Increased susceptibility of \textit{db/db} mice to rosiglitazone-induced plasma volume expansion: role of dysregulation of renal water transporters

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1Department of Pharmacology, Peking University, Beijing, China; 2Internal Medicine, University of Utah and Veterans Affairs Medical Center, Salt Lake City, Utah; 3Institute of Hypertension, Sun Yat-sen University School of Medicine, Guangzhou, China; 4Department of Pharmaceutical Sciences, College of Pharmacy, University of South Florida, Tampa, Florida; and 5Guangdong Provincial People’s Hospital and Guangdong Academy of Medical Sciences, Guangzhou, China

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Zhou L, Liu G, Jia Z, Yang KT, Sun Y, Kakizoe Y, Liu M, Zhou S, Chen R, Yang B, Yang T. Increased susceptibility of \textit{db/db} mice to rosiglitazone-induced plasma volume expansion: role of dysregulation of renal water transporters. \textit{Am J Physiol Renal Physiol} 305: F1491–F1497, 2013. First published September 4, 2013; doi:10.1152/ajprenal.00004.2013.—Thiazolidinediones (TZDs), which are synthetic peroxisome proliferator-activated receptor subtype-\(\gamma\) (PPAR\(\gamma\)) ligands, the synthetic insulin-sensitizing thiazolidinediones (TZDs) compounds, are used for glycemic control in patients with diabetes mellitus (13). Currently, two TZDs, rosiglitazone (RGZ) and pioglitazone, are available, since RGZ was withdrawn from the market in Europe and its use is restricted in the United States due to concerns about the increased risk of myocardial infarction in several clinical trials (18). TZDs lower blood glucose by increasing insulin sensitivity, improving lipid profile, attenuating microaluminuria, decreasing blood pressure, and inhibiting inflammation in animal models and diabetic patients (6, 10, 14). Despite the favorable effect on glycemic control, these agents are associated with body weight gain and fluid retention, which is presented as edema in extremities in at least 5% of patients and can progress to pulmonary edema and congestive heart failure (4, 20, 29). TZD usually leads to a 6–7% increase in blood volume in healthy volunteers (1, 2). The blood volume expansion can cause blood cell dilution, resulting in reduction of hematocrit (Hct). The changes in Hct have been used as a surrogate marker for TZD-induced plasma volume expansion. Viswanathan et al. (27) recently showed that of 260 patients on RGZ, 70% had plasma volume expansion as reflected by a drop of Hct. The severity of the side effect increases in patients treated with TZD in combination with insulin therapy (15). The fluid retention is often resistant to loop diuretics until the TZD therapy is discontinued (12, 17, 28).

The mechanism of TZD-induced fluid retention has been extensively investigated. Abundant evidence supports increased renal tubular transport as a major contributor of TZD-induced fluid retention although extrarenal mechanisms also play a role. Within the kidney, PPAR\(\gamma\) is predominantly expressed in the inner medulla and in inner medullary collecting duct (17, 18); an important site for the control of fluid metabolism. Collecting duct-specific deletion of PPAR\(\gamma\) attenuates plasma volume expansion induced by RGZ or pioglitazone (9, 30). ENaC is composed of three subunits (\(\alpha\), \(\beta\), and \(\gamma\)) and serves a major route for Na\(^+\) reabsorption across the apical membrane of the CD. There are conflicting reports on PPAR\(\gamma\) regulation of ENaC. ENaC\(\gamma\) transcription was originally shown to be upregulated by PPAR\(\gamma\) activation (9), but the expression of all three ENaC subunits was subsequently found to be suppressed by RGZ (5). Other reports showed that activation of other transporters, such as Na\(^+/H^+\) exchanger-3 (NHE3), NCC, and aquaporin (AQP)-2 and -3, may also play a contributory role (24). However, most of these studies employed normal animals, which may have limited the relevance to the clinical setting of diabetic patients treated with a TZD. The goal of the present study was to systematically examine the RGZ-induced fluid retention response in \textit{db/db} vs. lean control mice to further investigate the underlying mechanism.

**METHODS**

**Animals.** Male leptin receptor-deficient obese-diabetic \textit{db/db} (B6.BKS(D)-LepRd\(\text{vJ}\)) and nondiabetic \textit{db}/\textit{m} lean mice were purchased

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from Jackson Laboratories (Bar Harbor, ME). All animals were housed at the University of Utah Comparative Medicine Center, maintained on a 12-h light/dark cycle, and provided with food and water ad libitum. All animal procedures were approved by the University of Utah Institutional Animal Care and Use Committee.

**RGZ treatment.** RGZ was incorporated into a chow-based diet (LabDiet Rodent Chow 5001; Purina) at a level of 80 mg/kg diet. RGZ maleate (BRL49653C) was obtained from GlaxoSmithKline (Harlow, UK). The gelled diets were made by melting agar (1% by weight) in water (60%), cooling, and adding the drug and ground chow (39%). The same gelled diets without the drug served as controls. The mice were fed ad libitum. The db/db and lean mice were acclimatized to the control diet for 7 days. After the 7-day acclimation period, animals were placed on the gelled diet with or without RGZ for 14 days. Based on a measured average food intake of 7 g·day⁻¹·mouse⁻¹ for lean mice and 14 g·day⁻¹·mouse⁻¹ for db/db mice, the average dose of RGZ administered in both strains approximated 20 mg·kg⁻¹·day⁻¹. Measurements of body weight and collection of 24-h urine was performed.

**Hct measurement.** Hct was measured before and after RGZ treatment. The sphenous vein was punctured using a 23-gauge needle, and one drop of blood (≈5–10 µl) was collected by using a 10-µl capillary glass (Idaho Technology, Salt Lake City, UT). One side of the tube was sealed with Hemato-Seal and then centrifuged for 2 min in a Thermo IEC (Boston, MA) microcentrifuge machine.

**Nanoparticle measurement of plasma volume.** Measurement of plasma volume is traditionally reliant on the use of a dye tracer such as indocyanine green and Evans blue, which suffer numerous drawbacks including binding plasma proteins. Eisner et al. (7) have recently developed a nanotechnology-based method for accurate measurement of plasma volume in mice. Briefly, conscious mice received the fluorescent nanoparticle atto647 via a single bolus injection of suspension (100 µg in 50 µl of saline) into the tail vein. Seven minutes after tail vein injection, ~50 µl of whole blood samples were collected via puncturing of the submandibular vein. After centrifugation at 4,000 rpm for 5 min, plasma was collected and subjected to fluorescence measurements by using FluostarOptima (BMG Labtech). Plasma volume was determined based on the dilution factors.

**Quantitative RT-PCR.** Total RNA was isolated from renal tissues using TRIzol. One microgram of total RNA was denatured at 65°C for 5 min, and cDNA synthesis was then performed at 42°C for 1 h using Superscript reverse transcriptase (BRL, Gaithersburg, MD). Oligonucleotides were designed using Primer3 software (available at http://frodo.wi.mit.edu). Quantitative (q)PCR amplification was performed using SYBR Green Master Mix (Applied Biosystems) and the Prism 7500 Real-Time PCR Detection System (Applied Biosystems). Cycling conditions were 95°C for 10 min, followed by 40 repeats of 95°C for 15 s and 60°C for 1 min. The sequences of the oligonucleotide primers for are as follows: AQP2, 5’-ggaaccttgctgtcactgtc-3’ (sense) and 5’-atcggtggaggcaaagatg-3’ (antisense); GAPDH, 5’-gcttcaactagaggaagggag-3’ (sense) and 5’-tcatggatgaccttggccag-3’ (antisense); and PPARγ, 5’-ctctttaggtgctagtggaggtt-3’ (sense) and 5’cagcaggtgtctggagtgt-3’ (antisense).

![Fig. 1. Effect of rosiglitazone (RGZ) on body weight gain. Top: photograph of representative lean and db/db mice treated with vehicle or RGZ for 14 days. Bottom: body weight gain in lean and db/db mice over 14 days of RGZ treatment. CON, control (vehicle treatment). Data are means ± SE; n = 7 per group.](http://ajprenal.physiology.org/)

**Fig. 1.** Effect of rosiglitazone (RGZ) on body weight gain. Top: photograph of representative lean and db/db mice treated with vehicle or RGZ for 14 days. Bottom: body weight gain in lean and db/db mice over 14 days of RGZ treatment. CON, control (vehicle treatment). Data are means ± SE; n = 7 per group.
Immunoblotting. Lysate of the whole kidney was stored at \(-80^\circ\text{C}\) until assayed. Protein concentrations were determined using a Coomassie reagent. An equal amount of the whole tissue protein was denatured at 100°C for 5 min, separated by SDS-PAGE, and transferred to nitrocellulose membranes. The blots were blocked overnight with 5% nonfat dry milk in TBS, followed by incubation for 1 h with rabbit polyclonal antibodies against AQP2 (gift from Dr. Mark A. Knepper). The blots were washed with TBS followed by incubation with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody. Immune complexes were detected using enhanced chemiluminescence methods. The immunoreactive bands were quantified using the Gel and Graph Digitizing System (Silk Scientific, Orem, UT).

Blood glucose and plasma triglyceride measurement. Blood glucose levels were measured by CONTOUR blood glucose monitoring system (Bayer Healthcare, Mishawaka, IN). Plasma triglyceride was determined using a LabAssay Triglyceride ELISA Kit (catalog no. 290-63701; Wako).

Data analysis. Data are summarized as means \(\pm\) SE. Statistical analysis was performed using two-way ANOVA with the Bonferroni correction or Student’s \(t\)-test as appropriate. \(P<0.05\) was considered statistically significant.

Fig. 2. Effect of RGZ on plasma volume. Lean and \(db/db\) mice were treated with RGZ for 14 days. Hematocrit (Hct; \(A\)) and nanoparticle measurement of plasma volume (\(B\)) were determined before and after 14-day RGZ treatment. Data are means \(\pm\) SE. Lean mice: \(n = 6–7\) per group.

Fig. 3. Effect of RGZ on cardiac hypertrophy. Shown is heart weight in lean and \(db/db\) mice after 14-day treatment with vehicle or RGZ. Data are means \(\pm\) SE. Lean CON: \(n = 3\); lean RGZ: \(n = 6\); \(db/db\) CON: \(n = 3\); \(db/db\) RGZ: \(n = 6\).

Fig. 4. Effect of RGZ on urinary Na\(^+\) excretion and urine volume. Top: urinary Na\(^+\) excretion of vehicle and RGZ-treated lean mice and \(db/db\) mice. Bottom: urine volume of vehicle and RGZ-treated lean mice and \(db/db\) mice. Data are means \(\pm\) SE; \(n = 4–5\) per group.
ACCELERATED FLUID RETENTION IN ROSIGLITAZONE-TREATED db/db MICE

RESULTS

Effect of RGZ on body weight gain. TZDs induce weight gain as a result of fluid retention, adipogenesis, or a combination of the two mechanisms (19). Starting from 4 mo of age, db/db and lean mice were administered with RGZ (20 mg·kg body wt⁻¹·day⁻¹) for 14 days and body weight was monitored (Fig. 1). The body weight gain was 2.6 and 2.9 times greater in db/db mice than that in lean controls at days 7 and 14, respectively (day 7: 5.09 ± 2.2 vs. 1.95 ± 3%, P < 0.05; day 14: 15.91 ± 3.01 vs. 5.51 ± 4.43%, P < 0.01).

Effect of RGZ on plasma volume. Since TZDs have no effect on erythropoiesis, the change in Hct has been used as a surrogate marker of TZD-induced fluid retention (11). We examined Hct before and after RGZ treatment and also employed a newly developed nanoparticle-based technology to directly evaluate the changes in plasma volume. At day 14, RGZ treatment consistently induced a fall of Hct in both strains of mice and the fall was greater in db/db than in lean group (db/db: 44.8 ± 2.6 vs. 54.7 ± 1.7%, P < 0.05; lean: 47.8 ± 1.3 vs. 51.4 ± 1.8%, P < 0.05; Fig. 2A). Subsequently, plasma volume was measured using a fluorescent nanoparticle. Consistent with the Hct data, RGZ treatment induced a 220% increase in plasma volume in db/db mice (4.67 ± 2.2 vs. 2.16 ± 0.65 ml, P < 0.05) contrasting to only 30% increase in lean mice (1.77 ± 0.41 vs. 1.22 ± 0.12 ml, P < 0.05; Fig. 2B).

Effect of RGZ on heart weight. TZDs induce congestive heart failure in diabetic patients and cardiac hypertrophy in animals, possibly as a consequence of expanding plasma volume (3). Therefore, we evaluated heart weight in db/db and lean mice treated with RGZ. RGZ-treated db/db mice exhibited a significant increase in heart weight compared with vehicle control (185.6 ± 18.7 vs. 154.0 ± 17.6 mg, P < 0.05). In contrast, no change in heart weight was found in lean controls (128.6 ± 12.0 vs. 125.7 ± 5.7 mg, P > 0.05; Fig. 3). Histological analysis did not reveal obvious changes associated with RGZ treatment in db/db mice compared with vehicle control (data not shown).

Effect of RGZ on blood glucose and plasma triglyceride levels. At baseline, db/db mice exhibited significant hyperglycemia compared with lean mice (230 ± 40 vs. 142 ± 17 mg/dl, P < 0.01). The baseline values of plasma triglyceride in db/db mice were slightly higher than those in lean mice, but this difference did not reach a statistical significance (100 ± 45 vs. 60 ± 17 mg/dl). A 14-day treatment with RGZ decreased blood glucose and plasma triglyceride levels in db/db mice to a value comparable to the lean control.

Effect of RGZ on electrolyte and fluid metabolism. After a 14-day RGZ treatment, lean mice exhibited a significant increase in urinary Na⁺ excretion and urine volume. In contrast, in db/db mice, neither parameter was significantly affected by
this treatment (Fig. 4). Plasma Na\(^+\) and plasma osmolality in lean mice remained unchanged after RGZ treatment (Fig. 5). However, in \(db/db\) mice, RGZ treatment induced parallel decreases in plasma Na\(^+\) concentration (122.9 ± 4.0 vs. 151.3 ± 7.1 mmol/l, \(P < 0.05\)) and plasma osmolality (304.2 ± 6.2 vs. 334 ± 3.5 mosmol/kgH\(_2\)O; Fig. 5), suggesting that the accumulation of water may have exceeded that of Na\(^+\).

Effect of RGZ on renal expression of Na\(^+\) and water transporters. qRT-PCR detected a significant reduction of renal AQP2 mRNA in lean mice following 14-day RGZ treatment. In contrast, the AQP2 mRNA expression was unchanged in \(db/db\) mice (Fig. 6A). By immunoblotting, AQP2 protein was detected as 35- to 50-kDa bands as the glycosylated form and a 29-kDa band as the nonglycosylated form (Fig. 6B). In response to RGZ treatment, the glycosylated AQP2 exhibited a reduction in lean but not \(db/db\) mice. The nonglycosylated AQP2 remained stable among different groups (Fig. 6, B and C). RGZ had no effect on renal AQP3 protein expression in lean controls but significantly increased it in \(db/db\) mice (Fig. 7). In contrast, we observed no change in renal protein expression of sodium transporters including NHE3 and NKCC2 in response to RGZ treatment irrespective of the mouse strain. Of note, \(db/db\) mice had decreased baseline protein expression of NHE3 but increased expression of NKCC2 compared with the lean controls (Fig. 8).

**Effect of RGZ on renal expression of PPAR\(\gamma\).** To address the possibility that increased renal PPAR\(\gamma\) expression may underlie the increased sensitivity of RGZ-induced fluid retention in \(db/db\) mice, we conducted qRT-PCR analysis of PPAR\(\gamma\) mRNA expression in the kidneys of lean and \(db/db\) mice after vehicle or RGZ treatment. The results showed that renal PPAR\(\gamma\) expression of \(db/db\) mice exhibited a tendency of a decrease rather than an increase and that the expression in either strain was unaffected by RGZ treatment (1.11 ± 0.1 in lean-RGZ vs. 1.03 ± 0.24 lean-control, \(P > 0.05\); 0.857 ± 0.11 in \(db/db\)-RGZ vs. 0.7 ± 0.1 in \(db/db\)-control, \(n = 3–7, P > 0.05\)). The data suggest that the renal expression level of PPAR\(\gamma\) may not be a major determinant of the differential response to RGZ treatment.

**DISCUSSION**

Despite intensive investigation, the mechanism of TZD-induced fluid retention remains incompletely understood. In particular, most of the previous studies employed healthy animals, which may have limited relevance to clinical practice involving all diabetic patients. Here, we investigated the difference in TZD-induced plasma volume expansion in \(db/db\) vs. lean control mice and further explored the underlying mechanism. Using Hct and nanoparticle-based method, we documented accelerated plasma volume expansion in response to 2-wk RGZ treatment in \(db/db\) mice vs. lean controls. Similar to a compensatory response to volume expansion, RGZ-treated
lean mice exhibited increased urinary Na\textsuperscript{+} excretion and urine volume. In contrast, the natriuretic and diuretic responses were also almost absent in db/db mice. Downregulation of renal AQP2 expression in response to RGZ treatment was seen in lean mice but not in db/db mice. Renal AQP3 protein expression was unaffected by RGZ treatment in lean mice but was elevated in db/db mice.

Several previous studies reported that obese Zucker rats gained more weight than the lean controls (22). This accelerated body weight gain is suggested to be solely accountable by increased fat mass through apolipoprotein E/very low-density lipoprotein receptor signaling in the adipocytes (25). However, there are no reports in the literature on differences of TZD-induced fluid retention in obese vs. lean animals. It remains uncertain as to whether obese animals exhibited increased susceptibility to TZD-induced plasma volume expansion compared with their lean controls; if so, what is the mechanism? To address this issue, we evaluated RGZ-induced fluid retention compared with their lean controls; if so, what is the mechanism? To address this issue, we evaluated RGZ-induced plasma volume expansion in db/db and lean mice by the determination of Hct and the use of fluorescent nanoparticles. Of note, measurement of plasma volume is traditionally reliant on the use of a dye tracer such as indocyanine green and Evans blue, which suffer numerous drawbacks including binding plasma proteins. Eisner et al. (7) have recently developed a nanotechnology-based method for accurate measurement of plasma volume in mice. RGZ treatment induced a small but significant drop in Hct in lean mice, and this drop was greater in db/db mice. In line with this finding, the nanoparticle method detected a 220% increase in plasma volume in db/db mice compared to a 30% increase in the lean controls. The two independent methods consistently demonstrated the increased susceptibility of db/db mice to RGZ-induced plasma volume expansion. Further support of this notion comes from the observation that RGZ induced cardiac hypertrophy in db/db but not in lean mice. TZDs increase the incidence of congestive heart failure in clinical trials (16) and induce cardiac hypertrophy in experimental animals including mice, rats, and dogs (1, 3, 8, 21). TZD-inducing cardiac hypertrophy is likely secondary to expanding plasma volume although the direct cardiac action of the compounds is possible (23). To our knowledge, our study is the first to vigorously evaluate the volume status in TZD-treated diabetic animals.

It has been reported that a short term TZD treatment induces Na\textsuperscript{+} and water retention. Song et al. (24) have demonstrated that RGZ treatment in Sprague-Dawley rats at 94 mg/kg for 3 days decreased urine output and urinary Na\textsuperscript{+} excretion by 22 and 44%, respectively. This treatment increased whole kidney expression of several Na\textsuperscript{+} transporters including α_{1}-subunit of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, NHE3, Na-Pi2, AQP1–3, and NKCC2 but not NCC, AQP4, α\textsubscript{1}-, β\textsubscript{1}-, or γ-ENaC. These findings suggest that increased Na\textsuperscript{+} and water reabsorption in multiple nephron segments may contribute to TZD-induced fluid retention. However, when TZDs were administered for longer than 5 days, renal expression of nonglycosylated form of AQP2 (26) and all three subunits of ENaC (5) was significantly downregulated, coinciding with normal urine volume. Therefore, the later reduction of AQP2 and ENaC is considered as adaptive response to reestablish Na\textsuperscript{+} and water balance. Unlike the wild-type animals in the previous study (26), db/m mice exhibited increased urine volume and urinary Na\textsuperscript{+} excretion after RGZ treatment. This may suggest that the heterozygous mutation of leptin receptor already accelerated TZD-induced fluid retention, leading to enhanced diuresis and natriuresis at later time points.

As discussed above the normal diuretic response seen in lean mice is necessary for reestablishment of Na\textsuperscript{+} and water balance. In contrast, this response was significantly blunted in db/db mice, which developed severe fluid retention in response to RGZ treatment. It seems reasonable to speculate that impairment of natriuretic and natriuresis may account for the accelerated fluid retention in db/db mice. We further found that db/db mice exhibited blunted downregulation of renal AQP2 expression and elevated AQP3 expression after RGZ treatment. The dysregulation of these water transporter proteins likely underlies the abnormal diuretic response in db/db mice.

In summary, the present study examined RGZ-induced fluid retention in db/db mice. These animals exhibited accelerated plasma volume expansion after 2-wk RGZ treatment compared with their lean controls. In response to plasma volume expansion, lean mice exhibited increased urine volume, accompanied with downregulation of AQP2. In contrast, these renal responses to RGZ treatment were blunted in db/db mice. These results suggest that impaired diuresis as a result of defective response of renal water transporters may in part account for the increased susceptibility of db/db mice to TZD-induced fluid retention.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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