CYP2J2 attenuates metabolic dysfunction in diabetic mice by reducing hepatic inflammation via the PPARγ

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Epoxyeicosatrienoic acids (EETs) and arachidonic acid-derived cytochrome P450 (CYP) epoxygenase metabolites have diverse biological effects, including anti-inflammatory properties in the vasculature. Increasing evidence suggests that inflammation in type 2 diabetes is a key component in the development of insulin resistance. In this study, we investigated whether CYP epoxygenase expression and exogenous EETs can attenuate insulin resistance in diabetic db/db mice and in cultured hepatic cells (HepG2). In vivo, CYP2J2 expression and the accompanying increase in EETs attenuated insulin resistance, as determined by plasma glucose levels, glucose tolerance test, insulin tolerance test, and hyperinsulinemic euglycemic clamp studies. CYP2J2 expression reduced the production of proinflammatory cytokines in liver, including CRP, IL-6, IL-1β, and TNF-α, and decreased the infiltration of macrophages in liver. CYP2J2 expression also decreased activation of proinflammatory signaling cascades by decreasing NF-κB and MAPK activation in hepatocytes. Interestingly, CYP2J2 expression and exogenous EET treatment increased glucose uptake and activated the insulin-signaling cascade both in vivo and in vitro, suggesting that CYP2J2 metabolites play a role in glucose homeostasis. Furthermore, CYP2J2 expression upregulated PPARγ, which has been shown to induce adipogenesis, which attenuates dyslipidemias observed in diabetes. All of the findings suggest that CYP2J2 expression attenuates the diabetic phenotype and insulin resistance via inhibition of NF-κB and MAPK signaling pathways and activation of PPARγ.

Diabetes mellitus is a metabolic disorder that when poorly managed or untreated results in hyperglycemia, dyslipidemia, oxidative stress, inflammation, and advanced glycation, which often lead to deleterious alterations in various tissues (47). Among the pathogenic factors in diabetes mellitus, numerous studies have focused on the role of inflammation in the initiation and progression of noninsulin-dependent type 2 diabetes mellitus (T2DM) (13, 55). Proinflammatory cytokines, including C-reactive protein (CRP), tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and interferon-γ (IFN-γ), act synergistically to inflict cytotoxic effects on pancreatic β-cells, and elevated levels of these cytokines are risk factors for developing T2DM (15, 46). TNF-α has also been shown to decrease insulin sensitivity and increase lipolysis in adipocytes (61). Since proinflammatory pathways such as ERK1/2 and JNK mediate insulin resistance, the activation of anti-inflammatory pathways may be a novel therapeutic approach for T2DM (23).

Although adipose tissue is a primary site of inflammation in T2DM, the role of inflammation in other metabolically active tissues, such as the liver, remains unclear in T2DM disease progression (23, 57). A causative relationship between adipose tissue inflammation, organ-specific inflammation, and T2DM insulin resistance is suggested by the increased hepatic expression of proinflammatory genes that are expressed mainly in adipose tissue (42). The proinflammatory cytokines, which are often produced in T2DM, have been shown to increase hepatic insulin resistance, and JNK1-dependent secretion of IL-6 by adipose tissue caused increased expression of liver suppressor of cytokine signaling-3, a protein that induces hepatic insulin resistance, suggesting that adipose tissue/liver cross-talk existed; inflammatory cytokines may underlie this cross-talk (39, 43). In particular, the activation of NF-κB that is observed in insulin resistance is critical to the pathogenesis of diabetes-related hepatic disease (3).

Cytochrome P450 epoxygenase 2J2 (CYP2J2), a P450 arachidonic acid epoxygenase, is widely expressed in heart, lung, blood vessels, liver, kidney, ileum, jejunum, and colon in humans (56). This enzyme metabolizes arachidonic acid to biologically active cis-epoxyeicosatrienoic acids (5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET) that have diverse biological properties within the cardiovascular system (8, 9, 60). EETs possess cardioprotective properties and are catalyzed by soluble epoxide hydrolase (sEH), which is detected in a variety of mammalian tissues, including the liver, kidney, intestine, and blood vessels to dihydroxyeicosatrienoic acids (DHETs) that lack vasoactive property. Then, DHETs were released from circulation and excreted to urine (26, 35). EETs stimulate endothelial cell growth and angiogenesis and inhibit apoptosis (54). EETs enhance cardiac functional recovery and decrease infarct size after ischemia-reperfusion injury (45). EETs also exert an anti-inflammatory effect via the suppression of NF-κB and IκB kinase activity (36). Additionally, CYP2J2 targeting to endothelial cells attenuates adiposity and vascular dysfunction in mice fed a high-fat diet (1). The anti-inflammatory, angiogenic, and antiapoptotic properties of EETs suggest that CYP2J2, via the production of EETs, may attenuate diabetes-related disease progression through multiple mechanisms.

Numerous therapeutic agents have been developed to treat and manage T2DM. Introduced in the late 1990s, the thiazolidinedione (TZD) class of drugs activates the nuclear peroxisome proliferator-activated receptor-γ (PPARγ) and has been shown to attenuate insulin resistance (11). Although the therapeutic effects of TZDs in adipose tissue and peripheral tissues...
and by what mechanism(s), CYP2J2 expression can attenuate CYP2J (58). In this study, we sought to determine whether, increased expression of CYP2J3, the rat homolog of human CYP2J was significantly decreased in streptozotocin (STZ)-treated rats (32). Fructose-induced diabetes is attenuated by increased expression of CYP2J3, the rat homolog of human CYP2J (58). In this study, we sought to determine whether, and by what mechanism(s), CYP2J2 expression can attenuate CYP2J.

**RESEARCH DESIGN AND METHODS**

Construction and preparation of recombinant adeno-associated virus. Recombinant adeno-associated virus (rAAV)-CYP2J2 and rAAV-green fluorescent protein (GFP) were packaged in human 293 cells (American Type Culture Collection, Manassas, VA) and purified by a single-step gravity-flow column purification method as described previously (54).

Animals. Male diabetic mice (db/db) aged 8–10 wk and age-matched control C57BL/6J mice were purchased from the Nanjing Animal Center. The mice were fed using standard rodent chow and water ad libitum in sterile cages with a 12:12-h light-dark cycle. rAAV-CYP2J2 or rAAV-GFP (5 × 10^11 plaque-forming units) was injected through the tail veins of 12-wk-old mice. After 8 wk, mice were then administered GW-9662 (an irreversible PPAR antagonist) (34) in drinking water (1 mg·kg^−1·day^−1) every day for 4 wk (20). Body weight, plasma glucose, triglycerides, and cholesterol were measured every 2 wk, and food intake was measured every week (2). All of the experiments were conducted according to institutional guidelines for the humane treatment of laboratory animals and conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996). The study was approved by the Institutional Animal Research Committee of Tongji Medical College.

**Spontaneous voluntary activity.** Spontaneous voluntary activity was analyzed in an observation cage (26 × 62 × 66 × 39 cm) with white opaque walls using the infrared photo detection method, coupled with an automated activity monitor (TruScanH; Coulbourn Instruments) (41). The system was designed to enable separate monitoring of horizontal (x-y move time) and vertical activity (rearing). The test session took 10 min and was replicated after 48 h to assess the extent of habituation to the test cage. To avoid odor traces, the test cage was cleaned with 70% ethanol before each mouse.

Glucose and insulin tolerance. After 12 wk of gene delivery, glucose and insulin tolerance tests were performed on animals that had been fasted overnight. Blood glucose concentrations were determined at 30, 60, 90, and 120 min after animals were injected with 2 g/kg body wt of glucose by gavage or intraperitoneal injection of human regular insulin (0.75 U/kg body wt), as described previously (28).

**Hyperinsulinemic euglycemic clamp study.** The clamp studies were described as described previously (12). Briefly, 7 days before the clamp studies, indwelling catheters were inserted into the right internal jugular vein and advanced into the superior vena cava. After overnight fasting, the basal rate of glucose turnover was measured by continuous infusion of [3-3H]glucose (HPLC purified; Perkin-Elmer, Boston, MA) at a rate of 0.05 μCi/min for 2 h. Then, a 120-min hyperinsulinemic euglycemic clamp was conducted. Insulin was infused as a bolus of 300 mU/kg over a period of 3 min, followed by continuous insulin infusion at the rate of 2.5 mU·kg^−1·min^−1 (Humin R; Eli Lilly, Indianapolis, IN) to raise the plasma insulin concentration to a physiological range (4 ng/ml). Blood samples (10 μl) were collected at 10- to 20-min intervals for immediate measurement of plasma glucose, and 20% dextrose was infused at various rates to maintain plasma glucose at basal concentrations (6.7 mM). Insulin-stimulated whole body glucose flux was estimated using a primed, continuous infusion of [3-3H]glucose (10 μCi bolus, 0.1 μCi/min) throughout the clamp study. To estimate insulin-stimulated glucose transport activity, 2-deoxy-[2-14C]glucose (Perkin-Elmer) was injected as a bolus (10 μCi) 75 min into the clamp. Blood samples (10 μl) for the measurement of plasma [3H] and [14C] activities were taken at the end of the basal period and during the last 45 min of the clamp. Additional blood samples were obtained for the measurement of plasma insulin and free fatty acid concentrations at the end of basal and clamp periods. At the end of the clamp, mice were anesthetized with pentobarbital sodium, and tissues were dissected, frozen immediately using liquid N2-cooled aluminum blocks, and stored at −80°C for subsequent analysis.

**Glucose flux calculation.** For the determination of plasma [3H]glucose, plasma was deproteinized with ZnSO4 and Ba(OH)2,

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5’–3’)</th>
<th>Reverse (5’–3’)</th>
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<td>GCCAAATCTCCACTTTTGCCACTG</td>
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<tr>
<td>CRP</td>
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<td>TNFα</td>
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PPARγ, peroxisome proliferator-activated receptor-γ; G6PC, glucose-6-phosphatase catalytic subunit; PECK, phosphoenolpyruvate carboxykinase; GCK, glucokinase; CRP, C-reactive protein. Mouse primer sets are in lightface; human primer sets are in boldface.

**Table 1. Real-Time PCR primers**
dried to remove $^{3}$H$_{2}$O, resuspended in water, and then counted in scintillation fluid (Ultima Gold; Perkin-Elmer) on a scintillation counter (Beckman, Fullerton, CA). Rates of basal and insulin-stimulated whole body glucose turnover were determined as the ratio of the [3-$^{3}$H]$\text{glucose}$ infusion rate (disintegrations/min) to the specific activity of plasma glucose (disintegrations·min$^{-1}$·mg$^{-1}$) at the end of the basal period and during the final 30 min of the clamp experiment, respectively. Hepatic glucose production was determined by subtracting the glucose infusion rate from the total glucose appearance rate. The plasma concentration of $^{3}$H$_{2}$O was determined by the difference between $^{3}$H counts without and with drying. Whole body glycolysis was calculated from the rate of increase in plasma $^{3}$H$_{2}$O concentration divided by the specific activity of plasma [3$^{3}$H]glucose, as described previously (59). Whole body glycogen synth ent was estimated by subtracting whole body glycolysis from whole body glucose uptake, assuming that glycolysis and glycogen synth ent accounted for the majority of insulin-stimulated glucose uptake. For the determination of individual tissue glucose uptake, tissue samples were homogenized and the supernatants subjected to an ion-exchange column to separate tissue 2-deoxy-[14C]$\text{glucose}$ (2-[14C]DG)-6-phosphate from 2-[14C]DG. Tissue glucose uptake was calculated from the area under curve of plasma 2-[14C]DG profile and muscle 2-[14C]DG-6-phosphate content, as described previously (59).

**Biochemical analysis.** Plasma glucose, triglyceride, and total cholesterol levels were measured using a commercial kit (Glucose CII-166 Test, Triglyceride E-Test, and Cholesterol E-Test). Plasma insulin levels were measured by an ultrasensitive mouse insulin ELISA kit (Mercodia). CRP, IL-1β, IL-6, and TNFα in serum and liver, IL-6, TNFα in adipose tissue, and skeletal muscle were measured by enzyme-linked immunosorbent assays (Lincoplex).

Fig. 1. Cytochrome P450 epoxygenase ($\text{CYP2J2}$) gene therapy decreased body weight, food intake, plasma triglyceride, and cholesterol levels and increased physical activity in $\text{db/db}$ mice. Mice were treated with recombinant adeno-associated virus ($\text{rAAV}$)-CYP2J2 for 12 wk and GW-9662 (GW; 1 mg·kg$^{-1}$·day$^{-1}$) for 4 wk, as indicated. Effect of $\text{rAAV}$-CYP2J2 transfection on body weight (A), plasma triglycerides (B), plasma LDL cholesterol (C), food intake (D), and distance traversed (E; cm). Data are shown as the mean ± SE of 6–8 mice/group. *$P < 0.01$ compared with control mice; **$P < 0.05$ compared with $\text{db/db}$/$\text{rAAV}$-CYP2J2 mice; #$P < 0.05$ compared with $\text{db/db}$ + GFP mice.
**Immunoblotting analysis.** Liver nuclear and cytoplasmic extracts were prepared using the NE-PER extraction reagent kit (Pierce Biotechnology). Western blot analysis was performed as described previously (58). Blots were incubated with primary antibodies [anti-CYP2J2, anti-sEH, anti-PPARγ, anti-phospho-NF-κB p65 (Ser536), anti-ERK, anti-phospho-ERK (Thr202/Tyr204), anti-JNK, or anti-phospho-JNK] at 4°C for overnight. Anti-CYP2J2 is from Dr. Darryl C. Zeldin’s laboratory, and it is specific for detection of CYP2J2 of human as a rabbit polyclonal IgG (19). Anti-sEH, anti-PPARγ, anti-NF-κB p65, and lamin B were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); other antibodies were purchased from Cell Signaling Technology (Beverly, MA).

**14,15-DHET levels by ELISA.** 14,15-dihydroxyeicosatrienoic acid (14,15-DHET), the stable EET metabolite in urine and liver tissues, was measured by an ELISA kit (Detroit R & D, Detroit, MI) per the manufacturer’s instructions.

**Immunohistochemical evaluation.** The infiltration of activated macrophages in liver tissue was assessed by immunohistochemistry staining of established macrophage markers, including F4/80 for mature macrophages, CD11C for classically activated (M1) macrophages, and CD206 for alternatively activated (M2) macrophages (6, 21, 50) to illustrate the hepatic inflammation response to diabetic pathology (27). Immunohistochemistry was performed as usual (63). Briefly, paraffin sections of livers were deparaffinized, blocked with 3% peroxidase solution followed by 10% normal goat serum, and then incubated in primary antibody for F4/80, CD11C, or CD206 at 4°C overnight; then they were incubated with biotinylated anti-rabbit secondary antibodies and 3,3-diaminobenzidine substrate. Finally, the paraffin sections of livers were deparaffinized, blocked with 3%

**Total RNA preparation and quantitative RT-PCR analysis.** Total RNA was isolated with Trizol (Invitrogen), and cDNA was synthesized using the Applied Biosystems cDNA Archive Kit. Quantitative RT-PCR was performed with SYBR green (Invitrogen), using primers listed in Table 1. Data are expressed as band intensity for the gene of interest normalized to glyceraldehyde-3-phosphate dehydrogenase.

**Cell culture.** Human hepatoma HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium containing 4 mM L-glutamine, 1 mM pyruvate, 0.05 mg/ml penicillin, and 0.05 mg/ml streptomycin supplemented with 10% fetal calf serum (BioWhitaker). For free fatty acid incubations, the medium was prepared as described previously (38), Palmitic acid (500 μM) treatment was done for 24 h, and insulin (100 nM) treatment was done for 10 min.

**ELISA.** Cells were plated in six-well cell culture plates at a density of 2 × 10⁵ cells/well and allowed to grow for 24 h. After transfection with rAAV-CYP2J2 or rAAV-GFP for 24 h, the cells were treated with GW-9662 (1 μM) or 14,15-EEZE (an EET antagonist;1 μM) for 30 min, followed by palmitate (PA; 500 μM). After 24-h stimulation with PA, CRP, IL-1β, IL-6, and TNfα in the culture, media were quantified by using an ELISA kit (eBioscience).

**Glucose uptake by cultured hepatocytes.** HepG2 were plated onto six-well plates at 4 × 10⁵ cells/well, and glucose uptake was measured as described previously (44). The cells were cultured in a 37°C CO₂ incubator in William’s E buffer with 10% FCS. Subsequently, cells were serum starved overnight in William’s E buffer with 0.1% BSA. The cells were washed twice with Krebs-Ringer-Pi-HEPES (KRPH) and treated with either 100 nM insulin or KRPH buffer (control) for 30 min at 37°C, followed by the addition of 10 mM 2-deoxy-o-glucose (2-DG) containing 0.3 mCi/ml 2-deoxy-O-[¹H]glucose. After a 10-min incubation, the cells were washed with ice-cold PBS three times, air-dried, then lysed with ice-cold 0.2 N NaOH. Radioactivity was determined by scintillation counting, and protein concentrations were determined using the Bio-Rad DC protein assay. 2-DG uptake was expressed

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**Fig. 2.** CYP2J2 gene therapy ameliorated glucose tolerance/insulin resistance in db/db mice. Effect of rAAV-CYP2J2 treatment on nonfasting blood glucose levels (A), nonfasting insulin levels (B), glucose tolerance (C), and insulin tolerance (D) in db/db mice. %Basal point to %time 0 glucose level. Results shown are the mean ± SE of 6–8 mice/group. *P < 0.01 compared with WT control mice; #P < 0.05 compared with db/db (A and B) or db/db + green fluorescent protein (GFP) mice (C and D); **P < 0.05 compared with db/db + rAAV-CYP2J2 mice.
as counts-min⁻¹-ng⁻¹-ml⁻¹ protein concentration. A similar protocol was used to measure 2-deoxy-o-[³H]glucose uptake in wild-type cells that were either pretreated with 100 µM ATP or KRPH buffer for 30 min before the insulin-stimulated 2-DG uptake assay was commenced.

14,15-EET administration. HepG2 cells were grown to 80–90% confluence and then pretreated with GW-9662 (1 µM) or vehicle for 30 min, followed by the addition of 14,15-EET (250 nM) in DMSO.

Data analysis and presentation of results. All results are expressed as means ± SE. Comparisons between groups were performed by a one-way ANOVA with post hoc analyses performed using the Student-Newman-Keuls test. Values in glucose and insulin tolerance tests were determined by two-way ANOVA. P < 0.05 was considered significant.

RESULTS

CYP2J2 gene therapy in mice. To assess the in vivo effect of CYP2J2 expression on T2DM, male db/db mice (12 wk old) were injected intravenously with rAAV-CYP2J2 or rAAV-GFP. After 12 wk, rAAV-CYP2J2 injection induced predominant protein expression of CYP2J2 in the liver, but CYP2J2 protein was also detected in the heart and less so in skeletal muscle, whereas brain, kidney, aorta, and adipose tissue had no detectable CYP2J2 protein levels (data not shown). Increased EET levels were confirmed by measuring urinary 14,15-DHET, a stable 14,15-EET metabolite. Because we know that epoxygenases result in the production of four regioisomeric EETs, we used 14,15-DHETs here to represent levels of EET production. 14,15-DHET was released from circulation, especially liver, endothelial cells, and cardiomyocytes, and then excreted through the kidney. Thus urinary elevated 14,15-DHET in rAAV-2J2 animals reflects increased EET levels from liver to some extent. rAAV-CYP2J2 treatment increased urinary 14,15-DHET levels from 8.32 ± 2.41 to 30.66 ± 3.82 ng/ml (n = 8, P < 0.05). rAAV-CYP2J2 treatment also increased hepatic 14,15-DHET levels from 3.38 ± 1.03 to 21.17 ± 1.2 µg/mg tissue (n = 8, P < 0.01). In addition, we measured the expression of CYP2J2 in livers of C57BL mice at 2, 4, 8, and 12 wk, and the results showed that CYP2J2 expression was stable. We also detected 14,15-DHET levels in the livers of C57BL/CYP2J2 and db/CYP2J2 mice at 2, 4, 8, and 12 wk, and the data showed that 14,15-DHET levels did not change during experimental time course (data not shown), further suggesting that CYP2J2 expression is stable during the time course. The PPARα antagonist used in this study (GW-9662) had no effect on either CYP2J2 protein expression or 14,15-DHET levels. rAAV-CYP2J2 and/or GW-9662 treatment had no effect on sEH expression or endogenous mouse CYP2J2 proteins levels, either of which could also result in increased 14,15-DHET (data not shown).

CYP2J2 gene therapy attenuates metabolic dysfunction in diabetic mice. Prior to gene therapy treatment, 12-wk-old male db/db mice had severe metabolic disorders, including hyperglycemia, hyperlipidemia, and hyperinsulinemia compared with C57BL/6 mice (Figs. 1 and 2). After an additional 12 wk, body weight was increased further in db/db mice (56.4 ± 5.2 g), whereas C57BL/6 mice had a minimal change in body weight (23.1 ± 3.6 g). Meanwhile, db/db mice had increased food intake (34.45 ± 2.36 g/mouse⁻¹-wk⁻¹) and were less active (884 ± 77.11 cm) during the dark-light cycle compared with C57BL/6 mice (19.64 ± 2.13 g/mouse⁻¹-wk⁻¹, 3.379 ± 212.9 cm). CYP2J2 gene therapy significantly attenuated the increase in body weight (Fig. 1A) with increased physical activity (Fig. 1E) and decreased food intake (Fig. 1D), decreased plasma triglycerides (Fig. 1B), and decreased LDL levels (Fig. 1C) compared with control db/db mice. PPARα antagonist (GW-9662) treatment attenuated the beneficial ef-
CYP2J2 gene therapy. In C57BL/6 mice, no significant change in body weight, triglycerides, or LDL cholesterol occurred with/without rAAV treatment or PPARγ antagonist (GW-9662) treatment (data not shown).

rAAV-CYP2J2 treatment significantly lowered nonfasting blood glucose levels in db/db mice (16.8 ± 3.1 vs. 28.4 ± 3.6 mmol/l, P < 0.01) and decreased insulin release (1.52 ± 0.09 vs. 2.21 ± 0.14 ng/ml, P < 0.01). GW-9662 treatment attenuated these effects by increasing blood glucose (22.1 ± 4.7 mmol/l) and decreasing insulin release (2.34 ± 0.11 ng/ml) in rAAV-CYP2J2-treated mice. Glucose and insulin tolerance tests performed at the end of the experiment showed that CYP2J2 expression improved blood glucose levels and glucose tolerance in db/db mice (Fig. 2C). The reduction in blood glucose levels in response to insulin was also much greater in rAAV-CYP2J2-treated db/db mice compared with untreated db/db mice (Fig. 2). By comparison, GW-9662 attenuated the mild glucose-intolerant state in rAAV-CYP2J2-treated db/db mice but had no significant effect on untreated db/db mice (Fig. 2, C and D).

CYP2J2 gene therapy increases insulin sensitivity in db/db mice. To assess insulin sensitivity, we performed hyperinsulinemic euglycemic clamps in conscious C57BL/6 and db/db mice with radioisotope-labeled glucose infusion. Diabetic mice showed severe insulin resistance, and a 70% reduction in the steady-state glucose infusion rate was required to maintain euglycemia in db/db mice compared with C57BL/6 mice (Fig. 3A). Additionally, basal hepatic glucose production of untreated db/db mice was almost ~50% higher than that of C57BL/6 mice and was reduced by rAAV-CYP2J2 treatment (P < 0.05; Fig. 3B). The glucose uptake, glycolysis, and glycogen synthesis in db/db mice decreased 61, 32, and 53%, respectively, compared with C57BL/6, which further confirmed severe insulin resistance in db/db mice (Fig. 3C). In contrast, insulin sensitivity in the rAAV-CYP2J2-treated db/db mice was markedly increased, as reflected by the twofold increase in the steady-state glucose infusion rate that was necessary to maintain euglycemia (Fig. 3A). The improvement in insulin-stimulated glucose metabolism could be attributed to a 46% increase in insulin-induced suppression of hepatic glucose production (Fig. 3B) and a 72% increase in insulin-stimulated whole body glucose uptake (Fig. 3C). Glucose uptake in liver was significantly increased in rAAV-CYP2J2-treated db/db mice (Fig. 3D). This suggests that CYP2J2 expression suppresses hepatic glucose production by decreasing glycogenolysis and gluconeogenesis, leading to increased hepatic insulin sensitivity. GW-9662 treatment attenuated all the observed beneficial effects of rAAV-CYP2J2 treatment.

CYP2J2 expression inhibits expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase catalytic subunit and stimulates expression of glucokinase and PPARγ. PPARγ transcript levels increased 4.5-fold in the liver follow-

Fig. 4. CYP2J2 gene therapy increased expression of peroxisome proliferator-activated receptor-γ (PPARγ), decreased gluconeogenesis-related genes in livers of db/db mice, and regulated glycogen synthesis. A: CYP2J2 therapy increased expression of PPARγ genes in livers of db/db mice. B–D: CYP2J2 therapy decreased expression of glucose-6-phosphatase catalytic subunit (G6PC; B), decreased expression of phosphoenolpyruvate carboxykinase (PEPCK; C), and increased glucokinase (GCK) expression (D) in livers of db/db mice. Data are shown as the mean ± SE of 6–8 mice/group. *P < 0.05 compared with untreated C57BL/6 mice; **P < 0.01 compared with untreated db/db mice; #P < 0.05 compared with untreated db/db + rAAV-CYP2J2 mice.
ing rAAV-CYP2J2 treatment of db/db mice compared with untreated db/db mice (Fig. 4A). rAAV-CYP2J2 treatment reduced glucose-6-phosphatase catalytic subunit (G6PC) and phosphoenolpyruvate carboxykinase (PEPCK) levels in the livers of db/db mice, and GW-9662 reversed these effects (Fig. 4, B and C). Glucokinase (GCK), critical in glycolysis, was significantly reduced in diabetic livers. GCK expression was restored to nearly nondiabetic levels by rAAV-CYP2J2 treatment in db/db mice, and GW-9662 suppressed this effect (Fig. 4D). This suggests that CYP2J2 expression may lead to inhibition of glucose production to maintain glucose homeostasis.

**CYP2J2 suppresses production of proinflammatory cytokines.** Insulin resistance is increased in the setting of inflammation, and therefore, the anti-inflammatory effects of CYP2J2 gene therapy were assessed. CRP, IL-6, IL-1β, and TNFα protein levels were increased in serum and liver and transcriptionally upregulated in the livers of diabetic mice (Fig. 5, A–D). IL-6 and TNFα protein levels were also increased in adipose tissue and muscle of diabetic mice, but not as dramatically as in the liver (Fig. 5, E and F). CYP2J2 expression decreased both protein and transcript levels of all four inflammatory markers, and these effects were most prominent in the liver compared with adipose and muscle tissue. We also evaluated the inhibitory effects of CYP2J2 expression on inflammatory cytokine production in HepG2 cells with PA-induced insulin resistance. PA treatment increased the expression of CRP, IL-1β, IL-6, and TNFα in HepG2 cells (Fig. 6, A–D). CYP2J2 transfection prior to PA treatment attenuated the expression of all four inflammatory markers. GW-9662 or 14,15-EEZE partially reversed the anti-inflammatory effects of CYP2J2 gene therapy in vivo or in vitro.

**CYP2J2 prevents hepatic macrophage infiltration.** To investigate whether CYP2J2 expression played an important role in hepatic inflammation, we stained for established markers of mature macrophages (F4/80) and M1- (CD11C) and M2-activated (CD206) macrophages. CYP2J2 expression decreased macrophage infiltration, including mature, M1, and M2 macrophages, in the livers of db/db mice (Fig. 7, A–D). Additionally, CYP2J2 decreased M1 macrophages more than M2 macrophages (Fig. 7E), and GW-9662 attenuated the effects of CYP2J2.

**CYP2J2 expression suppresses inflammatory signaling pathways.** We assessed the effects of CYP2J2 expression on inflammatory signaling in the liver (22). Liver PPARγ protein levels were significantly upregulated in db/db mice by CYP2J2 expression (Fig. 8, A and B), and rAAV-CYP2J2 treatment suppressed NF-κB nuclear content in the livers of db/db mice (Fig. 8, C and D).

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**Fig. 5.** CYP2J2 gene therapy inhibited inflammatory cytokine production in the liver of db/db mice. CYP2J2 gene therapy decreased serum C-reactive protein (CRP; A) and serum IL-1β, IL-6, and TNFα levels. CYP2J2 expression decreased IL-1β, IL-6, and TNFα levels in livers of db/db mice (C) and downregulated CRP, IL-1β, IL-6, and TNFα mRNA expression in livers of db/db mice (D). CYP2J2 gene therapy decreased IL-6 and TNFα levels in adipose tissue (E) and skeletal muscle (F). Data are shown as the mean ± SE of 6 different animals. *P < 0.05 compared with C57BL/6 mice; **P < 0.05 compared with db/db mice; #P < 0.05 compared with db/db + rAAV-CYP2J2 mice.
A and C). CYP2J2 expression also attenuated the increased phosphorylation of ERK1/2 and JNK in db/db mice (Fig. 8, A, D, and E). GW-9662 attenuated most of the anti-inflammatory properties of CYP2J2 but had only a marginal effect on PPARγ expression. To corroborate the in vivo results, the anti-inflammatory properties of CYP2J2 expression were assessed in PA-induced inflammation in HepG2 cells. Similar to in vivo results, CYP2J2 expression increased PPARγ expression, attenuated NF-κB nuclear content, and decreased the phosphorylation of ERK1/2 and JNK in HepG2 cells. Consistent with both the in vivo and in vitro effects of CYP2J2 expression, treatment with the CYP2J2 metabolite 14,15-EET decreased the phosphorylation of ERK and JNK in PA-treated HepG2 cells (Fig. 8, D and E).

Additionally, to confirm that CYP2J2 effects are EET-dependent, 14,15-EEZE was used in PA-induced inflammation in HepG2 cells, and we found that CYP2J2’s effects on inflammatory cytokines and inflammation-signaling pathways were partially blocked by 14,15-EEZE (Fig. 8F), suggesting that CYP2J2 anti-inflammation’s effects are EET-dependent.

**CYP2J2 expression prevents PA-induced impairment of insulin signaling.** The metabolic activity of insulin was evaluated by 2-deoxy-D-[3H]glucose uptake in HepG2 cells. PA decreased insulin-induced glucose uptake by ~60%. PA-induced repression of glucose uptake was attenuated by CYP2J2 expression, and the effect was inhibited by GW-9662 treatment (Fig. 9A) and 14,15-EET inhibitor 14,15-EEZE. The latter suggested that CYP2J2 plays a role in insulin sensitivity in hepatocytes through EETs (Fig. 9B). Insulin-induced Akt phosphorylation was also decreased by PA treatment in HepG2 cells, and CYP2J2 expression reversed this effect completely (Fig. 9, C and D). The data support a protective role of CYP2J2 on insulin resistance in hepatocytes.

**DISCUSSION**

In the current study, we assessed the effects of CYP2J2 gene therapy in the liver on insulin resistance and diabetes in db/db mice and PA-treated hepatocytes (HepG2). rAAV-CYP2J2...
treatment in mice and HepG2 cells resulted in CYP2J2 protein expression and a corresponding increase in EET production. Both in the liver and in HepG2 cells, CYP2J2 expression enhanced glucose uptake and decreased glucose production by reducing expression of key enzymes associated with gluconeogenesis. Reduced production of inflammatory cytokines and macrophage migration by CYP2J2 expression are mediated partly by attenuated NF-κB nuclear content. Importantly, both CYP2J2 gene therapy and exogenous EETs inhibited the activation of inflammatory pathways in vivo and in vitro. These anti-inflammatory properties of CYP2J2 expression contribute to the alleviation of severe diabetic phenotype and insulin resistance in db/db mice.

Induction of diabetes decreased renal CYP2C44 and CYP4A expression in wild-type mice in previously published literature (17). Moreover, in STZ-treated rats, expression of CYP4A, CYP2C11, CYP2C23, and CYP2J was decreased significantly (32), and we also showed that CYP2J expression was decreased in the livers of diabetic db/db mice (Fig. 1F). In addition, sEH expression was lower in adipose tissue than in liver and kidney, but total adipose sEH activity was selectively increased in obese mice fed a high-fat diet (14). Because it is known that sEH activity is considered a major determinant of EET's bioavailability (25), it is speculated that adipose EET level was reduced in obese mice.

Numerous studies have identified an association between systemic inflammation and increased risk of developing T2DM, suggesting that inflammatory processes may contribute to the pathophysiology of the disease (16). Although the molecular mechanisms involved are poorly understood, activation of proinflammatory signaling molecules such as IKKβ/ NF-κB, activating protein-1, and JNK signaling molecules could interact with insulin-signaling pathways via inhibitory phosphorylation of insulin receptor substrate-1 (5, 62). NF-κB, a key regulator of hepatic inflammation, upon exposure to proinflammatory stimuli, promotes macrophage localization and activation (3). Macrophage recruitment to the liver results in an positive feed-forward loop for secretion of proinflammatory cytokines (49). However, endogenous epoxygenases are immunomodulators regulating monocyte/macrophage activation, depending on the underlying activation state (7). In the current study, we further validated the link between diabetic phenotypes and circulating levels of inflammatory markers and show that CYP2J2 expression decreased both the levels of inflammatory cytokines and macrophage recruitment.

Increasing evidence suggests that white adipose tissue, skeletal muscle, and the liver all suffer from severe inflammation and perturbed metabolism in T2DM. The metabolic dysfunction observed in the liver and adipose tissue is consistent with insulin resistance (IR) (48). IR often leads to further increases in inflammation of the liver (40). In addition, liver gluconeogenesis dysfunction, which is observed in diabetes-related hepatic disease, is a primary mechanism leading to fasting hyperglycemia in T2DM and leads to progressive hepatic insulin resistance in T2DM (33). In this study, we show that CYP2J2 gene therapy resulted in a significant decrease in CYP2J2, HEPATIC INFLAMMATION, AND DIABETES

Fig. 7. CYP2J2 prevented macrophage infiltration in liver of db/db mice. A: representative images of immunohistochemical staining for macrophage antibody; F4/80, CD11C, and CD206 reveal stronger overall staining of macrophages in livers of db/db mice than in the rAAV-CYP2J2-treated db/db mice (magnification: ×200). B–D: CYP2J2 overexpression markedly attenuated insulin resistance-induced macrophage (B), M1 macrophage (C), and M2 macrophage (D) infiltration. *P < 0.01 compared with control mice; **P < 0.05 compared with db/db + rAAV-CYP2J2 mice; #P < 0.05 compared with untreated db/db mice. E: CYP2J2 overexpression prevents more M1 than M2 migration. Quantitative image analysis of 10 staining-positive areas was performed in both db/db and db/db mice in the same section. Values are expressed as the decreased %positive cell over total cells per 0.1 mm² in db/db2J2 compared with db/db mice. *P < 0.05 compared with CD11C- cell reduction.

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blood glucose and blood insulin levels in diabetic mice. The antihyperglycemic effect of CYP2J2 appeared to be mediated partially through decreased expression of PEPCK and G6PC in the liver, both of which are critical in gluconeogenesis. In our study, CYP2J2 was also expressed in the heart and muscle, whereas rAAV-CYP2J2 was liver-aimed expressed, which means that liver-specific CYP2J2 expression impacts heart and muscle CYP2J2 expression. Furthermore, reduced inflammation in liver-specific CYP2J2-expressed db/db mice suggested that other tissues might also be impacted. In vivo studies showed that inflammation cytokine levels in adipose tissue and skeletal muscle were obviously reduced (Fig. 6, E and F), suggesting that there might be cross-talk between liver and muscle and adipose tissue, possibly through hepatic production of circulating substances. In our study, it is speculated that CYP2J2 overexpression and subsequent increased circulating EET levels contributed to the inhibition of inflammatory re- sponse and improvement of insulin sensitivity in both skeletal muscle and adipose tissue. Furthermore, we have demonstrated that treatment of HepG2 cells with exogenous 14,15-EET inhibitor 14,15-EEZE inhibited the effects of CYP2J2 overexpression on insulin-induced glucose uptake. Additionally, inflammatory cytokines and inflammation-signaling pathways were reduced by 14,15-EEZE, and exogenous 14,15-EET attenuated PA-induced ERK1/2 phosphorylation (D), and inhibited JNK phosphorylation (E) in db/db mouse livers. Densitometry values shown are means ± SE from the experiment carried out from 4 to 5 independent experiments. *P < 0.05 vs. control; **P < 0.05 vs. db/db mice; #P < 0.05 vs. db/db + rAAV-CYP2J2 mice.

Fig. 8. CYP2J2 gene therapy inhibits inflammatory signaling pathways in the livers of db/db mice, with similar results in in vitro CYP2J2 expression or in vitro EET treatment. A: total and nuclear protein extracts from mouse livers and cells were analyzed by Western blot, as indicated. CYP2J2 gene therapy increased PPARγ expression (B), decreased NF-κB nuclear context (C), inhibited ERK1/2 phosphorylation (D), and inhibited JNK phosphorylation (E) in db/db mouse livers. Densitometry values shown are means ± SE from the experiment carried out from 4 to 5 independent experiments. *P < 0.05 vs. control; **P < 0.05 vs. db/db mice; #P < 0.05 vs. db/db + rAAV-CYP2J2 mice.

The antidiabetogenic effects of PPARγ agonists have been documented in several studies (31). The PPAR receptors have emerged as important molecules that functionally link lipids, metabolic diseases, and immunity (52). PPARγ has several anti-inflammatory effects, including reduction of NF-κB activity, reduction in proinflammatory molecule production, and promotion of anti-inflammatory molecule production (24). The TZDs, via activation of the PPARγ receptor, have proven benefits of attenuating insulin resistance and hyperglycemia associated with T2DM (11). In this study, CYP2J2 gene therapy significantly increased PPARγ expression and inhibited NF-κB activation, which led to reduced hepatic inflammation. Most of the beneficial, antidiabetogenic effects of CYP2J2 expression were attenuated by PPARγ receptor antagonism (GW-9662), suggesting that CYP2J2 expression ameliorated inflammation and reduced severity of diabetic-
related dysfunction at least partially through the activation of the PPARγ receptor.

Previous studies have shown that EET-induced PPARγ signaling contributed to the attenuation of insulin resistance in T2DM animal models (37, 51). In this study, PPARγ antagonism markedly attenuated the beneficial effects of both CYP2J2 expression and EETs in terms of insulin resistance and inflammation. To further validate that the beneficial effects of CYP2J2 expression are EET dependent, we assessed the effects of CYP2J2 overexpression on insulin resistance in palmitic acid-treated hepatocytes. The ability of exogenous EETs to mimic the effects of CYP2J2 expression and the ability of the EET antagonist to attenuate the effect of CYP2J2 expression further confirmed that the attenuation of insulin resistance in this model was EET dependent. Furthermore, CYP2J2 gene delivery attenuated diabetic pathophysiology in db/db mice by attenuating the inflammatory response seen in T2DM.

In conclusion, our study suggests that modulating CYP2J2 expression and/or endogenous levels of its metabolites may represent a novel approach to treatment of insulin resistance and T2DM. The effects of CYP2J2 expression were associated with increased insulin sensitivity via reduced activation of NF-κB and MAPK signaling pathways in liver. The ability of CYP2J2 gene therapy to exert a broad spectrum of beneficial effects in the db/db diabetic model via the PPARγ receptor provides additional mechanistic insights into pathogenesis of T2DM. In addition, our previous studies demonstrated that CYP2J2 or -2C overexpression can enhance cancer (10, 29) but not carcinogenesis. Thus, patients with cancer will be contraindicative for gene therapy or EET administration in future. Of course, there are relatively few clinical studies that evaluate cancer with CYP therapeutic outcome, which still needs to be explored further. In summary, this study, along with numerous other studies over the last decade, highlights the therapeutic potential of modulating CYP2J2 epoxygenase metabolite levels in the management of diabetes.

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DISCLOSURES

The authors have nothing to disclose

AUTHOR CONTRIBUTIONS

R.L. performed experiments; R.L. and A.G. prepared figures; R.L. drafted manuscript; X.X., C.C., Y.W., and D.W.W. conception and design of research; X.X., Y.W., A.G., and D.W.W. interpreted results of experiments; C.C. analyzed data; A.G., D.C.Z., and D.W.W. edited and revised manuscript; D.C.Z. and D.W.W. approved final version of manuscript.

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