humidity and a 14/10-h light/dark cycle. Because diabetes is male sex limited in the models studied (1,12), only males were used. Eight to 12 males of each strain at ~8 weeks of age were split into two groups. A control group of males continued to receive the maintenance (control) diet (NIH-31 containing 6% fat; Purina Test Diets, Richmond, IN). The remaining one-half received the same diet supplemented with 50 mg rosiglitazone/kg (a gift from Dr. S. Smith, GlaxoSmithKline). Mice were maintained on the irradiation-sterilized diets for 14 weeks. At experiment termination (22 weeks of age), the mice were killed by CO₂ inhalation, and blood was collected by heart puncture using a heparinized 25-gauge needle and syringe. Livers were collected, weighed, and homogenized immediately for enzyme assay. A piece of each liver was isolated for histologic analysis. For comparative purposes, a separate study group of five F1 males at 8 weeks of age were placed for 6 weeks on the NIH-31 diet alone or supplemented with 0.001% CL316,243, a β_3 -adrenergic receptor agonist (gift of Dr. Kurt Steiner, Wyeth Research, Princeton, NJ) previously shown to exert potent antihyperglycemic/antihyperlipemic effects in the NZO/HLLt strain (13). Also, to compare compounds within the TZD class, troglitazone (a gift of Dr. H. Horikoshi, Sankyo, Tokyo) was prepared in pelleted NIH-31 diet at a concentration of 2 g/kg diet based on a previous report (14). Groups of five NON/Lt, (NZO \times NON)F1, and RCS8 males were placed on this diet from 6 to 16 weeks of age.

Clinical markers. Nonfasting plasma glucose sampled at 8 A.M. was measured by a Glucose 2 analyzer (Beckman Instruments, Fullerton, CA). Plasma insulin in the same samples was measured by Luminex bead assay (Linco).

Histology studies. Mice were necropsied at 22 weeks of age (14 weeks on the diet). Livers were fixed in Bouin's solution, and sections were stained by a standard protocol with periodic acid-Schiff reagent. Percentage of fatty area in liver was determined morphometrically using the MetaMorph Offline program (Universal Imaging, Downingtown, PA).

Cell fractionation and enzyme assays. Livers (1 g each) from nonfasted males at 22 weeks necropsy were cut into small pieces, then homogenized in a glass-Teflon homogenizer in 5 vol of 50 mmol/l Tris-HCl (pH 7.4), 150 mmol/l NaCl, 1.0 mmol/l phenylmethylsulfonyl fluoride, 1.0 mmol/l EDTA, 2.0 mmol/l dithiothreitol, and 0.25 mol/l sucrose. Homogenates were centrifuged at 600g for 5 min to remove unbroken cells. This supernatant (“total homogenate”) was further centrifuged at 12,000g for 10 min to obtain a crude mitochondria pellet. The mitochondrial-free supernatant fraction was transferred to a new tube and centrifuged at 100,000g for 1 h to obtain the postmitochondrial fraction (cytosol) from the supernatant and microsomes from the pellet. Protein

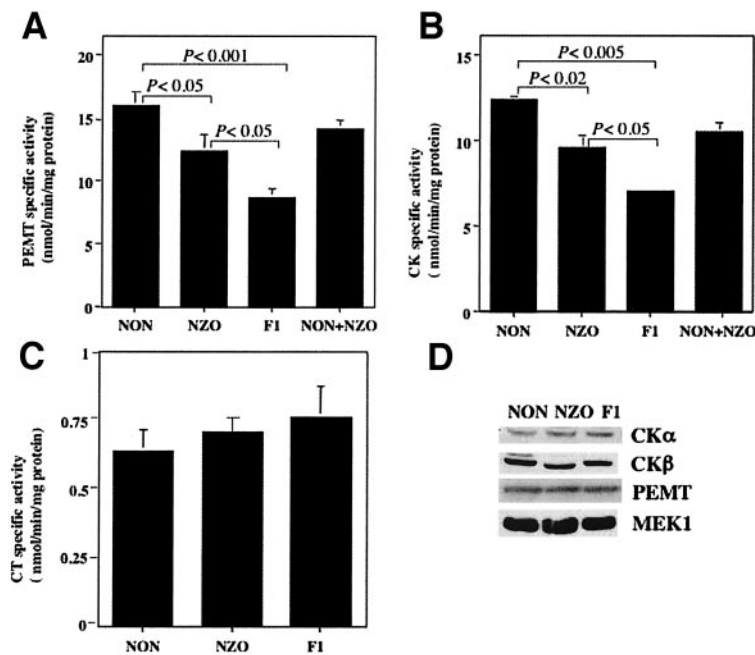


FIG. 1. The F1 metabolic milieu depresses hepatic PEMT and choline kinase (CK) activities but not cholinephosphate cytidyltransferase (CT) activity. *A*: Specific activity of PEMT in total liver homogenates. *B*: Specific activity of choline kinase in postmicrosomal fractions. *C*: Specific activity of cholinephosphate cytidyltransferase in total liver homogenates. Each bar represents the mean \pm SE with four to five mice of each strain. Note that 1:1 admixture of NON + NZO homogenates for PEMT and choline kinase did not exhibit the suppressed activity evident in the same two fractions from F1 liver. *D*: Western blot analysis showing comparable levels of enzyme protein among the three genotypes compared (NON, NZO, and F1). This blot is representative of three independent replications. MEK1, MAP/ERK-1 kinase.

concentration was determined (Total Protein; Sigma, St. Louis, MO) and normalized before performing enzyme assays and Western blot analyses. All assays were performed at saturating concentrations of substrate. PEMT activity in 100- μ g protein aliquots of total homogenate was assayed as described previously (15) at pH 9.2 with 2 mmol/l phosphatidylmethylethanolamine (Avanti Polar Lipids, Alabaster, AL) as substrate and 200 μ mol/l *S*-adenosyl-L-methionine (Sigma) and *S*-adenosyl-L-[Me- 3 H]methionine (2 μ Ci/reaction) (code TRK865; Amersham Pharmacia, Piscataway, NJ) as cofactor. The specific activity was estimated by the nanomoles of [3 H]phosphatidylcholine formed per minute per milligram of protein. Choline kinase activity was determined in 400- μ g protein aliquots of 100,000 g supernatant as described previously (16) at pH 8.7, with 10 mmol/l MgCl₂, 10 mmol/l ATP, 0.25 mmol/l choline chloride (Sigma), and [Me- 3 H]choline chloride (2 μ Ci/reaction) (code TRK 593; Amersham Pharmacia). Specific activity was estimated by the nanomoles of [3 H]phosphocholine formed per minute per milligram of protein. Cholinephosphate cytidyltransferase-specific activity was measured in total homogenates with [3 H]phosphocholine (15 μ Ci/reaction, 7–8 μ Ci/ μ mol) as substrate as described previously (7). Cholinephosphate cytidyltransferase-specific activity was estimated by nanomoles of [3 H]CDP-choline formed per minute per milligram of protein.

Western blot analysis. An aliquot (100 μ g protein) of the total homogenate was resolved by SDS-PAGE (10% gel), then transferred on to a polyvinylidene fluoride membrane and incubated with rabbit antibody against PEMT2 (1:1,000) (15). For choline kinase analysis, a 100- μ g protein aliquot of the 100,000 g high-speed supernatant (postmicrosomal fraction) of liver homogenates was incubated with affinity-purified subunit-specific rabbit antisera (100 \times dilution for anti-choline kinase- α and 200 \times dilution for anti-choline kinase- β) (17), a gift of Dr. K. Ishidate (Tokyo Medical University, Tokyo). Horseradish peroxidase-conjugated anti-rabbit IgG and enhanced chemiluminescence were used for subsequent signal detection procedures (Roche, Indianapolis, IN).

RESULTS

Unusual suppression of both arms of the phosphatidylcholine biosynthetic pathway in F1 livers. Although F1 hybrid mice generated by outcross of two inbred progenitor strains are generally considered more “robust,” the combination of NON/Lt and NZO/HILt genomes actually produced a “negative heterosis” in two of three critical enzymes in hepatic phosphatidylcholine biosynthesis. As shown in Fig. 1A and B, both PEMT-specific and choline kinase-specific activities were significantly lower in total homogenates of F1 liver than in total homogenate from either the NON/Lt or NZO/HILt parental strains. A comparable depression of either of these enzymatic activities

could not be modeled by a 1:1 mixing of liver total homogenates from NON and NZO directly. Instead, an intermediate activity for both PEMT and choline kinase was observed (Fig. 1A and B), as would be expected for additive activity contributions from each parental source. As shown in Fig. 1C, this unexpected hybrid inhibitory effect on PEMT and choline kinase activities did not extend to cholinephosphate cytidyltransferase (the rate-limiting enzyme in the CDP-choline pathway). There were no significant activity differences distinguishing either parental strain from the F1. Despite the significantly reduced PEMT and choline kinase enzymatic activities distinguishing F1 from the two parental strains, Western blots using antibodies against PEMT or against each of the two choline kinase subunits, choline kinase- α and choline kinase- β , showed no differences in enzyme protein concentration among the three strains (Fig. 1D). Thus, the F1 metabolic milieu was selectively producing post-transcriptional changes potentially affecting phosphatidylcholine production via the PEMT pathway and downregulating activity of one enzyme (choline kinase) in the CDP-choline pathway.

Rosiglitazone treatment suppressed all three key phosphatidylcholine-biosynthetic enzymes in F1 livers. The F1 males are distinguished from both parental strain males by a higher frequency of diabetes development (1). Although diabetic hyperglycemia and hyperlipemia were reversed by supplementing the diet with rosiglitazone, this therapy was accompanied by marked hepatosteatotic side effects (3). This effect was not limited to rosiglitazone, because troglitazone fed over the same time frame at 2 g/kg diet produced the same degree of hepatosteatosis (data not shown). The finding that two out of three phosphatidylcholine biosynthetic enzyme activities assayed in F1 liver were unexpectedly reduced below both parental strains, coupled with our previous observation of increased triglyceride accumulation in livers of rosiglitazone-treated F1 males (3), suggested that increased F1 hepatosteatotic sensitivity after TZD treat-

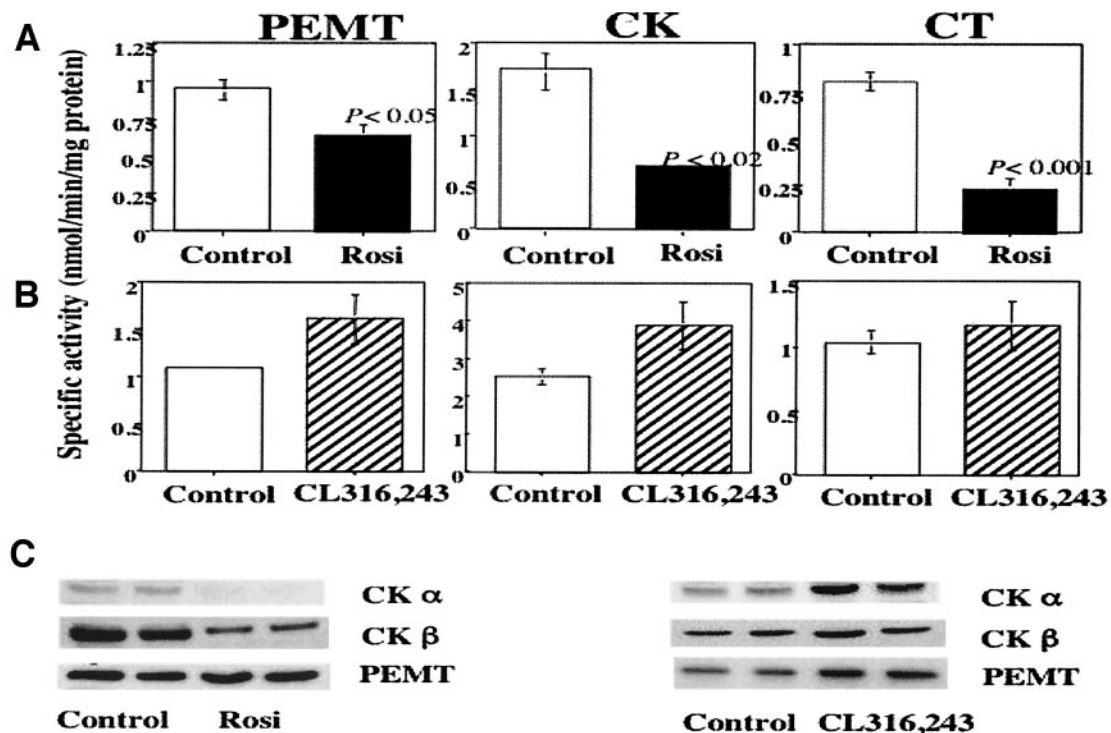


FIG. 2. *A:* Chronic rosiglitazone (Rosi) treatment (14 weeks) further suppresses already reduced hepatic PEPT and choline kinase (CK) activities and markedly reduces cholinephosphate cytidyltransferase (CT) activity in F1 liver homogenates. Data are means \pm SE with three to four males per treatment. *B:* This effect is TZD specific in that the β_3 -adrenergic receptor agonist, CL316,243 (0.001% [wt/wt] in the diet), a potent suppressor of both hyperglycemia and hyperlipemia in F1 males, produced no inhibitory effect on any of the three phosphatidylcholine biosynthetic enzymes. *C:* Western blot analysis of choline kinase- α , choline kinase- β , and PEPT from F1 livers treated with rosiglitazone or CL316,243. Results shown are from two individual mice from each group.

ment entailed a further drug-mediated impairment of these enzymatic functions. Data in Fig. 2 strongly support this. All three enzyme activities measured in untreated (“control”) F1 liver homogenates were significantly inhibited by chronic exposure to rosiglitazone at a dosage of 50 mg/kg diet (Fig. 2A). This included not only PEPT and choline kinase activities already significantly reduced in untreated “control” F1 livers (Fig. 1A and B), but also cholinephosphate cytidyltransferase activity that, in samples of untreated control F1 liver, did not differ significantly from parental activities. Western blot showed that rosiglitazone markedly suppressed concentrations of both choline kinase subunits (choline kinase- α and choline kinase- β) in the F1 liver, whereas PEPT concentration were quite comparable between control and rosiglitazone group (Fig. 2C, left panel). In contrast to rosiglitazone, CL316,243 significantly stimulated the expression of choline kinase- α subunit and slightly increased PEPT concentration (Fig. 2C, right panel). The regulation of protein expression correlates well with the actual enzyme activities. (Fig. 2A and B). Even though the rosiglitazone concentration used in the present study was fourfold lower than reported previously (3), histologic assessment of samples of the F1 livers used in the current experiments revealed macrovesicular hepatosteatosis (Fig. 3) as severe as reported previously using the higher drug dosage. A null mutation in the PEPT gene in mice produces increased sensitivity to development of hepatic steatosis (4,5). None of the phosphatidylcholine biosynthetic enzymes measured were completely ablated in rosiglitazone-treated F1 mice. Yet the ability of rosiglitazone to impair already inhibited PEPT and choline kinase activities, as well as to signifi-

cantly reduce activity of cholinephosphate cytidyltransferase, reveals an unusual genotype-drug interaction that compromises the liver’s phosphatidylcholine biosynthetic pathways. This impairment was limited to TZD, because treatment with CL316,243, an even more effective antihyperlipemic and antihyperglycemic agent in the F1 model (13), did not impair activity of any of the three enzymes. Although mean activities of all three were elevated above male mice on control diet, the differences did not achieve statistical significance after the 6-week treatment period (Fig. 2B).

Phenotypic screen of RCS sensitivity to rosiglitazone-enhanced steatosis. RCSs represent unique tools for dissecting drug-genome interactions. Introgression of different combinations of selected NZO-derived QTLs onto the diabetesity- and steatosis-resistant NON parental background produced a panel of different gene combinations for testing the genetic basis of the steatosis in the rosiglitazone-treated mice.

After 14 weeks of rosiglitazone treatment, NON males on control diet maintained normoglycemia. Rosiglitazone treatment did not produce an increase in body weight, although plasma insulin was significantly reduced (Table 1). However, with the exception of RCS1, most RCS strains, similar to the F1 males, responded to rosiglitazone-stimulated body weight gain (Table 1). As previously reported, RCS2 showed resistance to diabetesity development, whereas RCS1, RCS8, and RCS10 all exhibited diabetesity syndromes of differential severity as manifested by increases in both plasma glucose and insulin concentrations (Table 1). Also, in all three, the insulin-sensitizing action of rosiglitazone was reflected by significant de-

TABLE 1
Comparative physiologic responses of parental NON/Lt males versus RCS males with 14 weeks rosiglitazone treatment

Strains	Treatment	n	Body wt (g)	Plasma glucose (mg/dl)	Insulin (ng/ml)
NON	Control	6	39.2 ± 1.4	198 ± 11.9	2.4 ± 0.4
	Rosiglitazone	6	38.5 ± 1.0	179 ± 12.5	1.3 ± 0.1*
RCS1	Control	6	39.3 ± 2.8	238 ± 22.6	3.8 ± 0.9
	Rosiglitazone	6	39.8 ± 1.0	184 ± 6.7*	1.5 ± 0.5*
RCS2	Control	4	38.4 ± 2.0	194 ± 29.4	1.9 ± 0.5
	Rosiglitazone	4	46.4 ± 1.8*	179 ± 11.0	3.0 ± 1.1
RCS8	Control	5	45.9 ± 0.7	284 ± 34.3	8.0 ± 6.4†
	Rosiglitazone	4	54.2 ± 0.6‡	227 ± 13.8	4.9 ± 1.0
RCS10	Control	6	46.0 ± 0.5	353 ± 23.7	3.2 ± 0.4
	Rosiglitazone	6	60.1 ± 3.8‡	173 ± 7.0‡	2.7 ± 0.5

Data are means ± SE at 20 weeks of age. *Rosiglitazone is significantly different from control group at $P < 0.05$. †Variability due to one outlier (plasma insulin = 30 ng/ml). ‡Rosiglitazone is significantly different from control group at $P < 0.01$.

creases in plasma insulin. Nevertheless, differences in anti-hyperglycemic effects were noted. Drug-mediated plasma glucose decreases were significant in both RCS1 and RCS10 mice, but the decline in treated RCS8 mice did not achieve significance (Table 1). Moreover, plasma insulin was much more variable in control RCS8 males. Although rosiglitazone treatment reduced mean plasma insulin concentrations and reduced variability in the measurement, the difference again failed to achieve statistical significance. This suggested that the diabetes syndrome developing in RCS8 was different from that in RCS1 and RCS10.

Differential sensitivity of RCS to rosiglitazone-mediated hepatic steatosis. Results of morphometric profiling of hepatic fat infiltration into livers at the end of rosiglitazone treatment are shown in Fig. 3. Comparisons are with the NON parental males that exhibited minimal lipidosis that was not exacerbated by the 14-week period of rosiglitazone treatment. This absence of a drug effect in NON is in contrast to the extreme sensitivity of the F1 to this adverse side effect. Livers of F1 males on control diet exhibited an elevated mean basal concentration of fat (mostly microvesicular). Basal microvesicular fat in RCS males fed control diet were all comparable with the NON basal level (Fig. 3), despite marked differences in the severity of the diabetes syndromes in the RCS mice (Table 1). Neither the diabetes-sensitive RCS1 nor the diabetes-resistant RCS2 differed from NON in terms of resistance to rosiglitazone-exacerbated lipidosis. The most diabetes-prone strains, RCS8 and RCS10, showing comparable basal microvesicular lipidosis on control diet (14.4 and 18%), both exhibited significant increases in macrovesicular fat accumulation in response to rosiglitazone treatment. However, only RCS8, which did not respond as well as RCS10 to the glucose-lowering action of rosiglitazone, showed the extreme hepatosteatic response indistinguishable from that observed in drug-hypersensitive F1 males (Fig. 3A). The rosiglitazone-mediated increase in mean fatty area in RCS8 liver (45% in rosiglitazone treated versus 17.3% in control) compared well with that in the F1 liver (51% in rosiglitazone treated versus 26% in control, Fig. 3B). In RCS10, basal lipidosis was comparable with RCS8 (14.4%), whereas the rosiglitazone-mediated in-

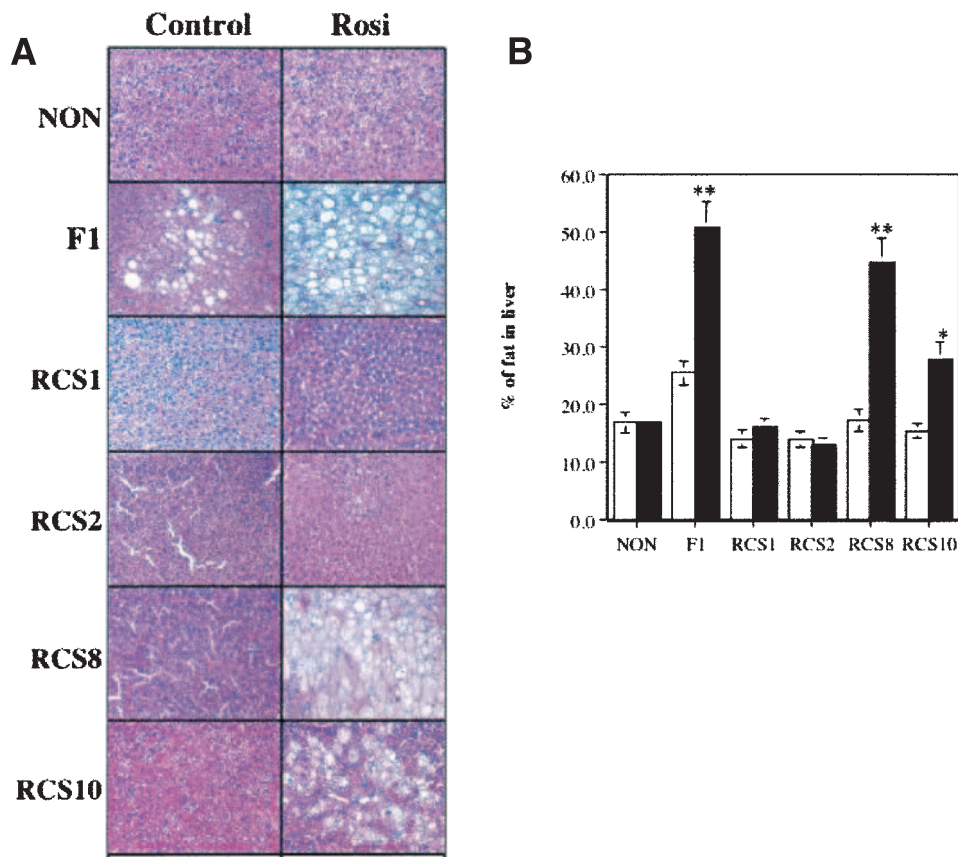


FIG. 3. A: Comparative differences in hepatic histology (periodic acid Schiff stain) of NON, F1, RCS1, RCS2, RCS8, and RCS10 males at the end of chronic rosiglitazone (Rosi) treatment (22 weeks of age). Liver specimens of males receiving control diet (left panel) showed no to mild lipid accumulation. Liver from rosiglitazone-treated RCS males (right panel) developed strain-specific macrovesicular lipidosis. B: Morphometric analysis shows that although both RCS10 and RCS8 exhibited steatotic responses, only RCS8 shows a response as extreme as the F1. □, untreated control group; ■, rosiglitazone-treated group. Data are means ± SE for four individuals. ** $P < 0.001$, * $P < 0.005$ indicates rosiglitazone group is significantly different from control group.

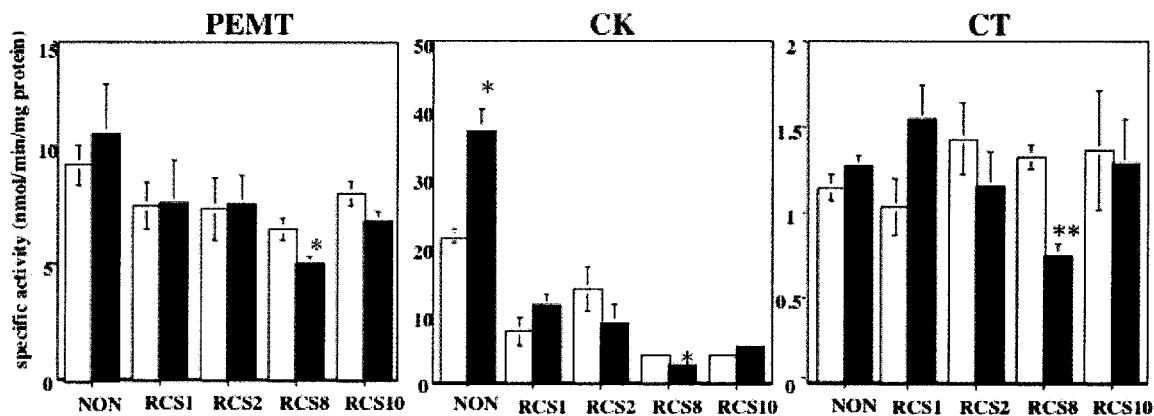


FIG. 4. Strain-dependent sensitivity to rosiglitazone-mediated inhibition of hepatic phosphatidylcholine biosynthetic enzymes. Male mice were treated with control diet or rosiglitazone-supplemented diet at 8 weeks of age. After 14 weeks of treatment, liver total homogenates or cytosol fractions (postmicrosomal fractions) were prepared and subjected to PEMT, choline kinase (CK), and cholinephosphate cytidylyltransferase (CT) activity assay as described in RESEARCH DESIGN AND METHODS. □, untreated control group; ■, rosiglitazone-treated group. A: Hepatic PEMT activity. B: Hepatic choline kinase activity. C: Hepatic cholinephosphate cytidylyltransferase activity. Data are means \pm SE for four individuals. ** $P < 0.001$, * $P < 0.02$ indicates rosiglitazone-treated group is significantly different from control group.

crease in mean fatty area (to 27.8%) was 37% lower than the rosiglitazone-mediated increase observed in RCS8 (Fig. 3B). This result correlated with the finding that treated RCS8 males exhibited the maximal elevation in plasma alanine:leucine aminotransferase, a clinical marker diagnostic of hepatotoxic stress (data not shown). Hence, among the panel of RCSs tested, RCS8 has the most potent combination of QTLs, fixed in the homozygous state, that predispose to the adverse drug response originally observed in F1.

Only RCS8 responds to rosiglitazone-mediated inhibition of phosphatidylcholine biosynthetic enzymes.

Data in Fig. 4 show that, among the panel of RCSs tested, only RCS8 responded to rosiglitazone with the same marked inhibitory effect on hepatic phosphatidylcholine biosynthetic enzymes shown for the rosiglitazone-treated F1 males described in Fig. 2A. In comparison with NON, all RCSs tested showed lower hepatic PEMT and choline kinase activities. After rosiglitazone treatment, a significant increase in choline kinase activity was observed in rosiglitazone-treated NON males. All of the control diet-fed RCSs, regardless of their constitutive sensitivity to diabetes development on this diet, exhibited the reduced PEMT and choline kinase activities, but not cholinephosphate cytidylyltransferase activity characteristic of the diabetes-developing F1. Yet only RCS8 showed the further depression in activity levels of all three enzymes in response to chronic rosiglitazone treatment (Fig. 4). This close correlation between the histologic documentation of heightened sensitivity of RCS8 to drug-exacerbated lipodosis and the RCS8-specific loss of activity for enzymes in both pathways of phosphatidylcholine biosynthesis clearly provide a mechanism for this RCS-specific drug sensitivity. It should be noted that a considerably higher concentration of troglitazone in the diet (2 g/kg diet) produced the same degree of hepatosteatosis in RCS8 males as did the rosiglitazone feeding at 50 mg/kg diet reported here. The same troglitazone diet had no effect on parental NON/Lt males (data not shown).

Genomic differences in RCSs. The genome-wide scans distinguishing the panel of RCSs used in this study are available online (http://www.jax.org/staff/leiter/labsite/type2_genomics.html), and the known diabetes QTL

markers have been published previously (12). The PEMT-encoding gene maps to one of these diabetes QTL regions on chromosome 11 (originally designated as *Nidd3*). Two linkage peaks on this chromosome were detected; this second peak contained another enzyme associated with phosphatidylcholine metabolism, phosphatidylcholine transfer protein, *Pctp*, that we have recently found to be mutated in NZO (H.-J.P., E.H.L., unpublished data). The choline kinase- β subunit encoding gene maps to another diabetes QTLs on chromosome 15 ("*Nidd*" designations were discontinued because of the large numbers of the complex epistatic interactions discovered among a multiplicity of QTLs identified [2]). Because the RCS8 response to rosiglitazone was unique among the panel tested (RCS1, RCS2, RCS8, and RCS10), comparative analysis of the genome-wide differences distinguishing RCS8 from the other three RCSs could potentially delineate the genetic basis for the increased sensitivity of this strain. The RCS8-unique set of NZO-derived genomic segments included virtually all of chromosome 16 (markers spanning 8.6–55 cM) and considerably smaller segments marked by single polymorphic simple sequence repeats on chromosome 6 (marked only by *D6Mit275*, 25.5 cM), chromosome 14 (markers *D14Mit195-D14Mit170*; 44.3–63 cM), and chromosome 17 (markers *D17Mit16-D17Mit11*; 17.4–22.8 cM). Hence, the adverse drug response in this strain may likely be controlled by a gene or genes on only one of these chromosomes, most likely chromosome 16. Reciprocal outcrosses between RCS2 (hepatosteatosis resistant) and RCS8 (hepatosteatosis susceptible) show that the F1 males inherit the high hepatosteatotic responsiveness to rosiglitazone in a dominant fashion (data not shown). This would be consistent with the high hepatosteatosis sensitivity of F1 males generated by the reciprocal NON/Lt \times NZO/HILt outcrosses.

DISCUSSION

Highly inbred strains of mice, such as NON/Lt and NZO/HILt, cannot accurately model for patient-specific drug responses in an outbred human population, whereas a genetically heterozygous F1 hybrid would be more representative in this regard. The present study used RCSs to

differentially distribute genetic susceptibility to TZD-exacerbated hepatosteatosis into a genetic background (NON/Lt) that is resistant to these effects. It is conceivable that the heightened sensitivity of the F1 and RCS8 males to TZD-exacerbated hepatosteatosis represents a component of insulin resistance uniquely present in these two stocks. We compared the antihyperglycemic and insulin-sensitizing action of troglitazone at 2 g/kg diet with rosiglitazone at 50 mg/kg diet in both the F1 and the derivative RCS8 stock. Troglitazone was much less effective either in suppressing hyperglycemia or in promoting pancreatic β -cell regranulation with insulin (data not shown). Nevertheless, both TZDs produced hepatosteatosis of equal severity. Detailed analysis of insulin resistance in TZD-treated RCS8 versus RCS10 males has not yet been done. Euglycemic-hyperinsulinemic clamp studies have shown that untreated RCS10 males are extremely insulin resistant (18), yet males of this strain are significantly more resistant than RCS8 to hepatosteatosis. Thus, we have no evidence at this point to conclude that intractable insulin resistance alone distinguishes steatosis-prone from steatosis-resistant RCSs.

Troglitazone, the first TZD marketed for treatment of type 2 diabetes, was withdrawn because of extreme hepatotoxicity in a very small percentage of patients taking the compound (19). Drugs of the later generation TZDs, of which rosiglitazone is an example, have been shown to reduce hepatic triglycerides in most patients rather than promote triglyceride accumulation and steatosis (20). Thus, availability of the panel of genetically characterized RCS models of diabetes with differential TZD sensitivities is useful for understanding the genetic basis for susceptibility to adverse drug responses. Such pharmacogenetic knowledge might distinguish that small percentage of patients who should not take this class of compounds. RCS8 and RCS10 exhibit comparable levels of obesity and basal hepatic lipidosis with a control diet, but only RCS8 experiences a drug-exacerbated steatosis as extreme as originally described in the F1. Interestingly, RCS10 rather than RCS8 is more comparable with the F1 in terms of the numbers of shared diabetes QTLs and, consequently, diabetes frequency (90–100%) and disease severity defined by nonfasting plasma glucose levels. RCS8 develops a lower frequency of diabetes with hyperglycemia not establishing consistently before 20 weeks of age (12). Thus, it was surprising that the moderate shifts in the glycemic status of RCS8 males before 20 weeks were not as responsive to rosiglitazone treatment as were the more hyperglycemic RCS10 males. Because the NON/Lt strain is completely resistant to rosiglitazone-exacerbated steatosis, the steatosis-promoting gene or genes must be NZO in origin. We surmise that this NZO-derived locus or loci promoting the phenotype of drug-exacerbated steatosis may be distinct from the set of NZO-contributed diabetes QTLs that synergize with certain NON-derived QTLs to increase frequency of diabetic hyperglycemia development in the F1 male. This inference is reinforced by the fact that the three steatosis-resistant RCSs collectively carry the spectrum of known diabetes QTLs. RCS8 has been fixed for known diabetes QTLs on chromosomes 1 and 11 (from NZO) and chromosomes 4 and 18 (from NON). The most notable genetic difference distinguishing

RCS8 from the others is its NZO origin of chromosome 16 in its entirety. The cholinephosphate cytidyltransferase- α subunit is encoded on this chromosome. The active form of cholinephosphate cytidyltransferase activity resides on cellular membranes. Subcellular fractionation showed that rosiglitazone-mediated reduction in cholinephosphate cytidyltransferase activity in total homogenate was primarily in a 100,000g cytosol and to a lesser extent, in the crude mitochondrial fraction (data not shown). We have sequenced cholinephosphate cytidyltransferase- α cDNA from NON and NZO and have not found coding polymorphisms. This is consistent with our finding of no differences in cholinephosphate cytidyltransferase enzymatic activities in NON, NZO, and F1 liver fractions from males fed a control diet. However, cholinephosphate cytidyltransferase- α differs from the cholinephosphate cytidyltransferase- β subunit (X chromosome linked and NON derived in all of the RCSs) in having a nuclear localization domain. Possibly, the rosiglitazone-specific suppression of cholinephosphate cytidyltransferase total activity in all subcellular fractions in F1 liver may entail a drug-specific interaction with cholinephosphate cytidyltransferase- α . Genetic disruption of cholinephosphate cytidyltransferase- α produces a hepatic phenotype similar to that observed in rosiglitazone-treated F1; e.g., 85% reduction of total cholinephosphate cytidyltransferase activity, reduced phosphatidylcholine levels, and triglyceride accumulation (9). In RCS8, not only is cholinephosphate cytidyltransferase activity reduced, but also this partial loss of cholinephosphate cytidyltransferase activity is accompanied by significant losses in both choline kinase and PEMT biosynthetic functions. In the case of the reductions in choline kinase enzymatic activity, our unpublished data suggest that, in the specific post-translational environment in the F1, coexpression of polymorphic alleles from NON and NZO reduce overall choline kinase catalysis by impairing α/β heterodimer formation or, alternatively, that the NZO/NON heterodimeric combinations are less thermodynamically favored compared with within-strain subunit dimeric combinations. At least four other loci on chromosome 16 associated with hepatic triglyceride metabolism and steatosis represent potential candidates. Among these are lipid defect 1 (*lpd*, 45 cM) and lipase, member H (*Liph*, 14.8 cM) (21,22). A QTL contributing to plasma glucose independent of body weight and mapping to chromosome 16 has also been reported in TallyHo mice, another diabetes model (23). A triglyceride hydrolase-encoding gene also maps to chromosome 16 (24). Finally, high expression of SOCS-1 (suppressor of cytokine signaling), another chromosome 16-encoded gene product has recently been shown in mice to contribute to heightened sensitivity to hepatic steatosis (25).

Although the PEMT pathway only accounts for 30% of phosphatidylcholine biosynthesis in liver, it is required for secretion of lipoproteins (8), so that PEMT inhibition significantly impairs the bulk incorporation of triglycerides into lipoprotein particles for export (26). In livers of mice with a genetically disrupted *Pemt* allele fed a standard diet, a diet deficient in choline, or a choline-enriched diet, there was hepatic steatosis and significantly higher frequency of apoptotic cells compared with wild-type controls (5). Similarly, reductions in multiple classes of plasma

lipoproteins were also observed in mice with a disrupted cholinephosphate cytidyltransferase- α gene (9). Thus, the remarkable finding that rosiglitazone treatment inhibited all three phosphatidylcholine biosynthetic enzymes assayed easily explains our previous analysis of the lipid metabolome, showing that rosiglitazone treatment removed plasma triglyceride into the liver where it accumulated rather than being metabolized in a normal manner (3). This rosiglitazone-mediated inhibition of enzymes in both arms of the phosphatidylcholine biosynthetic pathway would very likely impair lipoprotein assembly and export and hence promote the observed hepatic triglyceride accumulation. Moreover, regardless of the mechanism for the decrease in cholinephosphate cytidyltransferase and PEMT activity as a result of rosiglitazone treatment, less diacylglycerol would be used in the biosynthesis of phosphatidylcholine. Under such conditions, the diacylglycerol would be acylated to triacylglycerol and this could lead to the observed hepatic steatosis.

It remains to be established whether this unusual pharmacogenetic effect is mediated via direct effects of peroxisome proliferator-activated receptor- γ (PPAR γ) at the hepatocyte or indirectly by primary drug effects on another tissue such as white fat. We previously were unable to detect upregulation in PPAR γ 1 and PPAR γ 2 gene transcription in livers of rosiglitazone-treated F1 males undergoing severe steatosis (3). We used computational means (TRANSFAC database for transcription factor binding sites [<http://www.biobase.de/pages/products/customer.html>]) to search for upstream consensus PPAR γ binding sites in the gene sequences for PEMT, choline kinase, and cholinephosphate cytidyltransferase in ENSEMBL. Although none were found, we cannot exclude the possibility of nonconventional PPAR γ binding sites capable of negative regulation (27,28). That reduced hepatic phosphatidylcholine production is a major component of rosiglitazone-exacerbated steatosis in F1 and RCS8 mice is inferred from results with a β_3 -adrenergic receptor agonist, CL316,243 (Fig. 2). We previously demonstrated that this compound completely suppressed hyperglycemia and hyperlipidemia in NZO/HILt males and eliminated the mild hepatic lipidosis (13). When PEMT and choline kinase activities were measured in F1 males whose diabetes syndrome was also effectively suppressed by CL316,243, the absence of steatosis correlated with a drug-mediated increase in mean activity levels rather than a decrease (Fig. 2). The increased activities were reflected by increased choline kinase and PEMT protein expression (Fig. 2). In contrast, rosiglitazone treatment failed to increase either activity and in fact suppressed choline kinase protein in Western blots (Fig. 2).

The insulin-resistant NZO parental strain donating obesity/diabetes QTLs to the RCS panel shows a dissociation of insulin's effects on genes involved in glucose and lipid metabolism. Insulin-suppressible genes associated with hepatic gluconeogenesis and glycogenolysis are not suppressed, but insulin-inducible genes associated with lipogenesis are markedly upregulated (29). A combined gene transcription and lipid metabolome analysis of rosiglitazone-treated F1 livers indicated that lipid uptake and rosiglitazone-stimulated de novo lipid biosynthesis were not adequately compensated by lipid export (3). In the

case of the RCSs tested in this report, the physiologic profiling of plasma glucose and insulin responses to rosiglitazone in Table 1 suggest different degrees of insulin resistance. Unlike RCS10, whose insulin resistance was clearly diminished by rosiglitazone treatment as reflected by a restoration of normoglycemia, RCS8 appeared less responsive despite a considerably milder mean hyperglycemia over the time period studied.

In summary, the RCS analysis reported herein has allowed biochemical dissection of the marked sensitivity of the F1 diabetes model to TZD-exacerbated steatosis. Our results indicate a constitutive impairment of hepatic phosphatidylcholine biosynthetic enzyme functions that is further exacerbated by TZD treatment. The fact that, unlike RCS8, the NON/Lt parental background strain, RCS1, RCS2, and RCS10 do not exhibit these extreme responses to TZD establishes this RCS panel as potentially useful pharmacogenetic screening tools. Clearly, there are well-known differences between humans and mice in terms of rosiglitazone effects on liver fat accumulation (20). Rosiglitazone treatment of humans is associated with decreases, not increases, in liver fat (20). In mice, inability to express PPAR γ in liver protects from rosiglitazone-mediated steatosis (30,31). Despite these differences distinguishing mice from humans, understanding the genetic basis for these differential drug responses in mice may provide both physiologic and genetic insights to guide selection of patients capable of tolerating long-term drug treatments without adverse side effects.

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