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PPARγ antagonists decrease Na^+ transport via the epithelial Na^+ channel (ENaC)

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ABSTRACT

The epithelial sodium channel (ENaC), thought to represent the rate limiting step for sodium absorption in the renal collecting duct. Consequently, ENaC is a central effector impacting systemic blood volume and pressure. Sodium and water transport are dysregulated in diabetes mellitus. Peroxisome proliferator-activated receptor gamma (PPARγ) agonists are currently used in the treatment of type 2 diabetes, although their use remains limited by fluid retention. The effects of PPARγ agonists on ENaC activity remain controversial. While PPARγ agonists were shown to stimulate ENaC-mediated renal salt absorption, probably via the Serum- and Glucocorticoid-Regulated Kinase 1 (SGK1), other studies reported that the PPARγ agonists-induced fluid retention is independent of ENaC activity. Here we confirmed that four chemically distinct PPARγ agonists (pioglitazone, rosiglitazone, troglitazone and 15-deoxy-$\Delta^{12,14}$–prostaglandin J_2 (PGJ_2)) do not enhance Na^+ transport in cultured renal collecting duct principal mpkCCD_{c14} cells, as assessed by short-circuit current measurements. However, the PPARγ antagonist T0070907, and to lesser extent the GW9662 were found to decrease Na^+ reabsorption across mpkCCD_{c14} cell layers. Furthermore, pretreatment of monolayers with T0070907 diminished the insulin-stimulated sodium transport. PPARγ agonist PGJ_2 did not enhance insulin-stimulated Na^+ flux via ENaC. We also show that PPARγ enhances ENaC activity when all three subunits are reconstituted in Chinese hamster ovary (CHO) cells. GW9662 inhibits ENaC activity when ENaC subunits are coexpressed in CHO cells with PPARγ. In contrast, rosiglitazone has no effect on ENaC activity. We conclude

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that PPARγ activity is important for maintaining basal and insulin-dependent transepithelial Na^+ transport and ENaC activity.

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INTRODUCTION

The epithelial Na^+ channel (ENaC) is an ion channel localized to the luminal plasma membrane of epithelial cells lining hollow organs involved in Na^+ homeostasis, such as the distal colon and renal nephron. Activity of ENaC is rate-limiting for Na^+ transport across many epithelial tissues, including reabsorption across the renal collecting duct. Thus, ENaC functions as a critical component of the negative feedback pathway coupling renal Na^+ handling to control of systemic fluid volume and blood pressure. The importance of this channel and its proper regulation to human health and disease is apparent when considering that gain-of-function mutations in the channel itself and its upstream regulatory pathways cause improper renal salt conservation associated with hypertension. In contrast, loss-of-function mutations in ENaC and its regulatory pathways lead to inappropriate renal salt wasting (reviewed in (Hummler and Horisberger, 1999;Rossier et al., 2002;Rossier and Schild, 2008;Schild, 2004)). Abnormal ENaC activity, moreover, is beginning to be recognized as contributing to the disease processes associated with cystic fibrosis and polycystic kidney disease, as well as several other disorders with abnormal epithelial cell transport (Mall et al., 2004;Rohatgi et al., 2003;Veizis et al., 2004).

Peroxisome proliferator-activated receptor gamma (PPARγ) is a type II nuclear receptor. PPARs form heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various genes (Willson et al., 2001). PPARγ plays an important role in adipocyte differentiation and implicated in the pathology of numerous diseases including obesity, diabetes,

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atherosclerosis and cancer. Similar to other nuclear hormone receptors, PPARs act as ligand-activated transcription factors. PPARγ activators such as antidiabetic drugs thiazolidinediones (TZDs) decrease glucose and lipid levels in patients with type 2 diabetes and also have antiatherosclerotic and antihypertensive effects (Tontonoz and Spiegelman, 2008). In the kidney, PPARγ, which is encoded by the *Pparg* gene, is abundant in the collecting duct (Yang et al., 1999). It was shown that PPARγ is highly expressed in human renal cortical collecting ducts (Hong et al., 2003), the A6, M1 and mpkCCD$_{c14}$ cells (Nofziger et al., 2005).

Inappropriate activation of ENaC in the collecting duct in response to stimulation of PPARγ signaling has been implicated in the pathological fluid retention associated with insulin-sensitizing thiazolidinediones (Guan et al., 2005). Moreover, it was proposed that PPARγ activators may increase renal Na^+ reabsorption by stimulating ENaC and SGK1 might be involved in this regulation (Hong et al., 2003;Saad et al., 2008;Song et al., 2004;Tiwari et al., 2008). However, recent studies have demonstrated that PPARγ agonists do not enhance basal or insulin-stimulated Na^+ transport via ENaC (Nofziger et al., 2005) and TZD-induced fluid retention is independent of ENaC activity (Vallon et al., 2009). Furthermore, Artunc and colleagues propose that SGK1 contributes to but does not fully account for the volume retention during treatment with the PPARγ agonist pioglitazone (Artunc et al., 2008). Thus, the possible importance of this mechanism to regulation of ENaC in the collecting duct remains obscure and these examples emphasize the need for further experiments to fully

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understand the cellular signaling pathways and mechanisms controlling ENaC activity. Our results show that TZDs do not enhance ENaC-mediated sodium reabsorption which is in agreement with recent works of Nofziger et al. (Nofziger et al., 2005) and Vallon et al, (Vallon et al., 2009). However, we have determined that two chemically distinct specific PPARγ antagonists, GW9662 and T0070907, decrease basal and insulin-stimulated Na^+ transport via ENaC. Thus, these results are consistent with regulation of ENaC and Na^+ transport across the collecting duct by PPARγ.

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MATERIALS AND METHODS

cDNA constructs and cell culture. CHO cells were obtained from ATCC, maintained with standard culture conditions (DMEM, 10 % FBS, 1x Penicillin-Streptomycin, 37^0C, 5% CO_2) and transfected using the Polyfect reagent (Qiagen; Valencia, CA) as described previously (Staruschenko et al., 2005). For expression of mouse ENaC (mENaC) in CHO cells, subunit cDNA transfection ratios of 1: 1: 1 were used with 0.1 – 0.3 µg of each cDNA per 35 mm dish transfected. The plasmids encoding α-, β- and γ-ENaC have been described previously (Pochynyuk et al., 2007;Staruschenko et al., 2004). To define successfully transfected cells 0.5 µg of green fluorescent protein was also added to cDNA mix. The mpkCCD$_{c14}$ principal cells were grown in defined medium on permeable supports (Costar Transwells; 0.4 µm pore, 24 mm diameter) as described previously (Bens et al., 1999;Staruschenko et al., 2007b). Cells were maintained with FBS and corticosteroids allowing them to polarize and form monolayer with high resistance and avid Na^+ reabsorption. The mpkCCD$_{c14}$ cells were kept on filter supports for at least 7 days in defined medium which was changed every second day. Growth medium was composed of equal volumes DMEM and Ham's F_{12}, 60 nM Na^+ selenate, 5 µg/ml transferrin, 50 nM dexamethasone, 1 nM triiodothyronine, 10 ng/ml EGF, 5 µg/ml insulin, 2% FCS and 100 µg/ml Pen/Strep. Cells were grown in 5% CO_2/95% air atmosphere incubator at 37°C. Typically after 7 days, a confluent transporting cell monolayer has been developed that could be assessed by recording open circuit voltage and transepithelial resistance. 18 hours before use in any

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investigation, medium of cells incubating on filter supports was replaced with a minimal medium (without drugs or hormones) that contained only DMEM and Ham's F_{12}. All chemicals were of reagent grade and were purchased from Calbiochem, BioMol, or Sigma unless noted otherwise.

MTT cell viability assay. For the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, mpkCCD$_{c14}$ cells were seeded into 12 well cluster plates at a subconfluent levels and allowed to grow for at least one week to form a monolayer. Standard MTT assay was modified to correspond to growing conditions of mpkCCD$_{c14}$ cells on permeable supports. Troglitazone (10 and 50 μM), GW9662 (50 μM), 15-deoxy-$\Delta^{12,14}$–prostaglandin J_2 (20μM) and vehicle were added to the cultured cells and incubated further for another 24 or 48 h. After treatment, MTT (Sigma-Aldrich) was added (0.5 mg/ml) and cells were incubated for further 4 h. The medium was aspirated and replaced with isopropanol to solubilize the formazan products followed by optical density readings at 570 nm. The cell viability as a percentage of the untreated cells was calculated from the absorbance values. For each measurement, treatment, and control, three replicate wells were recorded.

Dual-luciferase reporter assay. Activity of the overexpressed PPARγ in response to its agonist/antagonists was assessed in CHO cell using PPARγ reporter (SABiosciences CCS-3026L). The PPAR reporter is a mixture of a PPAR-responsive luciferase construct and a constitutively expressing Renilla construct (40:1). The PPAR-responsive luciferase construct encodes the firefly luciferase reporter gene under the control of a minimal (m)CMV promoter and

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tandem repeats of the PPAR transcriptional response element. This construct monitors both increases and decreases in the transcriptional activity of PPAR. The constitutively expressing Renilla construct encodes the Renilla luciferase reporter gene under the control of a CMV immediately early enhancer/promoter and acts as an internal control for normalizing transfection efficiencies and monitoring cell viability. Cells were co-transfected with PPAR-reporter and negative control along with PPARγ expression vector in 48-well plate. After 18 hours of transfection cells were treated with pioglitazone (15 μM), GW9662 (10 and 100 μM) or T0070907 (50μM). Dual Luciferase assay was performed on PerkinElmer Victor^3 1420 multilabel counter using the Promega dual luciferase reporter kit (E1910). Promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization. Experiments were done at least in triplicates and results represent the relative luciferase activity that was normalized to untreated control.

Electrophysiology. Whole-cell macroscopic current recordings of mENaC expressed in CHO cells were made under voltage-clamp conditions using standard methods (Karpushev et al., 2008;Staruschenko et al., 2004). Current through ENaC was the inward, amiloride-sensitive Na^+ current with a bath solution of (in mM) 160 NaCl, 1 $CaCl_2$, 2 $MgCl_2$ and 10 HEPES (pH 7.4) and a pipette solution of (in mM) 120 CsCl, 5 NaCl, 5 EGTA, 2 $MgCl_2$, 2 ATP, 0.1 GTP, 10 HEPES (pH 7.4). Whole-cell macroscopic current recordings of ASIC1a expressed in CHO cells were performed under voltage-clamp conditions using standard methods (Staruschenko et al., 2007a). The pipette solution contained

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(in mM): 120 CsCl, 5 NaCl, 2 $MgCl_2$, 5 EGTA, 2.0 ATP, 0.1 GTP and 40 HEPES (pH 7.4). Cells were continuously superfused with an extracellular solution containing (in mM): 150 NaCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES and 10 MES adjusted to pH 7.4. Test solutions adjusted to acidic pH with HCl were made from this standard bath solution. ASICs were activated by rapid exchange of the standard bathing solution for one with acidic pH. Current recordings were acquired with an Axopatch 200B (Axon Instr.; Union City, CA) interfaced via a Digidata 1440 (Axon Instr.) to PC running the pClamp 10.2 suite of software (Axon Instr.). All currents were filtered at 1 kHz. Both a family of test pulses (500 ms each), stepping by 20 mV increments form a holding potential of 30 to 60 mV to –100 mV, and voltage ramps (300 ms) from 100 to –120 mV were used to generate current-voltage (*I-V*) relations and to measure ENaC activity at –80 mV. Whole cell capacitance was routinely compensated and was approximately ~8 pF for CHO cells. Series resistances, average 2–4 megohms, were also compensated.

Transepithelial Na^+ current across mpkCCD_{c14} cell monolayer was calculated as described previously (Staruschenko et al., 2007b). In brief, current was calculated using Ohm's law as the quotient of transepithelial voltage to transepithelial resistance under open circuit conditions using a Millicel Electrical Resistance System with dual Ag/AgCl pellet electrodes (Millipore Corp., Billerica, MA) to measure voltage and resistance. To determine the net Na^+ transport through ENaC, 10μM amiloride was added to the apical cell surface at the end of each experiment.

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Chemicals. Pioglitazone (Alexis Biochemicals, San Diego, CA) and rosiglitazone were dissolved in equal volumes of DMSO/EtOH and added to the monolayers as aliquots of stock solutions (2.5 mg/ml and 5 mg/ml for pioglitazone and rosiglitazone, respectively). GW9662 was dissolved in EtOH at 3.45 mg/ml. T0070907, troglitazone and 15-deoxy-$\Delta^{12,14}$–prostaglandin J_2 were dissolved in DMSO at 10 mg/ml for T0070907, and 50 mM for troglitazone and 15-deoxy-$\Delta^{12,14}$–prostaglandin J_2, respectively. Rosiglitazone, GW9662, T0070907 were from Cayman Chemical (Ann Arbor, MI). Troglitazone and 15-deoxy-$\Delta^{12,14}$–prostaglandin J_2 were from Sigma (St. Lois, MO).

Statistics. All summarized data is reported as mean ± SEM. Data was compared with either the Student's (two-tailed) *t*-test or a one way ANOVA. $P \leq 0.05$ considered significant.

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RESULTS

Effect of PPARγ agonists/antagonists on PPARγ activity and cell viability.

To investigate the actions of some compounds used in this study we initially evaluated the functional potency of these drugs as PPARγ agonists/antagonists. PPARγ activity was measured using dual luciferase reporter assay designed to measure transcriptional activity of PPARs. CHO cells were co-transfected with PPAR-reporter or negative control along with $PPAR\gamma_1$ expression vector. After 18 h of transfection medium was changed to growth medium and cells were treated with pioglitazone (15 μM), GW9662 (10 and 100 μM) or T0070907 (50 μM). Dual Luciferase assay was performed 18 h after the treatment, and promoter activity values were expressed as arbitrary units using a Renilla reporter for internal normalization. As shown in Figure 1, pioglitazone induced PPARγ agonistic activity. In contrast, GW9662 and T0070907 significantly decreased PPARγ activity. The effect of GW9662 was concentration-dependent. 10 and 100 μM of GW9662 decreased relative luciferase activity to 82 and 28 %, respectively (Fig. 1).

To test cytotoxicity effects of PPARγ agonists/antagonists on $mpkCCD_{c14}$ cells we performed MTT assay. MTT assay measures the conversion of MTT into purple-colored MTT formazan by the redox activity of living cells, and a decrease in cellular MTT formazan could be the index of grow inhibition and cell toxicity. Data obtained revealed that 50 μM of troglitazone induced significant inhibition of cell vitality in 24 h. However, lower concentration of troglitazone (10

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μM), GW9662 (50 μM) and 15-deoxy-$\Delta^{12,14}$ –prostaglandin J_2 (PGJ_2; 20 μM; data not shown) had no effect on cell vitality (Fig. 2). In addition, transepithelial resistance (an indication of cellular viability) was monitored throughout the entire duration of all electrophysiological measurements of Na^+ flux in the $mpkCCD_{c14}$ cells.

PPARγ agonists do not enhance sodium transport.

To examine the effect of PPARγ agonists on Na^+ absorption, we performed electrophysiological measurements of transepithelial Na^+ flux in the $mpkCCD_{c14}$ cells. For these experiments, we used monolayers of $mpkCCD_{c14}$ cells with robust but not maximal transepithelial transport (12.3 ± 0.3 μA/cm^2, n = 30) and resistance (1.70 ± 0.06 MΩ, n = 30). These basal values were similar to that reported previously (Nofziger et al., 2005;Pochynyuk et al., 2008;Soundararajan et al., 2005). Open circuit current in control experiments or after treatment with PPARγ agonists was normalized to starting levels. Amiloride (10 μM) was added to the apical membrane at the end of experiments to confirm that the transepithelial current was mediated by ENaC. The majority of basal ion transport in the $mpkCCD_{c14}$ cells is amiloride-sensitive, indicating that it is predominately due to reabsorptive Na^+ movement via ENaC.

Figure 3 summarizes relative short-circuit current (I_{sc}) through the $mpkCCD_{c14}$ cells in response to treatment for 8 and 24 h with four chemically distinct PPARγ agonists, including Pioglitazone (15 μM; Pio), Rosiglitazone (15 μM; Rosi), Troglitazone (10 μM; Tro) and 15-deoxy-$\Delta^{12,14}$ –prostaglandin J_2 (20 μM; PGJ_2). None of the agonists had an effect on ENaC-mediated Na^+ transport.

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Moreover, we have examined the effect of PPARγ agonists on short-term (1, 2 and 4 h). However, we also did not observe differences in the amount of basal Na^+ transport (data not shown). 48 h treatment with Troglitazone and PGJ_2 similarly had no effect on Na^+ flux in the $mpkCCD_{c14}$ cells. Thus, these findings demonstrate that none of studied PPARγ agonists change Na^+ flux in the $mpkCCD_{c14}$ cells.

PPARγ antagonist GW9662 decreases Na^+ transport in the $mpkCCD_{c14}$ cells.

$MpkCCD_{c14}$ cells express PPARγ transcript as it was recently shown by qPCR on RNA isolated from confluent $mpkCCD_{c14}$ cells and measured basal amounts of PPARγ mRNA expression (Nofziger et al., 2005). GW9662 is an irreversible PPARγ antagonist and is 10- and 600-fold less potent in binding PPARα and PPARδ, respectively (Collino et al., 2005). GW9662 does not loose its activity in cell cultures and is a valuable tool for determining specific PPARγ receptor-mediated functions in different biological systems. Figure 4A summarizes the time-course of relative inhibition of Na^+ current through $mpkCCD_{c14}$ cells in response to treatment the cells with PPARγ antagonist GW9662 (10 μM). Application of amiloride (10 μM) to the apical membrane at the end of experiments is shown by arrow. Figures 4B-D show the dose-response for GW9662-induced changes in transepithelial Na^+ current at 4 (B), 8 (C) and 24 (D) hours after treatment, respectively. As shown in these summary graphs, GW9662 modestly, but significantly decreases Na^+ transport at all studied concentrations in the range from 0.1 μM to 100 μM.

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T0070907 markedly decreases Na^+ transport.

T0070907 is a potent and selective antagonist of PPARγ. T0070907 covalently binds to Cys^{313} of PPARγ, inducing conformational changes that block the recruitment of transcriptional cofactors to the PPARγ/RXR heterodimer (Lee et al., 2002). T0070907 treatment (50 μM) caused a rapid reduction of I_{sc} by approximately 20 % compared to control cells 1 h after application of antagonist and reached maximum (~ 90 %) at 24 h. The effect of T0070907 was dose-dependent. 10 μM T0070907 had no effect on relative Na^+ transport across monolayers of $mpkCCD_{c14}$ principal cells neither on short (1-2 h) or long (24-48 h) treatment. Importantly, that 50 μM of T0070907 did not cause cytotoxicity. After 48 hours of treatment I_{sc} slightly recovered compare to cells treated for 24 h with T0070907 indicating the metabolic turnover of this ligand (Fig. 5). Thus, these results indicate that PPARγ activity is important in maintaining basal transepithelial Na^+ transport in $mpkCCD_{c14}$ cells.

PPARγ specifically increases ENaC activity.

To investigate the action of PPARγ on ENaC, we reconstituted the channel in CHO cells in the absence and presence of co-expressed $PPAR\gamma_1$. Mouse ENaC was reconstituted by co-expressing α, β and γ channel subunits together. To define successfully transfected cells GFP was also added in cDNA mix. Figure 6A shows typical currents from voltage clamp experiments performed on a cell expressing the channel alone (up) and in a cell expressing both the channel and $PPAR\gamma_1$ (bottom) before and after treatment with amiloride (Amil., 10 μM). Currents were elicited by applying test pulses from 60 mV to -100

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mV with 20 mV steps. Figure 6B shows summary of ENaC activity measured as amiloride-sensitive current density at –80 mV in the presence of increasing quantities of co-transfected PPARγ$_1$ cDNA. The effect of PPARγ$_1$ was saturated at ~1.0 μg cDNA per 35 mm dish. PPARγ$_1$ significantly increased ENaC activity from 207 ± 42 to 904 ± 104 pA/pF when was coexpressed at 1.0 μg cDNA. The number of experiments for each cDNA level is between 8 and 16.

We wondered whether the actions of PPARγ on ENaC were specific. To address this question, we tested the effects of PPARγ$_1$ on acid-sensing ion channel ASIC1a. Similarly to ENaC, acid-sensing ion channels also belong to the amiloride ENaC/Deg superfamily (Schild, 2004). Standard protocols and solutions for measuring ASIC1a channel activity were used for these experiments. As summarized in Figure 6C, the current densities for ASIC1a in the absence and presence of PPARγ$_1$ (333 ± 57 and 359 ± 74, respectively) were not different. These results demonstrate that PPARγ signaling has specific actions on ENaC compared to ASIC1a.

GW9662 decreases PPARγ-induced increases in ENaC activity.

Next we tested whether PPARγ antagonist GW9662 affect ENaC activity overexpressed in CHO cells. Figure 7A shows ENaC currents before (arrows) and after treatment with amiloride in a cell expressing the channel alone (up) and in a cell expressing both the channel and PPARγ$_1$ in the absence (middle) and presence (bottom) of treatment with GW9662 (10 μM). Currents were elicited by voltage ramping from 60 mV down to -100 mV (holding potential is 40 mV). GW9662 did not have a direct effect on ENaC alone (Fig. 7B). As summarized in

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Figure 7C, expression of PPARγ$_1$ with ENaC significantly increased ENaC activity from 401 ± 103 to 931 ± 135 pA/pF. Pretreatment of CHO cells for 4 h with GW9662 (10 μM) resulted in a significant decrease in ENaC activity to 357 ± 74 pA/pF. 1 μM of GW9662 had only a little effect on ENaC activity when treated for 4 h. For these experiments, plasmid encoding the PPARγ$_1$ was transfected at saturating level (1 μg; see Fig. 6B). We interpret these results as evidence that GW9662 affected PPARγ-induced increases in ENaC activity. In contrast, as shown in Figure 7D pretreatment of CHO cells for 24 h with rosiglitazone (15 μM) had no effect on ENaC activity when all three subunits were coexpressed with half-dose of PPARγ$_1$ (0.35 μg; see Fig. 6B). Similar results were obtained with ciglitazone (15 μM; 24 h), another synthetic PPARγ agonist (data not shown).

Pretreatment of monolayers with PPARγ antagonist inhibits the insulin-stimulated sodium transport.

Insulin activates ENaC via a variety of mechanisms. PPARγ plays a critical role in regulating insulin sensitivity and glucose homeostasis. As shown in Figure 8A, addition of 100 nM insulin to polarized mpkCCD$_{c14}$ principal cells with steady-state basal transport rates significantly increased Na^+ reabsorption in a time-dependent manner above basal levels. Addition of amiloride to the apical membrane completely abolished the transepithelial current that was stimulated by insulin, indicating that this hormone increases Na^+ reabsorption via ENaC. The addition of PPARγ antagonist T0070907 (50 μM) to the mpkCCD$_{c14}$ cell monolayers, significantly diminishes the increase in sodium transport induced by insulin (100 nM; basolateral) stimulation (Fig. 8A). Similar to experiments

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presented in Figure 5, current through monolayer started to recover after 24 hours treatment with T0070907 showing that effect of PPARγ antagonist was not caused by cytotoxicity of the drug. Thus, the results in Figure 8A are consistent with PPARγ is important for insulin-stimulated sodium reabsorption. To test potential insulin-sensitizing effects of the PPARγ agonists, a submaximal (10 nM) insulin concentration was used to stimulate cells in the presence or absence of PGJ_2 (20 μM;). However, PGJ_2 treatment did not increase the amount of Na^+ flux elicited by insulin (Fig. 8B).

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DISCUSSION

Several laboratories have provided important information about relationship between PPARγ, SGK1 and ENaC. PPARγ agonists have been shown to have significant therapeutic benefits in type 2 diabetic patients; however, these agents may cause fluid retention and correspondingly edema in susceptible individuals. Recent studies have demonstrated that mice with collecting duct (CD) specific knockout of PPARγ were resistant to the rosiglitazone-induced increases in body weight and plasma volume expansion found in control mice expressing PPARγ in the CD. Moreover, rosiglitazone stimulated sodium transport in primary cultures of CD cells expressing PPARγ (Zhang et al., 2005). Furthermore, it was shown that CD-specific deletion of *Pparg* decreased renal Na^+ avidity and increased plasma aldosterone. Treatment of cultured CD with thiazolidinediones (TZDs) increased amiloride-sensitive Na^+ absorption and γ-ENaC mRNA expression through a PPARγ-dependent pathway (Guan et al., 2005). Fluid retention and sodium reabsorption in response to PPARγ agonists is clearly observed in the kidney (Chen et al., 2005;Song et al., 2004;Tiwari et al., 2008).

A couple of investigations have shown contradictory results indicating the failure of PPARγ agonists to affect ENaC activity. Noftziger et al. demonstrated that two PPARγ agonists, pioglitazone and GW7845 did not directly enhance basal or insulin-stimulated Na^+ transport via ENaC in the A6, M-1 and mpkCCD_{c14} cell lines (Nofziger et al., 2005). Similarly to this study, we failed to show any changes in sodium reabsorption in response to different PPARγ

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agonists including pioglitazone, rosiglitazone, troglitazone and 15-deoxy-$\Delta^{12,14}$–prostaglandin J_2. Although activity of ENaC was unaltered by PPARγ activation, two chemically distinct selective PPARγ antagonists, GW9662 and T0070907 significantly decreased ENaC-mediated Na^+ reabsorption in mpkCCD$_{c14}$ cells. Moreover, T0070907 inhibited the insulin-stimulated sodium transport. T0070907 was more potent then GW 9662 with respect to sodium transport probably because T0070907 has higher selectivity for PPARγ over all other subtypes whereas GW9662 has been reported to have some PPARα agonist activity. Guan et al. have shown that pioglitazone-induced increase in apical to basolateral Na^+ flux was completely blocked by GW9662. However, GW9662 (1 μM) by itself had no effect on basal Na^+ flux (Guan et al., 2005).

In addition, Vallon et al. recently published elegant study using mice with CD-specific conditionally inactivated αENaC (Scnn1a^{loxloxCre}) (Rubera et al., 2003) and patch-clamp experiments in wild type mice to assess the effect of PPARγ agonists on ENaC activity in isolated CD (Vallon et al., 2009). The authors propose that TZDs-induced fluid retention and weight gain are mediated by nonselective cation channels (NSC) in inner medullary CD (IMCD) and ENaC-mediated Na^+ reabsorption in the CD is not critical for this effect. However, this study does not explain TZD-dependent increase of γ-ENaC mRNA expression using real-time RT-PCR (Guan et al., 2005) or cell surface expression measured with cell surface biotinylation of α-ENaC after 4 hr and α-ENaC mRNA after 24 hours treatments with TZDs in human cortical CD cells (Hong et al., 2003).

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Besides, the PPARγ is reported to regulate Serum and Glucocorticoid regulated kinase 1 (SGK1), a protein kinase that is known as a key regulator of ENaC (Chen et al., 1999). PPARγ agonists have been shown to stimulate the transcription of the SGK1, which might enhance the surface expression of ENaC (Hong et al., 2003). It was proposed that PPARγ agonists may increase renal Na^+ reabsorption by stimulating SGK1 and ENaC (Vallon and Lang, 2005). Artunc et al. using mice lacking SGK1 (*sgk1*$^{-/-}$) also have shown that SGK1 contributes to the volume retention during treatment with pioglitazone. Although, authors conclude that SGK1 does not fully account for this effect (Artunc et al., 2008). Similarly, it was recently shown that PPARγ agonists enhance the expression of NHE3 (Na^+/H^+ exchanger), AQP1 (Aquaporin water channel) and AQP7 in human proximal tubule cells through SGK1-dependent pathways (Saad et al., 2008). Furthermore, a role of PPARγ in regulation of cAMP-regulated chloride channel (cystic fibrosis transmembrane regulator, CFTR) was proposed. It was shown that PPARγ expression is altered in tissue lacking the CFTR. PPARγ expression in cftr$^{-/-}$ mice is downregulated at the RNA and protein levels and its function is diminished (Ollero et al., 2004). These changes may be related to the loss of function of CFTR and may be relevant to the pathogenesis of metabolic abnormalities associated with cystic fibrosis. Recently Noftziger et al. showed that PPARγ agonists inhibit vasopressin-mediated anion transport in the MDCK-C7 cells. The PPARγ agonist-induced decrease in anion secretion is the result of decreased mRNA levels of CFTR (Nofziger et al., 2009).

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Thus, the exact mechanism by which PPARγ agonists induce fluid retention is not completely clear but is likely to be multifactorial. It is clear that ENaC is not a sole channel responsible for fluid retention and sodium reabsorption in response to PPARγ agonists. Other channels and transporters such as NSC in IMCD (Vallon et al., 2009) or Na-K-ATPase, NHE3 and Na-K-2Cl (Song et al., 2004) are most likely involved in this process. Furthermore, PPARγ agonists enhance NO production through the increased expression of endothelial and neuronal nitric oxide (NO) synthase in the kidney, which would shift the sodium balance to increased excretion (Dobrian et al., 2004) and induce cyclooxygenase-2 (COX-2), an enzyme producing PGE_2 and PGI_2 in several cell lines, which also could favor sodium excretion (Bishop-Bailey and Warner, 2003).

The function of PPARs is modified by the precise shape of their ligand-binding domain induced by ligand binding and by a number of coactivators and corepressor proteins. It is well known that endogenous ligands for the PPARγ include free fatty acids and eicosanoids. In addition, the effect of PPARγ agonists on the cytochrome P450 4A (CYP4A) pathway was recently shown and authors propose that pioglitazone downregulates CYP4A, leading to sodium retention (Yoshioka et al., 2008). CYP epoxygenase-dependent metabolites such as 11,12-EET play an important role in the regulation of ENaC channel activity: inhibition of CYP epoxygenase increased, whereas stimulation of CYP epoxygenase-dependent metabolism decreased ENaC activity. For example, the inhibitory effect of arachidonic acid on ENaC was suppressed by low Na^+ intake

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(Sun et al., 2006). We hypothesize that eicosanoids and free fatty acids might be involved into PPARγ-regulated changes in fluid retention.

To place the current findings in the context with that reported previously by others, our results appear to be most supportive of an idea that ENaC is involved into PPARγ-regulated changes in fluid retention. The possible mechanisms underlining the regulation of ENaC via PPARγ remain obscure and require additional studies.

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FOOTNOTES

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LEGENDS FOR FIGURES

Fig. 1. Effect of GW9662, T0070907 and pioglitazone on PPARγ activity. CHO cells were transfected, treated and analyzed as detailed in the materials and methods. Luciferase data are normalized to control cells. Values are means ± s.e.m. of either three or six independent experiments. Concentrations of PPARγ agonists/antagonists are presented. $^{*}p < 0.05$, versus control.

Fig. 2. Growth inhibitory effects of GW9662 and troglitazone on viability of mpkCCD$_{c14}$ cells. Cells were treated with GW9662 (50 μM) and either 10 or 50 μM of troglitazone for 24 and 48 h. Grow inhibition was monitored by MTT assay. Absorbance at 495 nm was measured and the percent of growth against untreated cells was calculated. Data represented as a viable fraction (%) of untreated cells from at least triplicate experiments.

Fig. 3. Effect of PPARγ agonists on basal ion transport in mpkCCD$_{c14}$ cells. Summary graph of the normalized I_{sc} in mpkCCD$_{c14}$ cells in response to PPARγ stimulation with Pioglitazone (15 μM; Pio), Rosiglitazone (15 μM; Rosi), Troglitazone (10 μM; Tro) and 15-deoxy-$\Delta^{12,14}$ –prostaglandin J_2 (20 μM; PGJ_2). Measurements were made at 8 and 24 hours after treatment. MpkCCD$_{c14}$ cells were serum-starved overnight. PPARγ agonists and vehicle (control) were added at time 0 and current was normalized to starting level. The numbers of experiments are 12, 6, 12, 6, and 6 for control cells and cells treated with pioglitazone, rosiglitazone, troglitazone and 15-deoxy-$\Delta^{12,14}$ –prostaglandin J_2, respectively.

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Fig. 4. Effect of GW9662 on I_{sc} in mpkCCD$_{c14}$ cells. A, Time course of decreases in relative Na^+ transport in response to PPARγ inhibition with GW9662 (10 μM). MpkCCD$_{c14}$ cells were serum-starved overnight. PPARγ antagonist and vehicle (control) were added at time 0 and current was normalized to starting level. Amiloride (10 μM; arrow) was added to the apical membrane at the end of experiment. The numbers of experiments are 24 and 26 for GW9662 and vehicle, respectively. B-D, Dose response for GW9662–dependent decreases in relative Na^+ transport across monolayers of mpkCCD$_{c14}$ principal cells at 4 (B), 8 (C) and 24 (D) hours after treatment. The numbers of experiments are 12, 6, 5, 6 and 6 for control experiments and cells treated with 0.1, 1, 10 and 100 μM GW9662, respectively. All other conditions are the same as in Fig. A.

Fig. 5. T0070907 rapidly and markedly decreases Na^+ reabsorption. Time course for T0070907–dependent decreases in relative Na^+ transport across monolayers of mpkCCD$_{c14}$ principal cells. Current relative to starting levels. The numbers of experiments are 9 and 6 for control and cells treated with T0070907 (50 μM), respectively. All other conditions are the same as in Fig. 4A.

Fig. 6. PPARγ activates ENaC overexpressed in CHO cells. A, Typical macroscopic current traces before (left) and after (right) amiloride (Amil; 10 μM) under voltage clamp conditions from CHO cells transfected with mouse ENaC alone and coexpressed with PPARγ$_1$. Currents elicited by test pulses with 20 mV steps from 100 to –120 mV from a holding potential of 40 mV. B, Dose-response curves showing the mean amiloride-sensitive current density at -80 mV for voltage-clamped CHO cells co-transfected with ENaC and increasing

quantities of PPARγ$_1$ cDNA (for each point n ≥ 8). C, Acid-activated current density at pH5 for CHO cells transfected with ASIC1a alone and coexpressed with PPARγ$_1$. The number of observations in each group is shown.

Fig. 7. ENaC activity is coupled to PPARγ. A, Overlays of typical macroscopic current traces before (*arrow*) and after 10 μM amiloride from voltage-clamped CHO cells transfected with mENaC alone (*top*) and with PPARγ$_1$ not treated (middle) and treated with GW9662 (bottom; 10 μM; 4 hours). B, Summary graph of ENaC activity in CHO cells when all three mENaC subunits are expressed in the absence and presence of pretreatment with GW9662 (10 μM; 4 hours). C, Summary graph of ENaC activity when mENaC is expressed alone and with PPARγ$_1$ (1μg cDNA) in the absence and presence of pretreatment with GW9662 (1 and 10 μM; 4 hours). D, Summary graph of ENaC activity when ENaC subunits are expressed alone and with PPARγ$_1$ (0.35 μg cDNA) in the absence and presence of pretreatment with rosiglitazone (15 μM; 24 hours). The number of observations in each group is shown. $^{*}p < 0.05$, *versus* mENaC alone; ** $p < 0.05$, *versus* mENaC + PPARγ$_1$.

Fig. 8. PPARγ is necessary for insulin to increase Na^+ reabsorption across principal cells. A, Time course for insulin-dependent increases in relative Na^+ transport across monolayers of mpkCCD$_{c14}$ principal cells in the absence and presence of PPARγ antagonist T0070907 (50 μM). Insulin (100 nM, basolateral) was added at time 0. Amiloride (10 μM; arrow) was added to the apical membrane at the end of experiment. B, Summary graph of the normalized I_{sc} in mpkCCD$_{c14}$ cells in the absence and presence of PPARγ agonist 15-deoxy-$\Delta^{12,14}$

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–prostaglandin J_2 (20 μM; PGJ_2). Insulin (10 nM, basolateral) was added at time 0.

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Figure 1

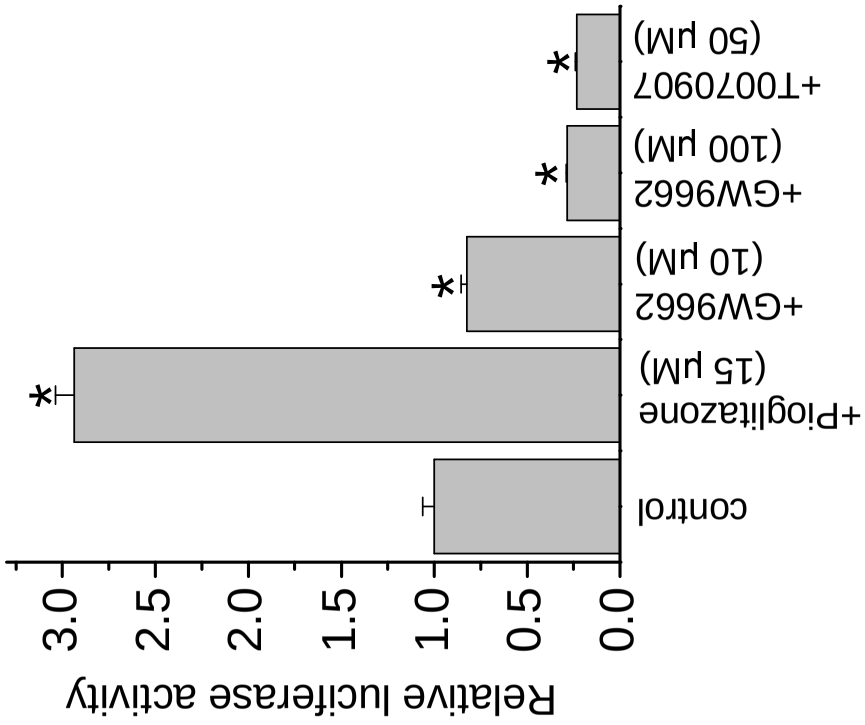


Figure 2

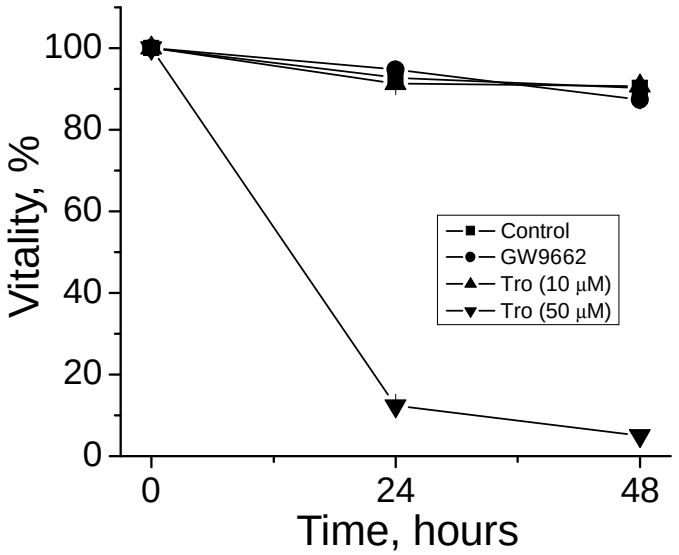
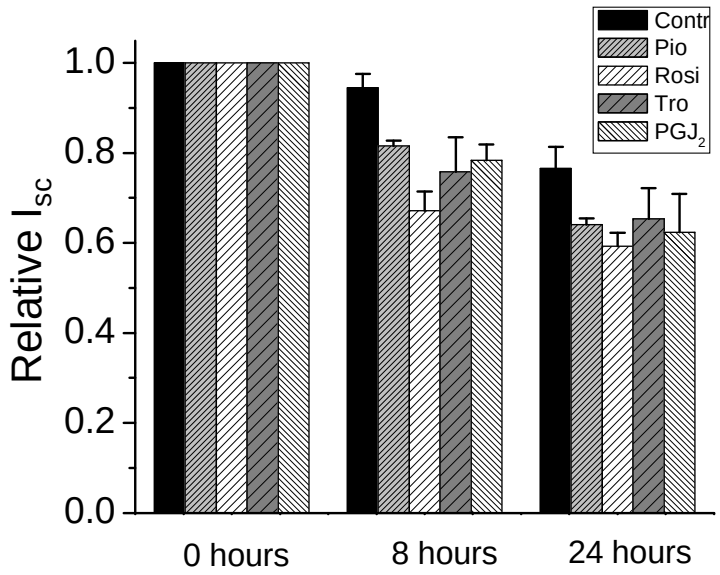


Figure 3



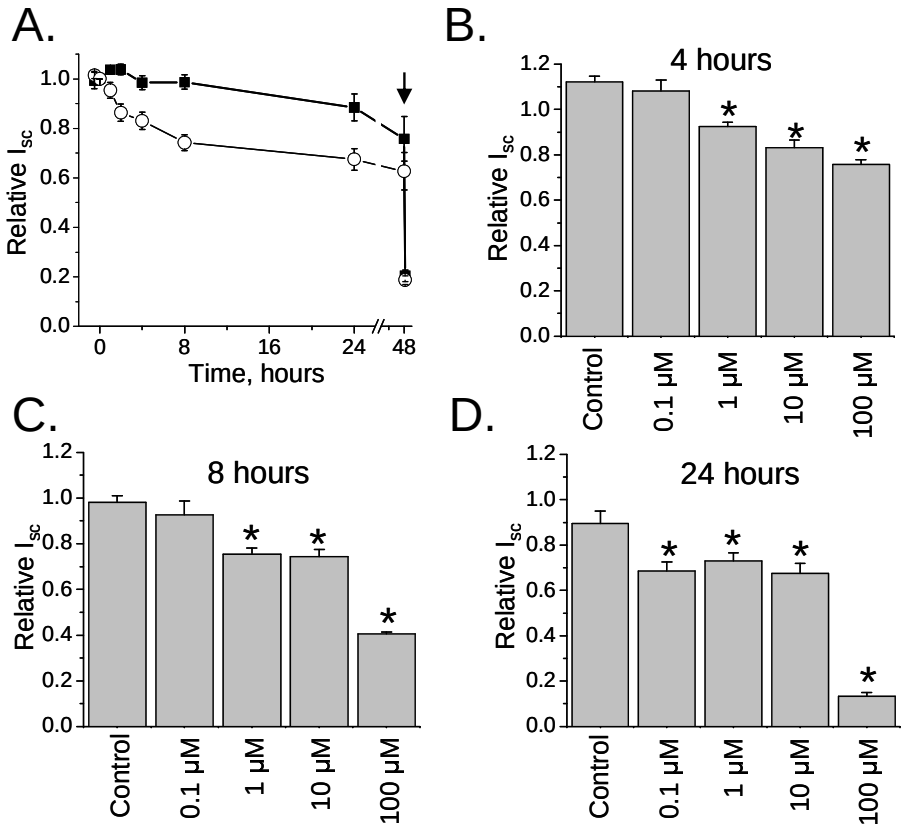


Figure 5