Cyclosporine stimulates the renal epithelial sodium channel by elevating cholesterol

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1Division of Nephrology, Department of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; 2Departments of Pharmacy and Cardiology, the 2nd Affiliated Hospital of Harbin Medical University, Harbin, People’s Republic of China; and 3Department of Physiology, University of Birmingham, The Medical School, Edgbaston, United Kingdom

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Wang J, Zhang Z, Chou C, Liang Y, Gu Y, Ma H. Cyclosporine stimulates the renal epithelial sodium channel by elevating cholesterol. Am J Physiol Renal Physiol 296: F284–F290, 2009. First published December 17, 2008; doi:10.1152/ajprenal.90647.2008.—Cyclosporine A (CsA) is an efficient immunosuppressant used for reducing allograft rejection but with a severe side effect of causing hypertension. We hypothesize that the renal epithelial sodium channel (ENaC) may participate in CsA-induced hypertension. In the present study, we used the patch-clamp cell-attached configuration to examine whether and how CsA stimulates ENaC in A6 distal nephron cells. The data showed that CsA significantly increased ENaC open probability. Since CsA is an inhibitor of the ATP-binding cassette A1 (ABCA1) transporter, we employed 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), another ABCA1 inhibitor, and found that DIDS mimicked the effects of CsA on ENaC basal and cholesterol-induced activity but without any additive effect if combined with CsA. CsA and DIDS also had an identical effect on reduced ENaC activity caused by cholesterol extraction. ABCA1 protein was detected in A6 cells by Western blot analysis. Confocal microscopy data showed that both CsA and DIDS facilitated A6 cells to uptake cholesterol. Since enhanced ENaC activity is known to cause hypertension, these data together suggest that CsA may cause hypertension by stimulating ENaC through a pathway associated with inhibition of ABCA1 and consequent elevation of cholesterol in the cells.

patch-clamp technique; confocal microscopy; cyclosporine A; ATP-binding cassette transporter A1; hypertension

The pot ent immunosuppressant cyclosporine A (CsA) leads to a dramatic improvement in the clinical outcomes after organ transplantation. However, introduction of CsA is associated with a significantly increased incidence of posttransplant hypertension that is irrespective of the transplanted organ (36) because hypertension also occurs in patients who have been treated with CsA for immunological problems (16). Previous studies that used animal models suggest that increased activity of sympathetic nerve contributes to the cyclosporine-induced hypertension (CIH) (27). However, studies on humans with administration of CsA show no change in sympathetic activity (15). It has been suggested that impaired vasodilation secondary to reduction in nitric oxide may be involved in CIH through inhibition of the endothelial nitric oxide synthase (eNOS) (17, 41). However, inconsistent results also exist, elucidating that CsA enhances rather than reduces nitric oxide synthesis in cultured endothelial cells (24) and that CsA has no effect on the expression of either eNOS or the inducible nitric oxide synthase in kidney, aorta, or heart (18). These inconsistent results inspire us to hypothesize that CsA may induce hypertension not only by causing defective vasodilation but also by targeting other organs.

The cytotoxicity of CsA to the kidney has been widely accepted. It has been suggested that CIH is associated with an early, subtle, renal defect in sodium excretion (10, 11). Several lines of evidence indicate that sodium retention may exist in CsA-treated dogs (8), rats (14), and healthy subjects (9). Therefore, the enhanced sodium reabsorption may contribute to the early stage of CIH. It is well known that the epithelial sodium channel (ENaC) plays an important role in regulating sodium reabsorption. However, it remains unknown whether CsA regulates ENaC in the cortical collecting ducts. Elevated ENaC activity caused by gain-of-function mutations in Liddle’s syndrome leads to severe volume-expanded hypertension (34). These defects illustrate the key role of ENaC in maintaining extracellular volume and blood pressure within a normal range (29). Clinical trials demonstrate successful improvement on control of blood pressure by decreasing ENaC activity in patients with salt-sensitive hypertension (31, 35). In vitro studies by measuring the short-circuit current suggest that ENaC in the isolated skin of Rana esculenta may be a target of CsA (22). Therefore, it is very likely that CsA may cause hypertension, at least in part, by stimulating ENaC function.

In the present study, by performing single-channel recordings from distal nephron cells, we show that CsA stimulates ENaC through a pathway associated with elevation of either membrane or intracellular cholesterol, probably due to inhibition of the ATP-binding cassette transporter (ABCA1), a flop-pase responsible for cholesterol outward transport (5, 32).

MATERIALS AND METHODS

Cell culture. The A6 cell line, which is originated from the distal nephron of Xenopus laevis, was purchased from American Type Culture Collection (Rockville, MD). Cells were cultured in plastic flasks in a modified DMEM/F12 media containing 100 mM NaCl, 20 mM NaHCO3, 60 U/ml penicillin, 60 U/ml streptomycin, 2 mM L-glutamine, 10% fetal bovine serum (Invitrogen, Carlsbad, CA), and 1 mM aldosterone (Sigma-Aldrich, St. Louis, MO) at 26°C and 4% CO2. Cells were removed from the flasks and plated on polyester membrane attached to Snapwell inserts (Corning Costar, Pittsburgh, PA) for 10–14 days to fully polarize before experiments.

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Patch-clamp single-channel recordings. Cell-attached recordings were performed using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA), as we recently described (43). Briefly, before the experiments, the Snapwell inserts were thoroughly washed with NaCl solution containing (in mM) 100 NaCl, 3.4 KCl, 1 CaCl2, 1 MgCl2, and 10 HEPES, adjusted pH to 7.4 with NaOH. The cell-attached configuration was established on the apical membrane of A6 cells with a glass micropipette, which was filled with NaCl solution (the pipette resistance is ~5 MΩ). Single-channel currents were obtained with zero applied pipette potential, filtered at 1 kHz, and sampled every 50 μs with pClampex 8.0 software. Experiments were conducted at 22–23°C. Signals were recorded for at least 10 min in control A6 cells and the cells treated with CsA and 4,4'-disothiocyanostilbene-2,2'-disulfonic acid (DIDS) for 1 h. Cholesterol and methyl-β-cyclodextrin (MβCD) were acutely applied to the basolateral bath. All these chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The total numbers of functional channels in the patch were estimated by observing the number of peaks detected on the current amplitude histogram during at least 10-min recording period. The open probability (P0) of ENaC was calculated, as we previously described (43).

Western blotting. A6 cells were cultured as described above. Cell lysate was loaded and electrophoresed on 10% SDS-PAGE gels for 60–90 min. Gels were blotted onto polyvinylidene fluoride (PVDF) membranes for 1.5 h at 50 V. After 1 h of blocking with 5% BSA-PBST buffer, PVDF membranes were incubated with primary antibody (1:2,000 dilution) of mouse anti-ABCA1 monoclonal antibody (Novus Biologicals, Inc) overnight at 4°C and then incubated with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG secondary antibody (1:10,000 dilution, GE Healthcare) for 1 h after four vigorous washes. Blots were developed by chemiluminescence using ECL Plus Western Blotting Detection System (GE Healthcare).

Confocal microscopy images. For testing the response of cells to the challenge by exogenous cholesterol, a fluorescent cholesterol analog, NBD cholesterol (Avanti Polar Lipids, Alabaster, AL) was used as we recently reported (43). The cells either in control conditions or after pretreatment with CsA or DIDS for 1 h were incubated with the apical membrane bathed in NaCl solution alone and the basolateral membrane bathed in NaCl solution containing 100 μg/ml NBD cholesterol for 10 min at room temperature. After thoroughly rinsing, the polyester membrane was excised and mounted on the glass slide. The fluorescent intensity in each experimental condition was respectively examined through a Leica confocal microscopy.

Statistical analysis. Data are reported as mean values ± SE. Statistical analysis was performed with SigmaPlot and SigmaStat software (Aspire Software, Ashburn, VA). One-way ANOVA and Student’s t-tests were used between different groups. Paired t-tests were used between two time periods before and after acute application of either cholesterol or MβCD with the same patch as a control. Results were considered significant if P < 0.01.

RESULTS

CsA stimulates ENaC in A6 distal nephron cells. To examine whether CsA affects ENaC at the single-channel level, we employed the patch-clamp technique. The cell-attached patches were formed on the apical membrane of A6 distal nephron cells cultured on filters. Since in the patients on CsA treatment CsA is delivered to the renal epithelial cells from the blood, to mimic the way of in vivo CsA delivery we applied CsA to the basolateral side of A6 cell monolayer. During the period from 12 to 15 min after application of 10 μM CsA to the basolateral bath, the mean ENaC P0 was increased from 0.14 ± 0.03 (control) to 0.23 ± 0.15 (12–15 min after CsA; Fig. 1A), but the increase was marginally significant (n = 11; P = 0.05). It seemed that the effect of CsA appeared gradually; it may take >15 min for CsA to reach its maximum effect. However, using the same patch as a control to evaluate ENaC activity in a recording period longer than 15 min is not very reliable because it has long been noticed that ENaC activity in a subset of cell-attached patches gradually declines, which is often referred to as “channel activity rundown.” Therefore, ENaC P0s were compared between two sets of cell-attached patches; one was from control A6 cells, whereas the other was

Fig. 1. Cyclosporine A (CsA) increases epithelial sodium channel (ENaC) open probability (P0) in A6 distal nephrons cells. A: representative ENaC single-channel currents recorded from two cell-attached patches; one shows that addition of 10 μM CsA to the basolateral bath stimulated ENaC (top), whereas the other shows that CsA had no effect on ENaC activity (bottom). The mean ENaC P0 during each 3 min period from 12 to 15 min for CsA to reach its maximum effect. However, using the same patch as a control to evaluate ENaC activity in a recording period longer than 15 min is not very reliable because it has long been noticed that ENaC activity in a subset of cell-attached patches gradually declines, which is often referred to as “channel activity rundown.” Therefore, ENaC P0s were compared between two sets of cell-attached patches; one was from control A6 cells, whereas the other was
from the cells treated with basolateral 10 µM CsA for 1 h. As shown in Fig. 1, B and C, ENaC Po was significantly increased in the cells treated with basolateral CsA for 1 h from 0.16 ± 0.04 (n = 9) to 0.40 ± 0.06 (n = 9; P < 0.01).

CsA stimulation of ENaC is mimicked by inhibition of ABCA1 with DIDS. Since CsA potently inhibits the ABCA1 transporter (19), we hypothesized that CsA may stimulate ENaC by inhibiting ABCA1. However, the ABCA1 in Xenopus laevis has not yet been cloned and sequenced. Therefore, this hypothesis cannot be tested by molecular knockdown of the ABCA1 transporter. Nevertheless, the experiment is feasible by using DIDS, another ABCA1 inhibitor (4). As we expected, hypothesis cannot be tested by molecular knockdown of the ABCA1 transporter. This is consistent with previous studies showing that the ABCA1 transporter is inhibited. These results provide a nice explanation for the contradictory results that extraction of cholesterol inhibits ENaC, but exogenous cholesterol is not very efficient in term of stimulating ENaC (44). If CsA stimulates ENaC by elevating cholesterol, CsA should also facilitate the possible effect of exogenous cholesterol on ENaC activity. Therefore, how exogenous cholesterol affects ENaC was determined in untreated A6 cells and the cells pretreated with CsA. Again, to test the possible involvement of the ABCA1 transporter, the experiments were also carried out in A6 cells pretreated with another ABCA1 inhibitor, DIDS. The data demonstrated that basolateral application of 100 µg/ml cholesterol had no acute effect on ENaC activity in untreated A6 cells with functional ABCA1 (Fig. 4A); ENaC Po remained unchanged: 0.13 ± 0.04 (before) vs. 0.13 ± 0.04 (5 min after cholesterol) (n = 7; P = 0.7). However, in the cells basolaterally pretreated with either 10 µM CsA or 100 µM DIDS, cholesterol at 5 min after addition to the basolateral bath significantly increased ENaC Po from 0.30 ± 0.08 to 0.47 ± 0.10 (n = 6; P < 0.01) and from 0.36 ± 0.07 to 0.61 ± 0.09 (n = 6; P < 0.01), respectively (Fig. 4, B and C). Combined with the confocal microscopy data showing that CsA and DIDS facilitate A6 cells to uptake exogenous cholesterol, these results suggest that CsA stimulates ENaC by elevating cholesterol through inhibition of the ABCA1 transporter. This is consistent with previous studies showing that the basolateral ABCA1 prevents cholesterol from freely entering into cells (33).

CsA and DIDS abolish inhibition of ENaC by methyl-β-cyclodextrin. methyl-β-cyclodextrin (MβCD) is a well known scavenger used to remove cholesterol from the cell membrane. To confirm the role of cholesterol in stimulating ENaC, we extracted membrane cholesterol by adding 20 mM MβCD to the basolateral bath. We found that extraction of cholesterol from the basolateral membrane significantly decreased ENaC Po from 0.26 ± 0.06 (before) to 0.07 ±

Fig. 2. 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) mimics the effect of CsA on ENaC Po. A: representative single-channel current of ENaC recorded under control conditions (top), after 1 h of treatment with 100 µM DIDS applied to the basolateral bath (middle), or after 1 h of treatment with both 100 µM DIDS and 10 µM CsA from basolateral membrane (bottom). B: summary plots show that ENaC Po was significantly increased after 100 µM DIDS treatment. Addition of CsA to DIDS did not further increase ENaC Po compared with DIDS alone. C: Western blotting analysis of A6 cell lysate detected by an antibody to mouse ABCA1 transporter. The two lanes represent two loadings: 25 µg (left) and 50 µg (right) of protein from the same sample showing consistent ABCA1 labeling in all three experiments.
0.02 (5 min after MβCD) in untreated A6 cells (Fig. 5A; \( n = 7 \); \( P < 0.01 \)). However, extraction of cholesterol lost its impact on ENaC when the ABCA1 transporter was inhibited by CsA or DIDS. ENaC \( P_O \) remained unchanged; 0.43 ± 0.07 (before) vs. 0.37 ± 0.05 (5 min after MβCD; \( n = 7 \); \( P = 0.1 \)) and 0.44 ± 0.08 (before) vs. 0.41 ± 0.07 (5 min after MβCD; \( n = 6 \); \( P = 0.1 \)) in the cells pretreated with either CsA and or DIDS, respectively (Fig. 5, B and C). These data indicate that CsA stimulates ENaC through a pathway closely associated with altered cholesterol in A6 cell membranes, as detailed in a hypothetical model proposed in DISCUSSION.

**DISCUSSION**

The present study shows that the immunosuppressive drug CsA stimulates ENaC in distal nephron cells through a pathway associated with inhibition of the ABCA1 transporter and consequent elevation of membrane or intracellular cholesterol (see Fig. 6). We propose that the ABCA1 transporter is involved because we show that the effects of CsA on both ENaC activity and cholesterol homeostasis can be dramatically mimicked by another ABCA1 inhibitor DIDS. We also propose that cholesterol plays a critical role in the signal transduction pathway downstream of the ABCA1 transporter because we demonstrate that inhibition of ABCA1 allows cholesterol to stimulate and also abolishes the effect of cholesterol extraction on ENaC activity. These arguments are supported by previous studies showing that ABCA1 acts as a floppase to flop cholesterol from the inner leaflet to the outer leaflet of the bilayer, subsequently transfers cholesterol to ApoA-I (5, 32, 42) and that in polarized renal epithelial cells ABCA1 distributes in the basolateral membrane to defend the invasion of elevated cholesterol from the blood to march into the cells (28). Our studies clearly indicate that cholesterol mediates CsA stimulation of ENaC. However, the mechanism for cholesterol to stimulate ENaC remains unclear.

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**Fig. 3.** Confocal microscopy optical cross (XZ) section of A6 cell monolayer cultured on filters, basolaterally treated with either 10 \( \mu M \) CsA (A) or 100 \( \mu M \) DIDS (B) for 1 h and then incubated with 100 \( \mu g/ml \) NBD cholesterol for 10 min. Images on the left show that NBD cholesterol was hardly observed in untreated (control) A6 cells. Images on the right show that a significant amount of NBD cholesterol was found in A6 cells pretreated with either CsA or DIDS. These images represent three experiments showing consistent results. The exact same parameters were used when each section was scanned.

**Fig. 4.** A: Basolateral addition of 100 \( \mu g/ml \) cholesterol alone had no effect on ENaC activity. Cholesterol significantly enhanced ENaC activity after basolateral pretreatment with either 10 \( \mu M \) CsA (B) or 100 \( \mu M \) DIDS (C). Representative ENaC currents are shown at left, whereas summary plots are presented at right.
Previous studies, by measuring short-circuit current across A6 cell monolayer, have consistently shown that extraction of cholesterol from the basolateral membrane can significantly reduce amiloride-sensitive current (1, 44). Since MβCD, which is used for extracting cholesterol, is not membrane-permeable, theoretically it should only extract the cholesterol in the outer leaflet of the bilayer. Although cholesterol is a small hydrophobic molecule, it may not be able to freely flip (inward movement) or flop (outward movement) between the two leaflets of the polarized epithelial cell membranes. However, as depicted in Fig. 6, a reduction of the cholesterol in the outer leaflet should be followed by a reduction of the cholesterol in the inner leaflet due to the ABCA1-mediated cholesterol flopping (outward movement). Conversely, when the ABCA1 transporter is inhibited by CsA or DIDS, MβCD may only decrease the cholesterol in the outer leaflet; the cholesterol in the inner leaflet may be unchanged. Since we show that MβCD no longer reduces ENaC activity when the ABCA1 transporter is inhibited, the cholesterol in the inner leaflet appears to be important for stimulating ENaC. Theoretically, inhibition of ABCA1 should only elevate the cholesterol in the inner leaflet of the basolateral membrane because of the basolateral localization of ABCA1 (28). However, our confocal microscopy data suggest that inhibition of ABCA1 also elevates the cholesterol in the apical membrane or submembrane pools, suggesting that efficient pathways exist for cholesterol migration between the apical and basolateral membranes. One possible pathway is that the submembrane pools may transport chole-

Fig. 5. A: extraction of cholesterol by basolateral addition of 20 mM MβCD significantly decreased ENaC P0. MβCD had no effect on ENaC activity in cells pretreated with either basolateral 10 μM CsA (B) or 100 μM DIDS (C). Representative ENaC currents are shown at left, whereas summary plots are presented at right.

Fig. 6. A hypothetical model of pathways for CsA stimulation of ENaC. In polarized distal nephron cells, ABCA1 is responsible for flopping (outwardly transporting) cholesterol (CHO) from the inner leaflet to the outer leaflet of the cell membrane. Abolishment of ABCA1 function by CsA or DIDS (1) causes cholesterol accumulation in the inner leaflet of the basolateral membrane (2) and probably also elevates cytosolic cholesterol levels. CsA or DIDS also facilitates the cells to uptake exogenous CHO by blocking ABCA1-mediated CHO flopping. The elevated CHO in the basolateral membrane migrates toward the apical membrane possibly via either intracellular trafficking (3a) or lateral diffusion along the inner leaflet passing through the tight junction (TJ) (3b). Subsequently, the increased Cho in the cytoplasm and/or in the inner leaflet of the apical membrane enhances ENaC activity.
terol in endosome between apical and basolateral membranes (39). The other is that cholesterol may laterally diffuse along the inner leaflet of membrane bilayer because it has been shown that tight junction, which restricts cholesterol moving cross through the outer leaflet of plasma membrane, cannot prevent its translocation along the inner leaflet between the apical and basolateral membranes (40). In addition, free cholesterol in the plasma membrane can be released into the cytoplasm and shuttles between apical and basolateral membranes by nonvesicular movement (26).

Our studies presented here have suggested the role of ABCA1 in mediating CsA stimulation of ENaC. However, other ABC transporters may be also involved because recent studies have shown that efflux of intracellular cholesterol is mediated not only by ABCA1 but also by P-glycoprotein (P-gp) (37). Coincidentally, CsA not only inhibits ABCA1-mediated lipid efflux (19) but also is a noncompetitive inhibitor of P-gp, which is referred to as ABCB1 (23), which can abolish P-gp-mediated relocation of cholesterol from the inner leaflet to the outer leaflet of the plasma membrane (13). Taken together, CsA may inhibit other ABC transporters, not only ABCA1 as we identified in this study, which works in a team to elevate cholesterol in the cell membrane or cytoplasm. The present study focused on the mechanism of how basolateral application of CsA stimulates ENaC. However, our unpublished data show that apical application of CsA also stimulates ENaC, leading to a direct extension of the present study to test a hypothesis that luminal CsA may stimulate ENaC by inhibiting other ABC transporters in the apical membrane.

Although CsA is also a calcineurin inhibitor, the stimulatory effect of CsA on ENaC activity should not be mediated by calcineurin because, unlike CsA, FK-506, another calcineurin inhibitor, either does not affect basal ENaC activity (45) or inhibits aldosterone-stimulated ENaC activity (30). Through inhibition of calcineurin, both CsA and FK-506 reduce Na+-K+-ATPase activity in cortical collecting duct (20, 38). Since it is known that inhibition of Na+-K+-ATPase reduces ENaC activity (6), CsA, if through its inhibitory effect on calcineurin, is expected to reduce rather than enhance ENaC activity. Previous studies have shown that CsA can also act as an activator of protein kinase C (PKC) (21). However, stimulation of PKC also reduces rather than enhances ENaC activity (3, 7). Therefore, even if CsA could inhibit calcineurin and stimulate PKC in A6 cells, these pathways should not account for the stimulatory effect of CsA on ENaC activity but counteract the effect, which may account for the long latency for CsA to stimulate ENaC.

The present study suggests that the immunosuppressant CsA may enhance sodium reabsorption by stimulating ENaC in distal nephron cells through inhibition of ABC transporters and elevation of membrane or intracellular cholesterol. Since we show that CsA facilitates the renal epithelial cell uptake cholesterol, the patients on CsA treatment with hypercholesterolemia may have a higher probability to develop hypertension due to cholesterol stimulation of ENaC than the patients on CsA treatment with normal plasma cholesterol levels. However, further studies are required to determine, in an animal model, whether CIH can be corrected by administration of amiloride, a potent ENaC blocker, and whether other inhibitors of ABC transporters can also cause hypertension. These prospective studies may provide important information for both the management of CIH and the guidance for clinical use of other ABC transporter inhibitors.

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