Rosiglitazone prevents sirolimus-induced hypomagnesemia, hypokalemia, and downregulation of NKCC2 protein expression

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Rosiglitazone (RGZ) is a thiazolidinedione used in the treatment of type 2 diabetes mellitus and is an agonist for peroxisome proliferator-activated receptor subtype γ (PPAR-γ) (8). The PPAR-γ is a member of the nuclear receptor superfamily of ligand-dependent transcription factors that regulate gene expression in response to the binding of a number of fatty acid metabolites (37).

There is evidence that molecular mechanisms underlie the fluid retention and edema associated with RGZ. Song et al. (31) demonstrated that administration of RGZ indeed results in rapid retention of sodium and water. This increase in sodium and water retention is accompanied by a drop in blood pressure, together with greater renal abundance of α1Na-K-ATPase, the Na-K-2Cl cotransporter NKCC2, NaPi-2, the sodium/hydrogen exchanger 3 (NHE3), and endothelial nitric oxide (NO) synthase, as well as of the collecting duct water channel aquaporin-2 (AQP2) (31).

In this study, we examined whether rats treated with SRL develop hypokalemia, hypomagnesemia, and polyuria, as well as investigating urinary excretion of potassium and magnesium. We tested the hypothesis that these disturbances are associated with changes in the renal protein expression of NKCC2, AQP2, and the epithelial Mg2+ channel TRPM6. We also tested the hypothesis that the coadministration of RGZ and SRL has a protective effect on the renal tubules.

METHODS

Experimental animals. Male Wistar rats weighing 200 to 250 g were obtained from the animal facility of the University of São Paulo School of Medicine. The rats were divided into four groups: control, consisting of rats fed a normal diet and given ad libitum access to water for 14 days (n = 10); SRL, consisting of rats fed a normal diet and receiving SRL (3 mg·kg body wt−1·day−1 in drinking water, ad libitum access) (2) for 14 days (n = 11); RGZ, consisting of rats fed a normal diet and receiving RGZ (92 mg/kg of food) (18, 31) for 14 days (n = 10); and SRL + RGZ, consisting of rats receiving the combination of SRL (3 mg·kg body wt−1·day−1 in drinking water, ad libitum access) and RGZ (92 mg/kg of food) for 14 days (n = 10). Throughout the intervention period, water and food intake were monitored on a daily, per animal basis.

The Ethics Committee of the University of São Paulo School of Medicine approved the experimental protocol.
Metabolic cage studies. At the end of the treatment period (on day 14, the last full day of drug or vehicle administration), all of the rats were moved to individual cages and maintained on a 12:12-h light-dark cycle (still being given ad libitum access to food and drinking water) for 24 h, during which time urine samples were collected. At the end of this 24-h period, the animals were killed and blood samples were collected by cardiac puncture.

Analysis of blood and urine samples. The volume of each 24-h urine sample was determined. Urine and blood samples were centrifuged, and the supernatants were analyzed. Urine osmolality was determined using a vapor pressure osmometer (model 5520; Wescor, Logan, UT). Plasma and urine levels of sodium and potassium were measured using flame photometry. The enzymatic colorimetric method (Labtest, Lagoa Santa, Brazil) was used to quantify levels of magnesium, calcium, phosphorus, and creatinine. Serum levels of SRL were determined using mass spectrometry detection.

Analysis of hemodynamic parameters. Another set of experiment was performed to analyze mean arterial pressure (MAP). Rats were divided into four groups as previously described: control (n = 5), SRL (n = 5), RGZ (n = 6), and SRL + RGZ (n = 4). Rats were anesthetized with intraperitoneal injections of pentobarbital sodium (50 mg/kg body wt) and placed on a temperature-regulated surgical table. A tracheotomy was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of membrane fractions for NKCC2, AQP2, and TRPM6 protein expression. Medulla and cortex samples were homogenized in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, 41 mM KOH; pH 7.5) containing protease inhibitors (cocktail protease inhibitor, Sigma, St. Louis, MO) using a Teflon pestle glass homogenizer (Schmid) or a Teflon pestle glass homogenizer (Schmidl), centrifuged at low speed (2,000 g) and placed on a temperature-regulated surgical table. A tracheotomy was performed, and a PE-50 catheter was inserted into the left femoral artery to record MAP.

Primary antibodies. The specific peptide-derived polyclonal antibody to NKCC2 was kindly supplied by Dr. M. Knepper (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD). The specific peptide-derived polyclonal antibody to TRPM6 was a gift from Dr. V. Chubanov (Institute for Pharmacology and Toxicology, Philipps University Marburg, Marburg, Germany). The specific peptide-derived polyclonal antibody to the AQP2 water channel was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of membrane fractions for NKCC2, AQP2, and TRPM6 protein expression. Medulla and cortex samples were homogenized in ice-cold isolation solution (200 mM mannitol, 80 mM HEPES, 41 mM KOH; pH 7.5) containing protease inhibitors (cocktail protease inhibitor, Sigma, St. Louis, MO) using a Teflon pestle glass homogenizer (Schmidl). The homogenates were centrifuged at low speed (2,000 g) for 15 min at 4°C to remove nuclei and cell debris. Subsequently, the supernatants were spun at 100,000 g for 1 h at 4°C using a Ti70i rotor (Beckman Coulter, Fullerton, CA) to produce a pellet containing membrane fractions enriched for both plasma membranes and intracellular vesicles. The pellets were suspended in isolation solution with protease inhibitors.

Preparation of intracellular and membrane fractions for NKCC2 protein expression. It has been shown that, under basal conditions, only a small fraction of total NKCC2 is present on the cell surface and that most of the NKCC2 protein is in the intracellular compartment (23). Therefore, we performed another set of experiments to study the total (intracellular and membrane) NKCC2 protein expression.

Table 1. Physiological data in control rats and in rats treated with SRL, SRL and RGZ, and RGZ

<table>
<thead>
<tr>
<th>Group</th>
<th>Cr.Cl, ml·min⁻¹·10⁻¹·g⁻¹</th>
<th>Food Intake, g/day</th>
<th>Water Intake, ml/day</th>
<th>ΔBody Weight, g</th>
<th>Serum SRL, mg/dl</th>
<th>Na, meq/l</th>
<th>K, meq/l</th>
<th>Mg, mg/dl</th>
<th>Ca, mg/dl</th>
<th>P, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>0.65 ± 0.04</td>
<td>26 ± 2.0</td>
<td>21 ± 1.5</td>
<td>67 ± 1.8</td>
<td>0.00 ± 0.00</td>
<td>144 ± 1.75</td>
<td>3.4 ± 0.12</td>
<td>1.69 ± 0.07</td>
<td>10.1 ± 0.35</td>
<td>6.5 ± 0.32</td>
</tr>
<tr>
<td>SRL (n = 11)</td>
<td>0.61 ± 0.11</td>
<td>26 ± 2.0</td>
<td>37.2 ± 10.3d</td>
<td>19 ± 2.4e</td>
<td>2.82 ± 0.33</td>
<td>141 ± 3.10</td>
<td>2.6 ± 0.08h,e</td>
<td>1.31 ± 0.03w,e</td>
<td>10.0 ± 0.26</td>
<td>5.2 ± 0.67</td>
</tr>
<tr>
<td>SRL + RGZ (n = 10)</td>
<td>0.72 ± 0.04</td>
<td>21 ± 1.7</td>
<td>28.3 ± 2.1h</td>
<td>37 ± 5.4h</td>
<td>2.98 ± 0.44</td>
<td>147 ± 0.80</td>
<td>3.0 ± 0.13d</td>
<td>1.52 ± 0.06</td>
<td>10.1 ± 0.28</td>
<td>5.7 ± 0.69</td>
</tr>
<tr>
<td>RGZ (n = 10)</td>
<td>0.59 ± 0.02</td>
<td>24 ± 3.6</td>
<td>26.4 ± 1.3</td>
<td>107.5 ± 8.2</td>
<td>0.0 ± 0.00</td>
<td>138 ± 1.0</td>
<td>3.5 ± 0.08</td>
<td>1.61 ± 0.06</td>
<td>10.8 ± 0.51</td>
<td>6.0 ± 0.23</td>
</tr>
</tbody>
</table>

Values are means ± SE. Physiological data in control rats, in rats treated for 14 days with sirolimus (SRL; 3 mg·kg·body wt⁻¹·day⁻¹ in drinking water), in rats treated for 14 days with SRL and rosiglitazone (RGZ; 3 mg·kg·body wt⁻¹·day⁻¹ in drinking water and 92 mg·kg·food, respectively), and rats treated for 14 days with RGZ (92 mg·kg·food). ΔBody weight: difference between the pretreatment and posttreatment body weight. ΔMean over the 14-day treatment period. Cr.Cl., creatinine clearance. *P < 0.001 vs. control. †P < 0.001 vs. RGZ. ‡P < 0.05 vs. SRL + RGZ. §P < 0.001 vs. control. †P < 0.0001 vs. control. ‡P < 0.0001 vs. RGZ.
Animals treated with RGZ or with the combination of SRL and RGZ. The results of clearance studies and renal hemodynamic function were comparable to those seen in the control group (Table 2). Therefore, normal tubular function was partially preserved in rats treated with the combination of SRL and RGZ. The RGZ group presented significantly higher urinary excretion of sodium, potassium, and magnesium than did the controls. In addition, the RGZ group gained more weight than did the control rats. In addition, there were no significant differences in urinary osmolality among the groups (Table 2).

Treatments with RGZ preserved normal tubular function in SRL-treated rats. Rats in the SRL + RGZ group presented significantly lower urinary excretion of sodium, potassium, and magnesium than did those in the SRL group (Table 2). Accordingly, serum levels of potassium and magnesium remained normal in the SRL + RGZ group (Table 1). In addition, urine volume in the SRL + RGZ group was comparable to that seen in the control group (Table 2). Therefore, normal tubular function was partially preserved in rats treated with the combination of SRL and RGZ. The RGZ group presented significantly higher urinary excretion of sodium, potassium, and magnesium than did the controls. In addition, the RGZ group presented significantly higher urinary excretion of sodium compared with the SRL + RGZ group (Table 2). Urinary excretion of potassium was higher in the RGZ group than in the control and SRL groups (Table 2). SRL treatment is not associated with changes in renal function. The results of clearance studies and renal hemodynamic analyses are presented in Table 3. Treatment with SRL did not result in any changes in creatinine clearance or MAP. Animals treated with RGZ or with the combination of SRL and RGZ did not present significant changes in creatinine clearance or MAP.

SRL treatment is associated with reduced protein expression of NKCC2 and AQP2. Apical NKCC2 is the major transporter for apical reabsorption of sodium and potassium by the thick ascending limb. In addition, the majority of magnesium reabsorption in the thick ascending limb is driven by Na-K-2Cl-mediated NaCl. As shown in Fig. 1 (semiquantitative immunoblot, using membrane fractions prepared from the entire medulla), NKCC2 protein expression in membrane fractions was markedly lower in the SRL group than in the control group (54.7 ± 2.4 vs. 99 ± 0.8%, P < 0.001). In contrast, expression was unaffected. The SRL-treated rats gained less weight than did the control and RGZ rats. However, this condition was ameliorated in the SRL + RGZ group rats (Table 1). The RGZ group gained more weight than did the control rats. In addition, there were no differences among the groups in terms of food intake (Table 1).

**Table 2. Urine osmolality, urine volume, and urinary excretion of sodium, potassium, magnesium, and phosphorus in control rats and in rats treated with SRL, SRL and RGZ, and RGZ**

<table>
<thead>
<tr>
<th>Group</th>
<th>Osmolality, mosmol/kg H2O</th>
<th>Volume, ml/day</th>
<th>UVNa, meq/day</th>
<th>UVK, meq/day</th>
<th>UVMg</th>
<th>UVCa</th>
<th>UVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 10)</td>
<td>772 ± 134</td>
<td>16 ± 3</td>
<td>0.48 ± 0.05</td>
<td>0.45 ± 0.04</td>
<td>0.28 ± 0.05</td>
<td>0.18 ± 0.02</td>
<td>16 ± 0.67</td>
</tr>
<tr>
<td>SRL (n = 11)</td>
<td>421 ± 78</td>
<td>31 ± 5</td>
<td>1.00 ± 0.12</td>
<td>1.12 ± 0.05</td>
<td>0.77 ± 0.07</td>
<td>0.39 ± 0.11</td>
<td>16 ± 1.5</td>
</tr>
<tr>
<td>SRL + RGZ (n = 10)</td>
<td>621 ± 146</td>
<td>19 ± 4</td>
<td>0.62 ± 0.07</td>
<td>0.75 ± 0.11</td>
<td>0.44 ± 0.06</td>
<td>0.16 ± 0.02</td>
<td>21 ± 3.12</td>
</tr>
<tr>
<td>RGZ (n = 10)</td>
<td>522 ± 53</td>
<td>21 ± 1</td>
<td>1.24 ± 0.17</td>
<td>0.83 ± 0.10</td>
<td>0.45 ± 0.05</td>
<td>0.30 ± 0.04</td>
<td>24 ± 1.56</td>
</tr>
</tbody>
</table>

Values are means ± SE. Urine osmolality, urine volume, and urinary excretion (UV) of sodium (Na), potassium (K), magnesium (Mg), calcium (Ca), and phosphorus (P) in control rats, in rats treated for 14 days with SRL (3 mg·kg body wt⁻¹·day⁻¹ in drinking water), in rats treated for 14 days with SRL and RGZ (3 mg·kg body wt⁻¹·day⁻¹ in drinking water and 92 mg/kg of food, respectively), and rats treated for 14 days with RGZ (92 mg/kg of food). A > P < 0.01 vs. control, b P < 0.01 vs. SRL + RGZ, c P < 0.05 vs. RGZ. d P < 0.05 vs. control. e P < 0.01 vs. SRL + RGZ. f P < 0.05 vs. RGZ. g P < 0.001 vs. control.

**Table 3. Hemodynamic measurements in control rats and in rats treated with SRL, SRL and RGZ, and RGZ**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>SRL</th>
<th>SRL + RGZ</th>
<th>RGZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP, mmHg</td>
<td>129 ± 4.0</td>
<td>124 ± 6.3</td>
<td>137 ± 14</td>
<td>131 ± 4.0</td>
</tr>
</tbody>
</table>

Values are means ± SE. Hemodynamic measurements in control rats, in rats treated for 14 days with SRL (3 mg·kg body wt⁻¹·day⁻¹ in drinking water), in rats treated for 14 days with SRL and RGZ (3 mg·kg body wt⁻¹·day⁻¹ in drinking water and 92 mg/kg of food, respectively), and rats treated for 14 days with RGZ (92 mg/kg of food). MAP, mean arterial pressure.

Fig. 1. Effect that administration of sirolimus (SRL), with and without rosiglitazone (RGZ), has on NKCC2 abundance in membrane fractions in the renal medulla. Top: densitometric analysis showing that, in the SRL group, there was a marked decrease in NKKC2 protein abundance, which remained near normal in the SRL + RGZ group. Bottom: immunoblot reacted with NKCC2.
of NKCC2 was partially preserved in the SRL + RGZ group compared with the SRL group (77.7 ± 7.2 vs. 54.7 ± 2.4%, P < 0.001). In the RGZ group, NKCC2 expression (103.5 ± 2.0%) did not differ significantly from that observed for the controls. These results were confirmed by those obtained for total (intracellular and membrane) NKCC2 protein expression, which was markedly lower in the SRL group than in the control group (52 ± 3.4 vs. 99.5 ± 1.5%, P < 0.05). As can be seen in Fig. 2, expression of total NKCC2 was preserved in the SRL + RGZ group compared with the SRL group (87.3 ± 12 vs. 52 ± 3.4%, P < 0.05), which is also in accordance with the Western blot results.

Densitometric analyses of NKCC2 protein were normalized by densitometric analysis of actin protein abundance.

As shown in Fig. 3 (semiquantitative immunoblot, using membrane fractions prepared from the entire medulla), AQP2 protein expression was lower in the SRL group than in the control group (54.2 ± 5.8 vs. 100.5 ± 1.7%, P < 0.001), whereas it was higher in the SRL + RGZ group than in the SRL group (77.2 ± 3.0 vs. 54.2 ± 5.8%, P < 0.001). However, RGZ treatment was not able to completely protect AQP2 protein expression (SRL + RGZ group: 77.2 ± 3.0%, P < 0.001 vs. controls). In the RGZ group, AQP2 expression increased compared with that observed in the control group (RGZ group: 165.3 ± 3.7%, P < 0.001). In addition, there was a significant difference between the RGZ group and the SRL + RGZ group in terms of AQP2 expression, which was greater in the RGZ group (P < 0.001).

**Renal protein expression of TRPM6 is greater in SRL-treated animals.** The decreased expression of NKCC2 in the SRL group suggests that downregulation of this transport protein plays a role in the pathogenesis of SRL-induced increase in urinary excretion of magnesium, inhibition of passive reabsorption of magnesium in the thick ascending limb potentially promoting hypomagnesemia. Active reabsorption of magnesium has been localized to the distal convoluted tubule. To determine whether SRL induced a defect in the active reabsorption of magnesium, protein levels of TRPM6 expression were determined. Cortical protein expression of TRPM6 was significantly greater in the SRL group than in the control group (127.2 ± 2.5 vs. 99.2 ± 0.5, P < 0.001) and RGZ group (97.3 ± 2.5, P < 0.001). Figure 4 shows that cortical protein expression of TRPM6 was lower in the SRL + RGZ group than in the SRL group (101.3 ± 2.0 vs. 127.2 ± 2.5%, P < 0.001), indicating that treatment with RGZ prevents the SRL-induced increase in cortical protein expression of TRPM6. Taken together, the significant alterations in expression of TRPM6 in the SRL group might represent a compensatory mechanism activated in response to the SRL-induced down-regulation of NKCC2 (Fig. 4).

**DISCUSSION**

In this study, we demonstrated that treatment with SRL decreases the expression of NKCC2, the principal sodium transporter in the thick ascending limb, as well as increasing urinary excretion of sodium, potassium, and magnesium. In addition, there was an SRL-induced reduction in the expression of AQP2, which can cause an increase in urine volume. Furthermore, we showed that expression of the TRPM6 increased after treatment with SRL; this increase representing either a direct stimulatory effect of SRL or a compensatory response and serving to limit renal magnesium wasting. Most importantly, RGZ prevented or attenuated all SRL-induced renal tubular defects, a finding that has potential clinical implications.

The NKCC2 cotransporter is found in the apical plasma membrane domains of the medullary and cortical segments of the thick ascending limb. In these segments, which are water impermeable, NKCC2 mediates apical NaCl transport (9, 13). In the present study, treatment with SRL resulted in lower NKCC2 expression. Although we were unable to identify the cause of this decrease, we found that it led to greater urinary excretion of sodium, potassium, and magnesium. This is consistent with previous observations that SRL administration is associated with hypomagnesemia and renal magnesium wasting (2, 19, 20). It is also possible that the lower NKCC2 expression in this model is related to factors similar to those responsible for the inhibition of AQP2 expression. In addition, the rats receiving SRL as monotherapy presented a significant decrease in plasma magnesium and an increase in urinary magnesium excretion. Magnesium moves passively across the epithelium or between the cells (paracellular pathway), driven by transepithelial-positive voltage in the thick ascending limb (33). Therefore, factors that influence NaCl transport, such as a decrease in NKCC2 expression, are expected to affect the transepithelial voltage and thus the passive transport of magnesium (25). In the present study, renal TRPM6 expression was increased in the rats receiving SRL alone. This is of note, since TRPM6 constitutes a magnesium-permeable channel located along the apical membrane of the distal convoluted tubule, to which active magnesium reabsorption is restricted (27, 35, 36). More recently, inhibition of TRPM6 was identified as the cause of tacrolimus-related renal magnesium wasting and consequent hypomagnesemia (22). The upregulation of TRPM6 reported in studies of diabetes is similar to that observed in the present study and, in both cases, might be a compensatory mechanism activated in response to increased solute load (17). In the present study, we also observed polyuria. In the normal state, urine is concentrated as a result of the combined functions of...
The loop of Henle and the collecting duct. The loop of Henle generates high osmolality in the renal medulla by driving the countercurrent multiplication process (24, 26). When vasopressin is present, the collecting duct permits osmotic equilibration between the urine and the hypertonic medullary interstitium (10, 16). The development of high osmolality in the renal medulla is dependent on the net NaCl absorption by the thick ascending limb. Here, we demonstrated marked decreases in the expression of NKCC2 in the thick ascending limb. This suppressed expression results in decreased active NaCl absorption by the thick ascending limb and is therefore predictive of decreased countercurrent multiplication of NaCl in SRL-treated animals. We also showed a 54% decrease in AQP2 expression. Such a decrease in collecting duct water channel expression might be due in part to an NKCC2-induced decrease in medullary interstitial hypertonicity, although a direct effect of SRL on the collecting duct cells cannot be ruled out (15).

Here, we demonstrated that SRL can downregulate NKCC2 and AQP2 protein expression. However, the mechanism by which SRL downregulates NKCC2 and AQP2 while upregulating TRPM6 remains unexplained. The SRL target protein, mTOR, is a serine/threonine kinase that is robustly regulated by a diverse array of upstream signals (6, 8). The isoform of Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) (NKCC1), which is expressed ubiquitously in animal cells, is significantly inhibited by rapamycin, indicating that mTOR plays a role in L6 rat skeletal muscle cells (38).

There are convincing data in the literature demonstrating RGZ-induced increases in the protein expression of sodium transporters and of AQP2 (11, 14, 31). However, there are few data demonstrating how such increases occur. It is not completely understood how RGZ affects the expression of all renal transporters, and there are few data available on this topic. Therefore, the mechanisms of the antagonistic effects of SRL and RGZ merit further study.

The RGZ group also presented significant increases in the urinary excretion of sodium, potassium, and phosphorus compared with control group. We hypothesize that the greater urinary excretion of these ions is associated with extracellular volume expansion. There are data in the literature indicating that epithelial sodium channel (ENaC) plays a role in thiazolidinedione-induced fluid retention (11). However, in studies evaluating primary mouse inner medullary collecting duct cells, no ENaC activity was detected, although a nonselective cation channel upregulated by pioglitazone was identified (34). The extracellular volume expansion resulting from increased sodium channel expression can also occur in response to an increase in AQP2 protein expression. In our RGZ group, protein expression of AQP2 was elevated, which might explain the extracellular volume expansion observed.

Electrolyte imbalances such as hypokalemia and hypomagnesemia are important in the pathogenesis of cardiovascular
and renal diseases (1, 5). Preventing hypokalemia is beneficial in several cardiovascular disease states including acute myocardial infarction, heart failure, and hypertension (5). Hypokalemia aggravates ischemic acute kidney injury in rats (29). It has also been shown that hypomagnesemia leads to hypertension, atherogenesis, and stroke (1). In previous studies, we demonstrated that hypomagnesemia induced by magnesium depletion in the diet potentiates postischemic acute kidney injury in rats (4). In another study, we showed that hypomagnesemia induced a decrease in GFR and in RBF in zidovudine-treated rats (28). More recently, we demonstrated that magnesium supplementation partially protected the postischemic rats with acute kidney injury, improving renal function by increasing endothelial NO synthase protein expression (3). Low serum magnesium levels have been associated with a faster rate of decline in kidney allograft function and with increased rates of graft loss in renal transplant recipients (12). Clinicians must remain alert to the possibility that these drug-induced electrolyte disturbances are potentially deleterious to patients.

In conclusion, SRL-induced downregulation of NKCC2 protein expression could be a critical factor in the pathogenesis of the increased urinary excretion of potassium and magnesium, which was partially prevented by coadministration of RGZ.

GRANTS

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induced fluid retention is independent of collecting duct αENaC activity. 


