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PPAR γ agonist-induced fluid retention depends on α ENaC expression in connecting tubules

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Short title: PPAR γ agonist-induced fluid retention

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Abstract

Background/Aims: Thiazolidinediones (TZDs, e.g. rosiglitazone (RGZ)) are peroxisome proliferator-activated receptor γ (PPAR γ) agonists used to treat type 2 diabetes. Clinical limitations include TZD-induced fluid retention and body weight (BW) increase, which are inhibited by amiloride, an epithelial-sodium channel (ENaC) blocker. RGZ-induced fluid retention is maintained in mice with α ENaC knockdown in the collecting duct (CD). Since ENaC in the connecting tubule (CNT) rather than in CD appears to be critical for normal NaCl retention, we aimed to further explore the role of ENaC in CNT in RGZ-induced fluid retention.

Methods: Mice with conditional inactivation of α ENaC in both CNT and CD were used (α ENaC lox/lox AQP2-Cre; “ α ENaC-CNT/CD-KO”) and compared with littermate controls (α ENaC lox/lox mice; “WT”). BW was monitored, and total body water (TBW) and extracellular fluid volume (ECF) determined by bioelectrical impedance spectroscopy (BIS) before and after RGZ (320 mg/kg diet for 10 days).

Results: On regular NaCl diet, α ENaC-CNT/CD-KO had normal BW, TBW, ECF, hematocrit, and plasma Na⁺, K⁺, and creatinine, associated with an increase in plasma aldosterone compared with WT. Challenging α ENaC-CNT/CD-KO with a low NaCl diet unmasked impaired NaCl and K homeostasis, consistent with effective knockdown of α ENaC. In WT, RGZ increased BW (+6.1%), TBW (+8.4%) and ECF (+10%), consistent with fluid retention. These changes were significantly attenuated in α ENaC-CNT/CD-KO (+3.4, 1.3, and 4.3%).

Conclusion: Together with previous studies, the current results are consistent with a role of α ENaC in CNT in RGZ-induced fluid retention, which dovetails with the physiological relevance of ENaC in this segment.

Introduction

PPARs are ligand-activated transcription factors that regulate a large number of genes by transcriptional activation and repression [1]. Thiazolidinediones (TZDs), such as pioglitazone or rosiglitazone (RGZ), are potent ligands and activators of PPAR γ and are insulin sensitizers used to treat type 2 diabetes [2]. Fluid retention is an important clinical limitation for TZDs, which occurs in up to 5% of treated diabetic patients, and, as a consequence, these agents are contraindicated in patients with New York Heart Association class III and IV congestive heart failure [3;4]. TZD-induced fluid retention and edema formation has been proposed to involve increases in vascular permeability [4-7] and vasodilation [8;9] as well as primary effects on the kidney. The latter may include an increase in sodium and water reabsorption due to upregulation of various renal transport regulating systems including the Na⁺-H⁺ exchanger NHE3, the Na-K-2Cl cotransporter NKCC2, the serum/glucocorticoid-regulated kinase 1, the α subunit of Na-K-ATPase, a non-selective cation channel, and/or the water channels aquaporin-2 (AQP-2) and AQP-3 [10-13].

Conditional knock-down of PPAR γ in the connecting tubules (CNT) and collecting ducts (CD) of mice (using Cre expression driven by the AQP-2 promoter) abrogated the ability of the TZDs - pioglitazone and RGZ - to increase plasma volume and body weight [14;15], indicating that the site of action may primarily reside in these segments. The epithelial sodium channel ENaC is expressed in the apical membrane of the CNT and CD and serves as a primary effector of regulated sodium reabsorption. Studies in mice [15] and in patients with type 2 diabetes [16] found that TZD-induced weight gain and fluid retention were attenuated by the ENaC inhibitor, amiloride, indicating a potential role of ENaC in this process.

ENaC is a heteromultimer and the α ENaC subunit appears critical for exporting the intact ENaC complex to the apical membrane [17]. Mice with conditional knockdown of α ENaC (Scnn1a) in both CNT and CD (using AQP2-promoter driven Cre) or in CD only (using Hox7-

promoter driven Cre) have previously been characterized and used to determine the quantitative role of ENaC in these segments for renal Na and K homeostasis. The studies found that Na and K homeostasis were only impaired when α ENaC was deleted in both CNT and CD whereas the sole knockdown in CD was ineffective [17;18]. This is consistent with the concept that it is ENaC in the CNT rather than in the CD that appears critical for normal NaCl retention and K excretion [19;20]. Potentially along these lines, RGZ-induced fluid retention still fully developed in mice in which α ENaC has been knocked down only in the CD (using Cre expression driven by the Hox7 promoter) [12]. Therefore, in the present study we used mice with AQP-2 promoter driven knockdown of α ENaC to test for a potential role of α ENaC in the CNT in TZD-induced fluid retention.

Methods

Animals: All animal experimentation was conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD) and was approved by the Institutional Animal Care and Use Committee of the Veterans Affairs San Diego Healthcare System. Female *Scnn1a*^{lox/lox} mice were bred with male *Scnn1a*^{lox/lox}/*APQ2:cre*^{+/-} mice to generate experimental *Scnn1a*^{lox/lox} mice (WT, used as control) and *Scnn1a*^{lox/lox}/*APQ2:cre*^{+/-} mice (αENaC CNT/CD KO) as previously described [18]. Age-matched adult male mice (22-30 weeks of age) were selected and housed in standard rodent cages on a 12:12-h light-dark cycle with free access to food (1% K⁺, 0.4% Na⁺, 4.4% fat; Harlan Teklad TD.7001) and water.

Body weight and fluid retention in response to rosiglitazone (RGZ): Basal body weight (BW), daily food intake and water consumption were determined while mice were kept in standard rodent cages. Mice were then anesthetized with ketamine (33.3 mg/ml, 2.5 ml/kg BW ip) and xylazine (3.33 mg/dl, 2.5 ml/kg BW ip) to determine total body water (TBW), extracellular fluid (ECF) and intracellular fluid (ICF) by bioimpedance spectroscopy (BIS) using the ImpediVet BIS1 system (ImpediMed, San Diego, CA) as previously described [21]. Using a set of carefully positioned subcutaneous needle electrodes, BIS determines body composition based on its electrical characteristics in response to the application of low amplitude alternating electrical currents [22]. The procedure and measurement takes about 5 minutes. After completion of BIS and while still under anesthesia, blood was collected by retro-orbital bleeding to determine hematocrit (Hct). Mice were subsequently allowed to recover for 5-7 days. Mice were then fed with repelletted diet containing RGZ (320 mg/Kg diet [12;14]) for 10 days. BW, food and water intake were measured every other day. On the last day, mice were anesthetized with ketamine and xylazine to determine body fluid volumes by BIS and subsequently hematocrit.

Functional confirmation of ENaC knockdown - response to low NaCl diet: Mice were maintained first on control diet (0.275% NaCl, 1% K⁺; Harlan Teklad TD.140039) and were then switched to low-NaCl diet (0.01% NaCl, 1% K⁺; Harlan Teklad TD.08601) for another 5 days. BW was monitored daily. Blood was withdrawn under isoflurane anesthesia before switching diets and on the 5th day of low salt diet to determine plasma Na⁺ and K⁺ (by flame photometer; Cole-Parmer, Vernon Hills, IL), creatinine (by isotope dilution LC-MS/MS in core laboratory of UAB-UCSD O'Brien Center for Acute Kidney Injury Research), and plasma aldosterone (by radioimmuno assay; DSL-8600; Diagnostic Systems Laboratories, Webster, TX).

Statistical analysis: Data are reported as means±SEM. Data from WT and KO were compared by ANOVA followed by two-tailed t test. When comparing parameters within genotype before and after treatment, paired t test was used. P< 0.05 was considered statistically significant.

Results

Basal phenotype of α ENaC CNT/CD KO mice and response to low salt diet

When fed a normal salt diet, BW and daily food intake and water consumption as well as hematocrit and plasma creatinine were not significantly different between WT mice and α ENaC CNT/CD KO mice (Table 1). BIS showed that basal TBW, ECF and ICF did not differ in WT and KO (Table 1), as were plasma concentrations of Na^+ and K^+ (Figs. 1b and 1c). This was associated with increased plasma aldosterone concentrations in α ENaC CNT/CD KO compared with WT (Fig. 1d). These data indicate that α ENaC CNT/CD KO were able to maintain relatively normal kidney function and Na^+ and K^+ homeostasis and body fluid volumes under normal salt intake, at least in part by upregulating aldosterone levels. When mice were placed on a low salt diet, α ENaC CNT/CD KO lost significantly more BW (Fig. 1a) and showed significantly higher plasma creatinine levels than WT (0.125 ± 0.014 vs 0.063 ± 0.002 mg/dl; $P < 0.001$). Whereas WT maintained plasma Na^+ or K^+ levels in response to a low salt diet, plasma Na^+ significantly decreased and plasma K^+ increased in α ENaC CNT/CD KO (Figs. 1b and 1c). Aldosterone was slightly increased by low salt diet in WT but showed a strong further increase in α ENaC CNT/CD KO. These data indicate that low salt diet unmasked impaired Na^+ and K^+ homeostasis in α ENaC CNT/CD KO consistent with previous studies performed in this mouse model [18] and support the concept that Na^+ retention and K^+ secretion depended on α ENaC expression in CNTs rather than CDs [17;18].

α ENaC CNT/CD KO attenuated RGZ-induced BW gain and fluid retention

In WT mice, RGZ increased BW by 6.1% at day 10 (Fig. 2a). This was associated with a significant reduction in hematocrit (Fig. 2b) and increases in TBW by 8.4% and in ECF by 10%, while the increase in ICF by 7.6% did not quite reach statistical significance ($P = 0.052$) (Fig. 3). α ENaC CNT/CD KO did not affect the RGZ reduction in hematocrit (Fig. 2b) but significantly attenuated the RGZ-induced increase in BW (3.4%) (Fig. 2a), TBW (1.3%) and ECF (4.3%) (each

P<0.05 vs. WT), and tended to reduce the increase in ICF when compared with WT (-0.1%, P=0.07 vs. WT)(Fig. 3). Daily food and water intake were not significantly affected by RGZ in either group (data not shown).

Discussion

Previous studies showed that the RGZ-induced fluid retention and increase in body weight were unaffected in mice with inactivation of α ENaC activity only in the CD [12]. Here we report that the RGZ-induced increase in body weight, TBW and ECF observed in WT mice were attenuated in a mouse model with inactivation of α ENaC activity in CNT and CD. Together these studies provided evidence that RGZ-induced fluid retention and increase in body weight depends in part on ENaC activity in the CNT of the kidney. The current studies employed α ENaC knockdown driven by the AQP-2 promoter, i.e., the same promoter that has previously been used to show the critical role of PPAR γ in these segments for TZD-induced fluid retention [14;15]. A role for ENaC in CNT in TZD-induced fluid retention dovetails with the physiological relevance of ENaC in this segment for Na⁺ reabsorption [17-20]. The findings are also in agreement with studies that found that the ENaC blocker, amiloride, and the aldosterone-antagonist, spironolactone, attenuated TZD-induced fluid retention in diabetic patients [16;23].

While α ENaC knockdown in CNT and CD attenuated the TZD-induced increase in BW, TBW and ECF, it did not affect the fall in hematocrit. Thus, the TZD-induced fluid retention was only partially prevented by the α ENaC knockdown in CNT and CD. This may be due to incomplete knockdown of α ENaC in the CNT in this model (α ENaC positive principal cells in late DCT reduced by about 70% – for details see [18]), potential effects of TZDs on ENaC in the late distal convoluted tubule, which together with ENaC in CNT are the primary sites of ENaC-dependent NaCl retention [20], and/or ENaC-independent mechanisms of TZD-induced fluid retention (see above).

It remains to be determined how PPAR γ activation enhances ENaC activity. Lower blood pressure could reduce renal perfusion pressure thereby inducing ENaC and increasing renal reabsorption of Na⁺ and fluid [13;24;25]. Moreover, TZDs increased amiloride-sensitive Na⁺ absorption as well as γ ENaC expression when applied to cultured WT mouse inner

medullary CD (IMCD) cells *in vitro* [15], indicating a potential direct effect of TZDs on ENaC. Long-term TZD treatment in patients with type 2 diabetes resulted in a decrease in plasma aldosterone concentrations and an increase in plasma atrial natriuretic peptide levels; these changes are consistent with a primary increase in sodium reabsorption and central venous volume expansion [23]. However, significant increases in the renal expression of the ENaC subunits α , β or γ could not be detected in response to TZDs [10;12;13] and one study even described a suppression of the γ -subunit of ENaC at the mRNA and protein level in the mouse kidney cortex and in a cortical collecting duct cell line [26]. Furthermore, *in vitro* electrophysiological studies in the A6, M-1, and mpkCCD(cl4) cell lines, which express viable and functional PPAR γ , found that both the basal and the insulin-stimulated Na⁺ flux via ENaC were insensitive to the PPAR γ agonists, pioglitazone and GW7845 [27]. Considering all these results together with the current data, one may speculate that the TZD-induced activation of ENaC occurs independently of a change in ENaC protein expression and may require the complexity of the *in vivo* setting that is not mimicked in cell lines. Further studies are needed to explore these hypotheses.

In summary and in the context of previous studies [12], the presented *in vivo* studies are consistent with the concept that ENaC-mediated Na⁺ reabsorption in the CNTs contributes to TZD-induced fluid retention and weight gain.

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Disclosure

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Table 1: Basal measurements on regular salt diet

Parameters	WT (n=12)	α ENaC CNT/CD KO (n=20)
BW (g)	33.4 \pm 0.6	33.6 \pm 0.4
Daily food intake (g)	4.2 \pm 0.2	4.4 \pm 0.1
Daily water intake (ml)	6.4 \pm 0.6	7.5 \pm 0.3
Hematocrit (%)	42.8 \pm 0.3	43.6 \pm 0.3
Plasma creatinine (mg/dL)	0.086 \pm 0.002	0.089 \pm 0.001
TBW (ml)	18.8 \pm 0.6	20.3 \pm 0.6
ECF (ml)	6.5 \pm 0.2	6.9 \pm 0.2
ICF (ml)	12.3 \pm 0.4	13.4 \pm 0.4

BW, body weight; TBW, total body water; ECF, extracellular fluid; ICF, intracellular fluid.

Figure legends

Fig. 1: When challenged with a low salt diet, α ENaC CNT/CD KO mice lost more body weight than WT mice (A). Plasma Na^+ and K^+ levels were similar between genotypes under normal salt diet. A low salt diet reduced plasma Na^+ and increased plasma K^+ levels only in α ENaC CNT/CD KO (B and C). Plasma aldosterone was moderately elevated in α ENaC CNT/CD KO vs. WT under normal salt diet; this difference was strongly increased in response to a low salt diet (D). $n=6$ in each group. * $P<0.05$ vs WT; # $P<0.05$ vs. normal salt in same genotype.

Fig 2: α ENaC CNT/CD KO attenuated the RGZ-induced increase in body weight versus WT (A), whereas the reduction in hematocrit was not affected (B). * $P<0.05$ vs. WT; # $P<0.05$ vs. basal within genotype. $n=12$ in WT; $n=18-20$ in KO.

Fig. 3: RGZ significantly increased TBW and ECF and tended to increase ICF in WT; this response was blunted in α ENaC CNT/CD KO. * $P<0.05$ vs. WT; # $P<0.05$ vs. basal within genotype. $n=11$ in WT; $n=20$ in KO.

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Fig. 1

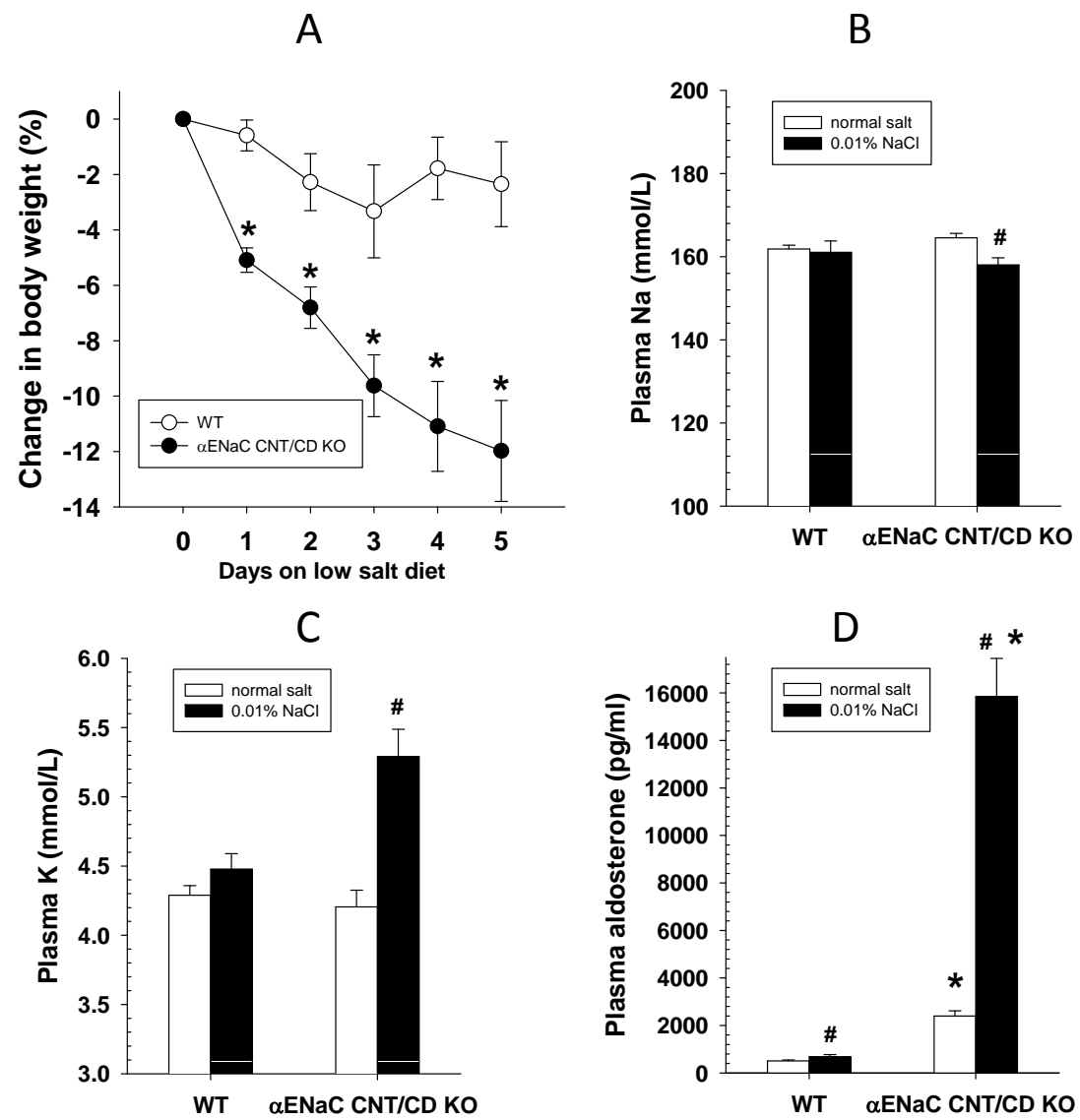


Fig. 2

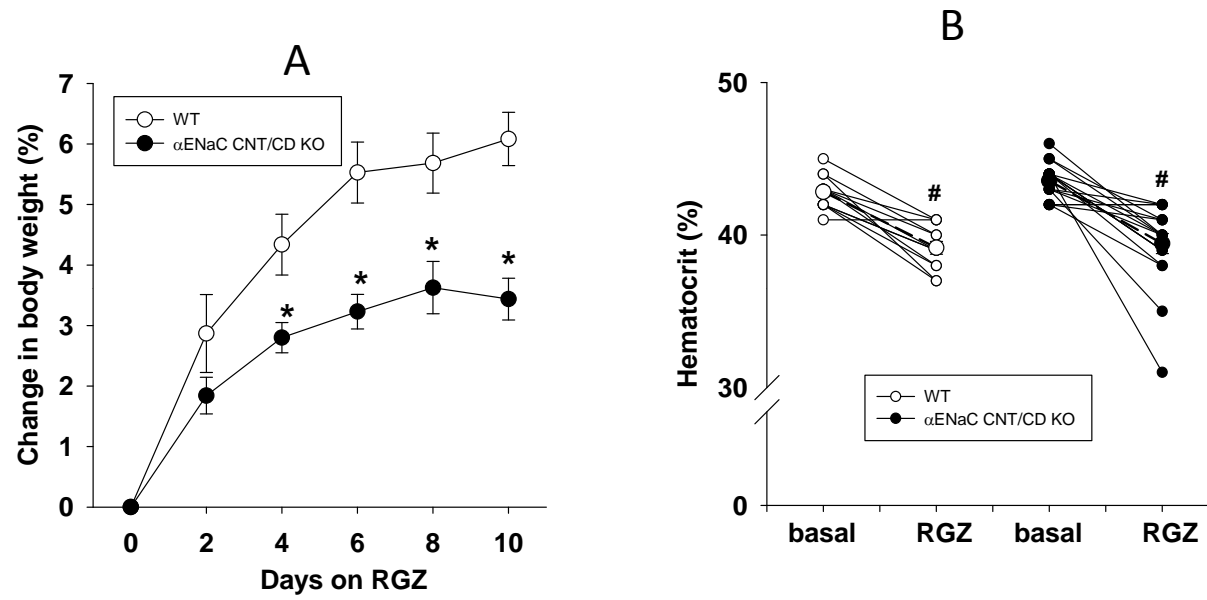


Fig. 3

