Sirolimus and cyclosporine A alter barrier function in renal proximal tubular cells through stimulation of ERK1/2 signaling and claudin-1 expression

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Martin-Martin N, Ryan G, McMorrow T, Ryan MP. Sirolimus and cyclosporine A alter barrier function in renal proximal tubular cells through stimulation of ERK1/2 signaling and claudin-1 expression. Am J Physiol Renal Physiol 298: F672–F682, 2010. First published December 2, 2009; doi:10.1152/ajprenal.00199.2009.—Alteration of the tight junction complex in renal epithelial cells can affect renal barrier function and perturb normal kidney homeostasis. The immunosuppressant drugs cyclosporine A (CsA) and sirolimus (SRL) used in combination demonstrated beneficial effects in organ transplantation but this combination can also result in increased adverse effects. We previously showed that CsA treatment alone caused an alteration of the tight junction complex, resulting in changes in transepithelial permeability in Madin-Darby canine kidney distal tubular/collection duct cells. The potential effect of SRL on transepithelial permeability in kidney cells is unknown. In this study, subcytotoxic doses of SRL or CsA were found to decrease the paracellular permeability of the porcine proximal tubular epithelial cells, LLC-PK1 cell monolayers, which was detected as an increase in transepithelial electrical resistance (TER). The cotreatment with SRL and CsA was found to increase TER in a synergistic manner. SRL treatment increased total cellular expression and membrane localization of the tight junction protein claudin-1 and this further increased with the combination of SRL/CsA. SRL and CsA treatment alone or in combination stimulated the phosphorylation of ERK1/2. The MEK-ERK1/2 pathway inhibitor, U0126, reduced the SRL, CsA, and CsA/SRL-induced increase in TER. U0126 also reduced the CsA and CsA/SRL-induced increase in the membrane localization of claudin-1. Alterations in claudin-2 and claudin-4 were also detected. However, the results suggest that the modulation in expression and localization of claudin-1 appears to be pivotal in the SRL- and CsA-induced modulation of the epithelial barrier function and that modulation is regulated by ERK1/2 signaling pathway.
	ransepithelial electrical resistance; immunosuppressive drugs; nephrotoxicity

Cyclosporine A (CsA) has proven to be an invaluable immunosuppressive drug in solid organ transplantation (13). However, its clinical use is limited by the major side effect of nephrotoxicity (8). In vitro CsA-induced nephrotoxicity has been shown to be associated with direct effects of CsA in proximal tubular cells (21, 38). In vivo, CsA acutely causes an increase in intrarenal resistance and hemodynamic alterations and CsA-induced hypertension is associated with sodium and water retention (11). Sirolimus (SRL), a macrolactone, is a potent immunosuppressive agent that may act synergistically with CsA to achieve powerful immunosuppression (3). Phase III studies of de novo treatment of recipients of renal allografts from living-related donors demonstrated that SRL either had a subclinical adverse effect on renal function or exacerbated the nephrotoxicity of CsA (28). New phase III studies demonstrated that early withdrawing of CsA from the SRL/CsA regimen resulted in improved renal function and renal histology (37).

The mechanism of the enhanced nephrotoxicity of this immunosuppressive combination (CsA/SRL) is not fully understood. CsA blocks the expression of the interleukin-2 gene in activated T cells by inhibiting calcineurin (17). The immunosuppressive actions of SRL are synergistic to those of CsA as SRL acts further downstream, influencing biochemical events later in the T cell cycle (46). One possibility for the enhanced nephrotoxicity of the combination of CsA/SRL is that the exacerbation of CsA nephrotoxicity may be due to pharmacokinetic interactions. SRL has been shown in vivo to increase CsA concentrations in whole blood and particularly in kidney tissue, thus augmenting CsA-induced renal dysfunction (41). Anglicheau et al. (4) demonstrated in vitro that SRL exerted an inhibitory effect on P-glycoprotein, increasing cellular CsA concentrations in a dose-dependent manner in normal human renal epithelial cells. The present in vitro experiments were designed to test the hypothesis that the enhanced nephrotoxicity of the combination of CsA/SRL may result from direct effects with renal proximal tubular epithelial cells.

Epithelial tissues serve as selective permeability barriers, separating fluid compartments with different chemical compositions. Renal epithelial tight junctions (TJs) mediate this barrier role and seal cells together to impede the paracellular leakage of small molecules. In renal tubules, the paracellular pathway plays an important role in vectorial transport with some selectivity for transport of ions. It has been shown that CsA and SRL treatments alone can induce magnesium wasting in a rat model probably due to improper reabsorption of this ion (2, 12).

In the mammalian kidney, the transepithelial electrical resistance (TER) and complexity of the TJs increase from the proximal tubule along the nephron to the distal tubule. The specific barrier and permeability characteristics of the proximal tubule epithelium are determined primarily by the functional state of the TJ complex that includes occludin, junctional adhesion molecule, zonula occludens-1 (ZO-1), and claudins (6, 42). Each claudin may have unique selectivity characteristics. An emerging model is that the fixed charges on the extracellular loops of claudins line aqueous pores and electrostatically influence the passage of soluble ions (14). Overexpression of claudins has been shown to both increase (27, 33, 47) and decrease (1, 18, 48) TER. In the proximal tubular epithelial cell line (LLC-PK1) used in this study, it has been shown that claudin-2, -15, and -16 selectively increase the permeability of Na+ through the TJs (23, 48), whereas the
knockdown of claudin-4 or -7 expression depressed the permeation of Cl\(^{-}\) (22, 48).

TJs are subject to physiological and pharmacological regulation and modulation (5, 19, 34). The MAPK family consists of a group of important intracellular mediators of signal transduction from cell surface to the nucleus, in response to various stimuli. ERK1/2 are connected to the regulation of growth and differentiation (9, 40). Several studies implicate a role for the ERK1/2 pathway in paracellular permeability but findings are conflicting. Wang et al. (49) demonstrated that activation of PKC caused the disruption of TJs through activation of MAPK in human corneal epithelial cells. Chen et al. (10) reported that inhibition of the MAPK pathway by MEK inhibitor, PD98059, resulted in the recruitment of occludin, claudin-1, and ZO-1 to the membrane of Ras-transformed Madin-Darby canine kidney (MDCK) cells, with a corresponding increase in TER. However, Lipschutz et al. (32) demonstrated that after hepatocyte growth factor treatment (HGF), ERK1/2 activation decreased claudin-2 expression in MDCK II and increased TER. This was blocked by the MEK inhibitor, U0126. Woo et al. (50) found that the dexamethasone-induced increase in TER across Can8 mammary cells was reduced by PD98059.

We previously showed that CsA alters the barrier function of MDCK cells and the mechanism may involve activation of ERK1/2 pathway (29). Further studies demonstrated that this CsA-induced modulation of paracellular permeability may be, at least in part, mediated by an increase in transforming growth factor-β (TGF-β) production through ERK1/2 activation (16). The present study was designed to test the hypothesis that SRL does not have a protective role in the CsA-induced effect in paracellular permeability and that the enhanced nephrotoxicity of CsA/SRL combination may be related to changes in epithelial barrier function. In this study, the effect of SRL, CsA alone, and CsA/SRL in combination on the integrity of the cellular TJs complex in renal proximal tubular cells was assessed through the measurement of TER and the expression of claudin-1, -2, and -4. The role of the ERK1/2 pathway in the CsA/SRL-induced modulation of barrier function was also investigated. Some of these findings have been previously presented at the ASN 2008 annual meeting and published in abstract form (Martin N et al., J Am Soc Nephrol PO141).

**MATERIALS AND METHODS**

**Cell culture.** The renal epithelial cell line LLC-PK\(_1\) (from pig proximal tubule) (26) was obtained from the European Collection of Animal Cell Cultures (Wiltshire, UK). Cells were cultured in Medium 199 (GIBCO) supplemented with 10% heat-inactivated fetal calf serum and penicillin/streptomycin (GIBCO). The culture was maintained at 37°C in a humidified incubator gassed with 5% CO\(_2\) and 95% air. The cells were passaged on a weekly basis (with a split ratio of 1:5).

**Cell treatment.** CsA and SRL were prepared as stock solutions (4.2 and 1 mM, respectively) in 100% EtOH. U0126 was prepared as a stock solution of 10 mM in DMSO. Cells were preincubated for 1 h with 10 μM U0126 before treatment with 4.2 μM CsA or 1 μM SRL either alone or in combination.

**TER.** LLC-PK\(_1\) cells were grown on 24-Well HTS-Transwell cell culture plates, which consisted of tissue culture-treated polycarbonate membranes with a pore size of 0.4 μm and an effective growth area of 0.33 cm\(^2\). The upper chamber (apical side) contained 0.25 ml medium and the lower one contained 1 ml medium. LLC-PK\(_1\) cells were seeded at a density of 2.5 × 10\(^5\) cells/well and the cells were seen to reach a stable TER representing a confluent monolayer 4 days postseeding. Confluent monolayers of cells were treated with various treatments on the apical and on the basolateral side of the filter. The point of addition of the drug was taken as time 0 and TER across the monolayers was monitored over a range of times under sterile conditions in a laminar flow hood using the REMS automated system (World Precision Instruments).

**Ion effects on TER.** To examine the effect of Na\(^+\) and Cl\(^{-}\) ions in TER, TER measurements were performed in P1 buffer containing 140 mM NaCl, 2 mM CaCl\(_2\), 1 mM MgCl\(_2\), 10 mM glucose, 10 mM HEPES, pH 7.3. To examine the effect of Na\(^+\) and Cl\(^{-}\) ions on TER separately, some of the TER measurements were performed in the modified P1 buffer with 140 mM NaCl replaced by 140 mM arginine-HCl or 140 mM sodium aspartate. At neutral pH, arginine is positively charged while aspartate is negatively charged in the solution. HCl dissociates into H\(^+\) and Cl\(^{-}\) in the solution and H\(^+\) will combine with OH\(^-\) to form water at pH 7.3. TER was normalized to the area of the filter after removal of background resistance of a blank insert on which no cells were seeded and which contained only medium. TER was thus measured as ohms × cm\(^2\) (Ω·cm\(^2\)). The results were expressed as the change in TER with respect to time-matched control [ΔTER (Ω·cm\(^2\))]. Each experiment was carried out three times in duplicate. A poly-L-lysine-positive control was used for TER measurements at the end of each experiment to determine that cells were responding in a normal manner.

**Cellular viability.** Confluent monolayers were treated for appropriate periods of time. Monolayers were incubated with 0.1 mg/ml of Resazurin reagent at 37°C for 90 min. Fluorescence was read at 545 nm on a Wallac Victor-V multiwell plate reader. The amount of fluorescence detected is proportional to the percentage of viable cells.

**Immunofluorescence.** LLC-PK\(_1\) cells grown on 18-mm coverslips were treated with the appropriate treatment for 48 h. The cells were fixed by 4% paraformaldehyde for 20 min. The fixed cells were then permeabilized for 20 min in 0.2% BSA/PBS Triton X-100, 100 mM glycine in PBS. Cells were washed three times in PBS and then blocked in 0.2% BSA/PBS for 1 h. The cells were then incubated in a monoclonal mouse anti-claudin-4 (Zymed Laboratories) in 0.2% (wt/vol) BSA/PBS for 1 h and Alexa 488-conjugated anti-mouse IgG antibody in 0.2% (wt/vol) BSA/PBS per well for 1 h. To stain the nuclei, cells were incubated with Dapi for 5 min. The whole process was carried out at room temperature. Coverslips were then mounted using the anti-quenching agent (Dako). The immunofluorescence was analyzed using a Zeiss Axioscan II epifluorescent microscope attached to a digital camera.

**Intracellular flow cytometry.** LLC-PK\(_1\) cells grown to 100% confluency on a six-well tissue culture plate were treated with the appropriate treatment for 48 h. Cells were then washed twice with ice-cold PBS and trypsinized. The cells were fixed overnight at 20°C with 70% ethanol added in a drop-wise manner. The cells were then washed twice in ice-cold PBS. The suspended cells were then divided into 10\(^6\) cell aliquots and then incubated with the primary antibody, rabbit anti-claudin-1 or mouse anti-claudin-4 on ice for 1 h. The cells were washed twice and the secondary antibody conjugated with FITC (Vector Labs, Burlingame, CA) was incubated on ice for another hour. After the cells were washed twice, the cells were analyzed with a Bectin-Dickson FacsCalibur flow cytometer. The staining profiles were recorded at the same FL1 PMT voltage.

**Preparation of Triton X-100-soluble and -insoluble fractions for TJs protein analysis.** This method has been adapted and modified from Yoo et al. (51). LLC-PK\(_1\) cells grown on a six-well tissue culture plate were washed twice with ice-cold PBS and then scraped in a Triton X-100 lysis buffer (1% Triton X-100, 20 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 1 mM Na\(_2\)VO\(_4\), 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 20 mM sodium pyrophosphate, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, 1.9 μg/ml aprotinin) and incubated at 4°C for 30 min, followed by centrifugation at 10,000 g for 20 min. The resulting supernatant was considered the Triton X-100/detergent-
soluble fraction. The remaining pellet was resuspended in SDS solubilization buffer (1% SDS, 20 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 1 mM sodium orthovanadate (Na3VO4), 1 mM PMSF, 50 mM NaF, 20 mM sodium pyrophosphate, 1 μg/ml pepstatin, 0.5 μg/ml leupeptin, 1.9 μg/ml aprotinin) and briefly sonicated. After sonication, the suspension was incubated at 4°C for 30 min, followed by centrifugation at 10,000 g for 20 min. The supernatant was considered the Triton X-100/detergent-insoluble fraction. Protein concentrations were determined by a BCA protein assay kit (Pierce) according to the manufacturer’s instructions and an equal amount of protein was analyzed.

Preparation of whole extracts for phosphorylated and whole cell MAPK protein analysis. Following the appropriate treatment, LLC-PK1 cells grown on a six-well tissue culture plate were washed twice with ice-cold PBS and then scraped in a RIPA buffer (90% RIPA buffer, 10% of 1 mM Na3VO4, 1% of 1 mM PMSF, 20% proteinase inhibitor cocktail) and incubated at 4°C for 30 min, followed by centrifugation at 12,000 g for 7 min. The resulting supernatant was stored in aliquots at −20°C until required for immunoblotting. Protein concentrations were determined by a BCA protein assay kit (Pierce) according to the manufacturer’s instructions and equal amounts were analyzed.

SDS-PAGE and Western blotting. Equal amounts of cell extracts were electrophoresed on SDS-polyacrylamide gels and proteins were transferred to nitrocellulose membrane. To ensure equal loading, membranes were stained with Ponceau-S. Membranes were blocked with 5% (wt/vol) milk proteins/Tris-buffered saline (TBS) and incubated overnight at 4°C with the primary antibody: rabbit anti-claudin-1, mouse anti-claudin-4, rabbit anti-claudin-2, rabbit anti-phospho-ERK1/2, or rabbit anti-ERK1/2. Bound antibody was detected with P1 buffer (140 mM NaCl). Similarly, the TER with P1 buffer across LLC-PK1 monolayers was found to be 116 ± 2.3 Ω·cm2. Treatment with subcytotoxic concentration of CsA (4.2 μM) or SRL (1 μM) decreased the paracellular permeability of LLC-PK1 cell monolayers, which was detected as an increase in TER up to 72 h (Fig. 1, A–B). CsA treatment effect on TER at 48 h (ΔTER: 163.5 Ω·cm2) was greater than that of SRL (ΔTER: 40.4 Ω·cm2). CsA/SRL cotreatment increased TER at 48 and 72 h in a synergistic manner (ΔTER: 306.6 and 414.6 Ω·cm2, respectively; Fig. 1C). Figure 1D shows the comparison between the different treatments. These effects in TER were not due to any effect on viability of the cells as can be seen in Fig. 1E. The concentrations of CsA and SRL used were chosen to approximate to the concentrations used in vivo and reflect concentrations in kidney but not to exert any gross cytotoxic effects in the renal cells.

Effect of CsA, SRL, and CsA/SRL cotreatment on TER in modified P1 buffer across LLC-PK1 monolayers. The increase in TER caused by CsA and/or SRL may reflect a decrease in Na+ or Cl− conductance (or both), since they are the major ions conducting the current in the culture medium. After 48-h treatment with CsA or CsA/SRL, the solution was replaced with P1 buffer (140 mM NaCl). Similarly, the TER with P1 was increased in CsA and further increased in CsA/SRL.

RESULTS

CsA, SRL, and the CsA/SRL cotreatment increased TER across LLC-PK1 monolayers. The TER of confluent control LLC-PK1 monolayers was found to be 116 ± 2.3 Ω·cm². Treatment with subcytotoxic concentration of CsA (4.2 μM) or SRL (1 μM) decreased the paracellular permeability of LLC-PK1 cell monolayers, which was detected as an increase in TER up to 72 h (Fig. 1, A–B). CsA treatment effect on TER at 48 h (ΔTER: 163.5 Ω·cm²) was greater than that of SRL (ΔTER: 40.4 Ω·cm²). CsA/SRL cotreatment increased TER at 48 and 72 h in a synergistic manner (ΔTER: 306.6 and 414.6 Ω·cm², respectively; Fig. 1C). Figure 1D shows the comparison between the different treatments. These effects in TER were not due to any effect on viability of the cells as can be seen in Fig. 1E. The concentrations of CsA and SRL used were chosen to approximate to the concentrations used in vivo and reflect concentrations in kidney but not to exert any gross cytotoxic effects in the renal cells.

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treatment after 48 h (Fig. 2). To determine the resistance to each ion separately, P1 buffer was then replaced with 140 mM arginine-HCl buffer to eliminate the contribution of Na⁺ ions or with 140 mM sodium aspartate to eliminate the contribution of Cl⁻ ions. Either in the presence of Cl⁻ without Na⁺ or in the presence of Na⁺ without Cl⁻, the increase in TER followed the same trend compared with that of P1, demonstrating that CsA and CsA/SRL treatment reduced the conductance of both Cl⁻ and Na⁺ ions across the epithelial cell monolayer (Fig. 2).

**Effect of CsA, SRL, and CsA/SRL on the localization of claudin-4 in the TJ complex and cell monolayer integrity in LLC-PK₁ cells.** As shown in Fig. 3, claudin-4 was normally distributed in control cells. No disruption of the cell monolayer or holes between cells could be detected in any treatment group. Following 48-h treatment with 4.2 μM CsA, 1 μM SRL, or CsA/SRL cotreatment, the fluorescent signal for claudin-4 tended to be more concentrated at the cell periphery than in control cells.

**CsA, SRL, and CsA/SRL cotreatment increased the expression of the TJ protein, claudin-1.** The effect of CsA, SRL, and CsA/SRL cotreatment on total cell expression of TJ proteins is shown in Fig. 4. Flow cytometry analysis showed that CsA (4.2 μM) and its combination with SRL (1 μM) increased the total cell claudin-1 after 48 h (Fig. 4, A and C). Although not significant, an increasing trend in the total cell expression of claudin-4 was observed after 48 h of CsA or CsA/SRL treatment (Fig. 4, B and D). Western blot analysis showed that CsA may increase claudin-1 distribution in the cytoskeleton-associated fraction (detergent-insoluble fraction) after 24 and 48 h of treatment (Fig. 5A). The treatment with either SRL alone or in combination with CsA may also increase claudin-1 localization in both the cytoplasmic and cytoskeleton-associated fraction (soluble and insoluble fractions). A trend for increase in claudin-4 was observed in both the soluble and insoluble fractions after 48 h of CsA, SRL, and combined CsA/SRL treatment (Fig. 5B). The levels of claudin-1 and -4 in the insoluble fraction appeared to be further increased when both treatments were applied in combination. Coomassie Brilliant Blue staining was used as sample loading control (Fig. 5C).

CsA, SRL, and CsA/SRL cotreatment stimulated ERK1/2 activation in LLC-PK₁ cells. Treatment of LLC-PK₁ cells with 4.2 μM CsA, 1 μM SRL, and their combination resulted in significant activation of ERK1/2 signaling at 24 and 48 h (Fig. 6). There was much greater activation of ERK1/2 with the combined treatment of CsA/SRL compared with either CsA or SRL alone. The MEK inhibitor, U0126 (10 μM), reduced basal activation of ERK1/2 at 48 h and also prevented the CsA, SRL, and the CsA/SRL-induced activation of ERK1/2 at 24 and 48 h (Fig. 7).

**Inhibition of ERK1/2 signaling pathway reduced the CsA, SRL, and CsA/SRL-induced increase in TER across LLC-PK₁ monolayers.** Cotreatment of LLC-PK₁ cells with the MEK inhibitor U0126 (10 μM) plus 4.2 μM CsA significantly reduced the CsA-induced increase in TER which was observed when cells were treated with CsA alone up to 48 h (Fig. 8A). Cotreatment of LLC-PK₁ cells with 10 μM U0126 plus 1 μM SRL significantly reduced the SRL-induced increase in TER which was observed when cells were treated with SRL alone at 48 h (Fig. 8B). Cotreatment of the LLC-PK₁ cells with 10 μM U0126 plus 4.2 μM CsA/1 μM SRL significantly reduced the CsA/SRL-induced increase in TER which was observed when cells were treated with CsA/SRL alone at 24 and 48 h (Fig. 8C). These changes were not accounted for by any significant alterations in cellular viability due to CsA, SRL, and CsA/SRL cotreatment combined with U0126 as demonstrated by the results in Fig. 8D.
In this study, we demonstrated for the first time that SRL can have direct adverse effects on the barrier function of renal proximal tubular cells. Furthermore, we demonstrated that the SRL effects are similar to those of CsA and the combination of SRL and CsA is synergistic in producing alterations in barrier permeability of renal proximal tubular cells. We previously showed that CsA treatment decreases paracellular permeability in MDCK cells (29) and here, we show similar effects in LLC-PK₁ cells. However, the effects of SRL on renal epithelial barrier function had not been previously investigated.

Regulation of cellular barrier permeability is a vital and complex process involving intracellular signaling and rearrangement of TJ proteins that influence the passage of soluble ions. Our data show that SRL and CsA alone and their combination induced increases in TER with rearrangement of TJ proteins, especially claudin-1, which may explain some of the nephrotoxic changes observed after CsA/SRL treatment. Moreover, this study shows how these treatments are able to enhance barrier function that could explain the enhancement of CsA nephrotoxicity observed clinically. Only few substances like TGF-β₁ (16, 24), EGF (7, 44), HGF (32), IL-7(30), or glucocorticoids (50) have been demonstrated to be able to enhance barrier function, whereas that a wide range of substances have been shown to perturb it.

These effects of SRL, CsA, and the combined CsA/SRL resulted from direct effects of the drugs on the tubular cells. In the in vivo setting, it had been suggested that the enhanced toxicity of a CsA/SRL combination may have been due to pharmacokinetic interactions. Napoli et al. (36) demonstrated that a pharmacokinetic interaction between SRL and CsA increased the concentration of both agents in whole blood and especially in renal tissue of rats. Angelicheau et al. (4) studied the role of P-glycoprotein (Pgp) in the CsA/SRL interaction in vitro. They found that the increased CsA nephrotoxicity in human renal epithelial cells may be due to inhibition of Pgp-mediated CsA cellular efflux by SRL. Here, we demonstrated direct effects of SRL on the epithelial function of renal proximal tubular cells. The CsA/SRL cotreatment produced a synergistic effect on TER. As the TER inversely reflects the conductance of ions and small molecules through the TJs, CsA and SRL treatment resulted in a better sealing or tightening of the TJs between the cells and a decrease in the paracellular permeability. While the synergistic effects of the combined CsA/SRL may have been contributed to by increased CsA intracellular levels in the presence of SRL, alternative explanations are more likely. The fact that SRL on its own, in the

DISCUSSION

Inhibition of ERK1/2 signaling pathway prevented the CsA, SRL, and CsA/SRL-induced modulation of TJ proteins claudin-1 and -2 in LLC-PK₁ cells. Western blot analysis showed that CsA, SRL, and their combined treatment increased claudin-1 localization in the insoluble fraction after 48 h of treatment (Fig. 9A). U0126 prevented the CsA, SRL, and CsA/SRL-induced increase of claudin-1 in the insoluble fraction (Fig. 9A, right, and D, dark gray bars). After 48 h of CsA, SRL, or combined treatment, a trend was observed for the increased claudin-4 localization in the insoluble fraction (Fig. 9B). However, ERK inhibition with U0126 did not prevent this trend in the distribution of claudin-4; however, in contrast to claudin-1, a trend was shown for increased localization of claudin-4 in the insoluble fraction (Fig. 9A, right, and E, dark gray bars). Claudin-2 distribution was also examined and found to be decreased in the insoluble fraction in cells treated either with CsA or CsA/SRL cotreatment compared with control at 48 h (Fig. 9C). Inhibition of ERK1/2 activation induced an increase of claudin-2 in the insoluble fraction in all the treatment groups (Fig. 9C, right, and F, dark gray bars).
absence of CsA, increased TER clearly indicates that SRL exerts effects without requirement for CsA. Furthermore, the fact that the combined treatment of CsA and SRL showed a synergistic effect rather than a simply additive effect would also support the hypothesis that the effect with the combined treatment was not just due to increased CsA in the cells. We did carry out additional experiments with increasing concentrations of CsA and SRL to test the effects of increased CsA. However, these experiments were complicated by the fact that increasing cytotoxicity was detected with increasing CsA and SRL concentrations. The concentrations used in the present study were chosen to investigate effects of CsA and SRL without significant cytotoxicity.

Here, we demonstrated that CsA and CsA/SRL reduced the conductance of both Na⁺ and Cl⁻ ions across the epithelial layer. These two drugs have been shown to produce hypomagnesemia associated with inappropriately high fractional excretion of magnesium (2). These authors suggested that the mechanism of hypomagnesemia and tubular injury induced by CsA or SRL was unlikely to be dependent on calcineurin as they compared these two drugs with another calcineurin inhibitor, tacrolimus, which also produced hypomagnesemia. Furthermore, CsA nephrotoxicity results in induced hypertension associated with sodium and water retention (11). As TER inversely reflects the ion conductance through the TJ (paracellular pathway), the alteration observed after CsA or CsA/SRL treatments highlights ion transport disturbance in this model in vitro. If similar tightness mechanisms in the barrier function occur in the kidney in vivo, it might explain some of the ion disturbances after CsA and SRL treatment.

We demonstrate that this CsA or CsA/SRL modulation of the barrier function was not due to an increase in the cell volume as demonstrated by immunofluorescence of cells. Some investigators reported that increases in TER could also be generated by a decrease in cell number, as cell density increases; there is an increase in intracellular space per unit area of monolayer (43). This is a controversial topic, since other investigators reported that a rise in cell number could generate an increase in TER (44). Here, in this study we showed by immunofluorescence similar junctional pattern and cell density under all treatments. Also, we showed that none of the treatments affected cell viability. Therefore, it is highly unlikely that alterations in cell number or size were responsible for the alteration in TER in these experiments.

Our initial results indicated that a possible mechanism by which CsA and SRL influence TJs function could be by
increased total cellular expression and altered localization of TJs proteins, claudin-1 and claudin-4. It is clear that there was an increase in the total cell expression of claudin-1 which was statistically significant as estimated by flow cytometry. Within the cells, the increases in claudin-1 and claudin-4 were primarily localized to the detergent-insoluble fraction after CsA or SRL treatment. The levels of these proteins were further increased when both treatments were applied in combination.

Fig. 7. Effect of ERK1/2 inhibition on CsA, SRL, or CsA/SRL cotreatment-induced ERK1/2 phosphorylation in LLC-PK1 cells. LLC-PK1 cells were grown to confluency on 6-well plates and pretreated for 1 h with 10 μM U0126, before treatment with 4.2 μM CsA or 1 μM SRL, either alone or in combination for 24 and 48 h. Whole cell lysates were then made using RIPA lysis buffer. Equal amounts of cellular proteins were separated electrophoretically on SDS-polyacrylamide gel and transferred to nitrocellulose membrane. Expression of phospho-ERK1/2 (A) was identified using a polyclonal rabbit anti-phospho-ERK1/2 and an anti-rabbit HRP-conjugated secondary antibody. The nitrocellulose was then stripped of the antibody and expression of whole cell ERK1/2 (B) was identified using a polyclonal antibody and an anti-rabbit HRP-conjugated secondary antibody. C and D: ratio of phospho-ERK/whole ERK band density at 24 and 48 h of blots from 3 independent experiments. ANOVA and multiple comparisons between different treatments were made using the Bonferroni posttest. Alternatively, the paired Student’s t-test was used to test for statistical significance between control and a treatment group. Results were expressed as means ± SE of 3 experiments. A probability of 0.05 or less was deemed statistically significant between different treatments (\#P < 0.05, ##P < 0.01, and ###P < 0.001) or time-matched control where indicated (*P < 0.05, **P < 0.01, and ***P < 0.001).

Fig. 8. Effect of the MEK1 inhibitor, U0126 (U0), on the CsA, SRL, and CsA/SRL-induced increase in TER across LLC-PK1 cell monolayers. LLC-PK1 cells were grown on 24-Costar Transwell filters for 4 days. Cells were then pretreated for 1 h with 10 μM U0126 before the treatment with 4.2 μM CsA (A), 1 μM SRL (B), or CsA/SRL (C) up to 48 h. Results are expressed as ΔTER compared with time-matched control cells and are given as means ± SE of 5 independent experiments, each performed in duplicate. Paired Student’s t-test was used to test for statistical significance between control and treatment groups. ANOVA was carried out and multiple comparisons between control and treatment groups were made using the Bonferroni posttest. A probability of 0.05 or less was deemed statistically significant (*P < 0.05, **P < 0.01, ***P < 0.001) compared with other treatment groups. D: cellular viability was measured at 48 h using the resazurin cell viability assay. Results are expressed as % control (100%) and are given as means ± SE of 3 independent experiments, each performed in triplicate. Paired Student’s t-test was used to test for statistical significance between control and treatment groups.
The levels of these proteins in the insoluble fractions would be the most relevant to any alterations in the TJs and corresponding changes in TER. Claudin-1 and -4 are related to the tightness of the epithelium in renal epithelial cells. Therefore, these results suggest that CsA and SRL are capable of modulating the kidney epithelial barrier via alterations in TJs protein expression and this correlates with the observed CsA and CsA/SRL-induced increase in TER. These findings are consistent with previous findings in other models relating to an increase in TER. Inai et al. (27) demonstrated that claudin-1-expressing MDCK cells exhibited approximately four times higher TER than wild-type MDCK cells. Sonoda et al. (45) reported that MDCK I cells incubated with a claudin-4-binding peptide (Clostridium perfringens enterotoxin) caused the removal of claudin-4 from the TJs. This resulted in a significant decrease in the TJ barrier as measured by TER and paracellular flux. In MDCK II cells, overexpression of claudin-4 has also been shown to increase TER (47). Kiuchi-Saishin et al. (31) described segmental distribution of various claudins in the mouse nephron showing that claudin-3 and -4 are expressed mainly in the “tight” epithelia of the kidney and that only claudin-1, -3, -4, and -8 are expressed in the collecting segment of the kidney nephron, the segment with the highest TER. Different studies described the role of the claudins in forming charge-selective channels in the paracellular pathway. Colegio et al. (14) demonstrated in MDCK cells that substitutions of negative for positive charges in claudin-4 increased the paracellular permeability of Na$^+$, defining claudin-4 as a Na$^+$ barrier. Claudin-2 has been defined as a cation pore. When claudin-2 was overexpressed in LLC-PK1 cells, the permeability for Na$^+$ was increased (48). In our cells, claudin-2 tended to decrease as the TER increased after CsA or CsA/SRL treatment. We are not aware of any studies defining claudin-1 either as a cation or an anion barrier. However, our studies demonstrated that claudin-1 might be acting as Na$^+$ or Cl$^-$ barrier and claudin-2 as a cation pore as has been previously demonstrated (48, 52).

Many different signal transduction pathways are involved in the regulation of TJs function and assembly. A recent review...
by Gonzalez-Mariscal et al. (20) compiles the different signaling pathways involved in TJ assembly and disassembly and reviews the different controversial reports about ERK1/2 signaling and TJ modulation. We previously showed that CsA modulates epithelial barrier function in MDCK cells by the ERK1/2 pathway (29). In LLC-PK1 cells, Mullin et al. (35) demonstrated that activation of ERK1/2 by transfection with an activated Ras mutant increased TER. This increase in TER was correlated with an increase in claudin-1, -4, and -7 and a decrease in claudin-2 levels. However, this activation of Ras increased also the permeability to relatively large and uncharged molecules (mannitol), whereas decreased the permeability to small charged solutes (ions). Another study by Kinugasa et al. (30) demonstrated that ERK-mediated mechanisms are required to assemble functional TJs in intestinal epithelial cells correlated with upregulation of claudin-1 and -2 gene expression.

Here, we demonstrate that SRL and CsA treatment alone or in combination activated the ERK1/2 pathway at 24 and 48 h in LLC-PK1 cells. It was clearly shown that SRL on its own activated ERK1/2 and further that the combination of SRL and CsA showed much greater activation of ERK1/2 than either drug alone. The inhibition of ERK1/2 by U0126 significantly reduced the SRL, CsA, and the SRL/CsA-induced increases in TER. This provides clear evidence that the SRL, CsA, and SRL/CsA treatment-induced increases in TER involve the ERK1/2 signaling pathway. To our knowledge, this is the first evidence that SRL can result in activation of ERK1/2 in kidney tubular epithelial cells. However, there have been a number of publications indicating cross talk or pathway interactions between MAPKs and mTOR (25, 39). A possible cross talk of relevance to our findings may involve protein tyrosine phosphatases. It has been shown in cortico-striatal slices and primary neuronal cultures that SRL completely blocked the translation of a striatal-enriched protein tyrosine phosphatase (STEP). As one of the substrates of STEP includes ERK itself, such inhibition of STEP translation would result in reduction of dephosphorylation of ERK (25). Work from the same laboratory also showed that STEP was phosphorylated and inactive under basal conditions (39). Activation of calcineurin led to dephosphorylation and activation of STEP with resultant dephosphorylation of ERK. If similar tyrosine phosphatases and signaling cross talk exist in kidney cells, these may be involved in the ERK1/2 activation seen in our experiments with SRL, CsA, and the combination of CsA/SRL.

With the establishment that ERK1/2 activation played a key role in regulating SRL, CsA and CsA/SRL induced barrier alterations as indicated by TER, the effect of U0126 on the TJ components was examined. Inhibiting ERK1/2 activation prevented the SRL, CsA, and CsA/SRL-induced increase of claudin-1 in the cytoskeleton-associated fractions. This clearly indicates an important role for claudin-1 in the SRL, CsA, and CsA/SRL-induced alterations in renal epithelial cells. The effects of ERK1/2 inhibition on claudin-4 were somewhat different in that inhibition of ERK1/2 did not prevent the increase in claudin-4. As inhibition of ERK1/2 prevented the increase in TER, the clear implication is that alterations in claudin-4 do not seem to be critical in the SRL, CsA, and CsA/SRL-induced changes in renal proximal tubular cells. The effect of U0126 on the localization of claudin-1 in the insoluble fraction coupled with the effect of U0126 on preventing the increase in TER clearly points to a mechanism of CsA and SRL involving the localization of claudin-1 in the insoluble fraction.

Inhibition of ERK1/2 activation after SRL, CsA, or CsA/SRL treatment also produced an increase of claudin-2 localization in the insoluble fractions. The exposure to CsA and CsA/SRL in the absence of ERK1/2 inhibition caused a downregulation in claudin-2 expression. Claudin-2 has been described as an anion pore and it is related to the leakiness of the epithelium (1, 15, 48). Furuse et al. (18) reported that MDCK I cells with a higher TER resistance than MDCK II lack claudin-2. When claudin-2 cDNA was introduced into MDCK I cells, the TER values of MDCK I expressing claudin-2 fell to the levels of MDCK II. Our data show that ERK1/2 negatively regulates claudin-2 expression. This is consistent with previous studies by Lipschutz et al. (32) in MDCK II cells where ERK1/2 activation decreased claudin-2 expression and transiently increased TER after HGF treatment. It would seem therefore that downregulation of claudin-2 was also involved in the SRL, CsA, and CsA/SRL-induced alterations in TER.

In conclusion, our study showed that CsA and CsA/SRL treatment caused a tightening of the paracellular pathway as evidenced by an increase in the TER across tubular epithelial cell monolayers. If similar effects occur systemically in vivo, with these two immunosuppressive drugs, the enhancement of barrier function in areas of the body may decrease luminal antigen presentation, decreasing immune activation and helping the immunosuppressive action. The fact that the inhibition of the activation of ERK1/2 after CsA and CsA/SRL cotreatment restored TER levels and altered the localization and/or expression of claudin-1 and -2 strongly suggests that the ERK1/2 signaling pathway is involved in TJs modulation after the treatment with these immunosuppressive drugs. These results including the finding that SRL alone can have some direct effect on renal proximal tubular cells and the synergistic effects of CsA/SRL combination on the epithelial function as assessed by TER and claudin expression and localization provide novel insights in the enhanced nephrotoxicity of CsA/SRL combination.

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DISCLOSURES
No conflicts of interest are declared by the authors.

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