Downregulation of Renal Sodium Transporters and Tonicity-Responsive Enhancer Binding Protein by Long-Term Treatment with Cyclosporin A

Sun Woo Lim,* Kyung Ohk Ahn,† Mee Rie Sheen,* Un Sil Jeon,* Jin Kim,‡ Chul Woo Yang,† and H. Moo Kwon*

*Department of Medicine, University of Maryland, Baltimore, Maryland; and Departments of †Medicine and ‡Anatomy, Catholic University of Korea, Seoul, Korea

Tonicity-responsive enhancer binding protein (TonEBP) is a transcriptional activator that is regulated by ambient tonicity. TonEBP protects the renal medulla from the deleterious effects of hyperosmolality and regulates the urinary concentration by stimulating aquaporin-2 and urea transporters. The therapeutic use of cyclosporin A (CsA) is limited by nephrotoxicity that is manifested by reduced GFR, fibrosis, and tubular defects, including reduced urinary concentration. It was reported recently that long-term CsA treatment was associated with decreased renal expression of TonEBP target genes, including aquaporin-2, urea transporter, and aldose reductase. This study tested the hypothesis that long-term CsA treatment reduces the salinity/tonicity of the renal medullary interstitium as a result of inhibition of active sodium transporters, leading to downregulation of TonEBP. CsA treatment for 7 d did not affect TonEBP or renal function. Whereas expression of sodium transporters was altered, the medullary tonicity seemed unchanged. Conversely, 28 d of CsA treatment led to downregulation of TonEBP and overt nephrotoxicity. The downregulation of TonEBP involved reduced expression, cytoplasmic shift, and reduced transcription of its target genes. This was associated with reduced expression of active sodium transporters—sodium/potassium/chloride transporter type 2 (NKCC2), sodium/chloride transporter, and Na\(^{+}\)/K\(^{+}\)-ATPase—along with increased sodium excretion and reduced urinary concentration. Infusion of vasopressin restored the expression of NKCC2 in the outer medulla as well as the expression and the activity of TonEBP. It is concluded that the downregulation of TonEBP in the setting of long-term CsA administration is secondary to the reduced tonicity of the renal medullary interstitium.


cyclosporin A (CsA) is a potent immunosuppressant with efficacy in improving the survival of solid-organ transplants and in treatment of autoimmune disorders. However, the use of CsA is limited by significant nephrotoxicity. The nephrotoxicity is reproduced in the rat model in which long-term administration of CsA is combined with sodium depletion. In this model, reduced GFR is observed along with histologic manifestations such as striped interstitial fibrosis, arteriolopathy, and tubular atrophy (1,2). In addition, tubular defects that are characterized by reduced urinary concentration and polyuria, magnesium wasting, distal tubular acidosis, and hyperkalemia develop.

Tonicity-responsive enhancer binding protein (TonEBP) is a transcriptional activator that is stimulated by ambient hypertonicity (3). TonEBP is highly expressed in the hypertonic renal medulla, where it regulates essential processes (for a recent review, see reference [4]). First, TonEBP protects cells from the stress of hypertonicity (hyperosmotic salinity) and high concentrations of urea. This is achieved by enhancement of the expression of genes that encode plasma membrane transporters and biosynthetic enzymes, such as aldose reductase (AR), that drive cellular accumulation of organic osmolytes. TonEBP also stimulates expression of heat-shock protein 70. Organic osmolytes and heat-shock protein 70 protect cells from the stress of hypertonicity and high concentrations of urea, respectively. Animals that are deficient in TonEBP develop severe medullary atrophy because the medullary cells fail to adapt to the hyperosmolality (5,6). Second, TonEBP regulates the urine-concentrating ability by stimulating transcription of aquaporin 2 (AQP2) (7) and UT-A urea transporter (8) in a manner independent of vasopressin.

There have been conflicting reports on the effects of CsA on TonEBP. Sheik-Hamad et al. (9) showed that CsA inhibited the nuclear translocation and transcriptional activity of TonEBP in cultured cells. This report also described that administration of CsA to rats resulted in an almost complete reduction in the renal expression of TonEBP target genes in correlation with increased apoptosis in the renal medulla. It was proposed that failure of the cellular adaptation to hypertonicity in the renal medulla as a result of the inhibition of TonEBP contributed to the CsA-induced nephrotoxicity.
Other reports provide contradictory views. CsA forms a complex with cyclophilin and calcineurin, also known as protein phosphatase 3 or Ca\(^{2+}\)/calmodulin-dependent serine/threonine protein phosphatase. Formation of the complex prevents calcineurin from dephosphorylating its substrates, including the NFAT transcription factors (10). TonEBP does not have a binding site for calcineurin, and CsA has no effect on nuclear translocation of TonEBP (11). We have shown that CsA or FK506 does not affect expression of TonEBP target genes (12). If inhibition of TonEBP leads to apoptosis because cells fail to adapt to hypertonicity as proposed (9), then the apoptotic cells should be limited to the hypertonic renal medulla. However, we observed far more apoptosis in the isotonic cortex than the hypertonic medulla in response to long-term CsA treatment (13). In fact, the majority of the pathologic changes, including interstitial fibrosis and arteriolopathy, were confined to the cortex.

In this study, we examined sodium transporters and TonEBP in the rat model of CsA nephrotoxicity. Short-term (7 d) treatment of CsA did not affect TonEBP or expression of its target genes in the kidney. However, long-term treatment (28 d), which resulted in overt nephropathy, was associated with reduced expression and cytoplasmic shift of TonEBP in the renal medulla. Our data suggest that the downregulation of TonEBP was caused by a reduction of the medullary tonicity as a result of reduced expression of sodium transporters: the sodium/potassium/chloride transporter type 2 (NKCC2) and Na\(^{+},K\(^{-}\)-ATPase.

Materials and Methods

Animals and Chemicals

The animal experiments were approved by the Animal Care Committee of The Catholic University. Male Sprague-Dawley rats (Charles River Co., Osan, Korea) that weighed 220 to 240 g were housed individually in metabolic cages (Nalge, Rochester, NY) in a temperature- and light-controlled room. Before experiments, rats were placed on a low-salt diet (0.05% sodium; Teklad Premier, Madison, WI) for 7 d. CsA (Novartis Pharma, Basel, Switzerland) was dissolved in PBS at 10 mM.

Experimental Design

Rats were randomized into four groups: vehicle (VH), CsA, VH + dDAVP, and CsA + dDAVP. Each rat received a daily subcutaneous injection of CsA (15 mg/kg body wt) or olive oil (VH, 1 ml/kg body wt) up to 28 d. On the 21st day, some rats (VH + dDAVP and CsA + dDAVP groups) were anesthetized with ketamine (40 mg/kg body wt) and xylazine (5 mg/kg body wt), and osmotic pumps (Alzet, Palo Alto, CA) that released 0.1 \(\mu\)g/h dDAVP were implanted. Twenty-four-hour urine samples were collected to measure volume and osmolality. Rats were killed after 7 or 28 d, and kidneys were analyzed as described next.

Immunohistochemistry

The kidneys were perfused briefly with PBS through the abdominal aorta to wash out the blood. Then they were perfused with periodate-lysine-paraformaldehyde solution, cut into sagittal slices of 1- to 2-mm thickness, and postfixed overnight in periodate-lysine-paraformalde-
medullary salinity/tonicity. We further hypothesized that the drop in medullary tonicity would lead to downregulation of TonEBP in the renal medulla. To test this hypothesis, we set out to (1) determine the osmolality of the inner medulla; (2) correlate the changes in medullary osmolality with changes in TonEBP and its target genes; and (3) examine the effect of dDAVP, which corrected the defect in urinary concentration that was induced by long-term CsA administration (13).

We attempted to measure changes in the osmolality of the inner medulla by analyzing homogenates of fresh tissues as described (17). In our hands, this method did not detect a decrease in the medullary osmolality: Whereas an increase in the inner medullary osmolality in response to water deprivation was detected readily, a decrease in osmolality in response to water diuresis was not (data not shown). Because the long-term CsA treatment resulted in a diuresis (Figure 1), we did not use this method. To maximize the sensitivity of detection, we decided to take advantage of specific antibodies and examine the active sodium transporters—NKCC2, NCC, and the α1 subunit of Na⁺,K⁺-ATPase—that drive sodium into the renal interstitium against the concentration gradient using energy in the electrochemical ion gradients across the plasma membrane (NKCC2, NCC) or hydrolysis of ATP (Na⁺,K⁺-ATPase). The single effect—active sodium chloride reabsorption in the thick ascending limb—of the countercurrent multiplier in the renal medulla is driven by the Na⁺,K⁺-ATPase and NKCC2. Other molecules, such as potassium and chloride channels, also participate in the process, but they are passive (i.e., do not use energy).

Male rats were treated with daily injections of CsA in combination with a low-sodium diet for 28 d as described previously (13). Figure 1 shows that significant polyuria and reduced

Figure 1. Effects of cyclosporin A (CsA) and 1-Desamino-[8-d-arginine] vasopressin (dDAVP) on urinary flow and osmolality. Rats received daily injections of vehicle (○) or 15 mg/kg body wt CsA (□) for 28 d. From days 21 to 28, half of the rats received infusions of dDAVP using osmotic pumps (▲, ■). Urine samples were collected every 24 h, and volume (A) and osmolality (B) were measured. Data are means ± SEM. *P < 0.05 versus vehicle or vehicle in combination with dDAVP; n = 6.

Figure 2. Effects of CsA on expression of sodium/potassium/chloride transporter type 2 (NKCC2), sodium/chloride transporter (NCC), and the α1 subunit of Na⁺,K⁺-ATPase. (A) Immunoblotting was performed on cortices (Co) and outer medullae (OM) of kidneys from rats that were treated with vehicle for 7 d (data not shown) or 28 d (VH) or with CsA for 7 d (7d CsA) or 28 d (28d CsA). Percentage of abundance in 7d CsA and 28d CsA relative to 7d VH and 28d VH, respectively, are shown below each blot in means ± SEM. *P < 0.05 versus VH; †P < 0.05 versus 7d CsA; n = 6; representative two lanes are shown from each group. (B) Immunohistochemistry of NKCC2 on Co (a through c) and OM (d through f) of kidneys from 28d VH (a and d), 7d CsA (b and e), or 28d CsA (c and f). Magnification, ×400.
urinary concentration developed on day 21 and continued until day 28, as expected.

In the cortex, expression of NCC and NKCC2 was reduced significantly by the CsA treatment on days 7 and 28, whereas expression of Na\(^{+},K^{+}\)-ATPase decreased only on day 28 (Figure 2A). The decreased expression of NKCC2 in the cortical thick ascending limbs was confirmed by immunohistochemical analysis (Figure 2B, a through c). The samples were processed exactly the same in staining and imaging for accurate comparison. Long-term treatment of CsA led to downregulation of all three transporters in the cortex with concomitant decrease in mRNA level (Table 1), indicating that reduced transcription was a major factor.

In the outer medulla, expression of NKCC2 showed biphasic changes: A dramatic increase on day 7 without changes in mRNA abundance followed by a decrease on day 28 along with mRNA (Figure 2, A and B [d through e]; Table 1). Expression of Na\(^{+},K^{+}\)-ATPase and its mRNA also decreased on day 28. On day 7, no changes in urine volume (Figure 1) or sodium excretion were reported (1) despite the large increased NKCC2, probably because the downregulation of NCC and NKCC2 in the cortex might have compensated the overly active NKCC2 upstream. These results demonstrate that changes in the sodium transporters occur before overt nephropathy in response to long-term CsA administration.

### Table 1. Effects of CsA on the renal abundance of mRNA

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Location</th>
<th>7d CsA</th>
<th>28d CsA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NKCC2</td>
<td>Co</td>
<td>55 ± 5(^b)</td>
<td>44 ± 8(^b)</td>
</tr>
<tr>
<td>NKCC2</td>
<td>OM</td>
<td>108 ± 8</td>
<td>89 ± 8(^c)</td>
</tr>
<tr>
<td>NCC</td>
<td>Co</td>
<td>40 ± 1(^b)</td>
<td>15 ± 2(^b,c)</td>
</tr>
<tr>
<td>Na(^{+},K^{+})-ATPase α1</td>
<td>Co</td>
<td>80 ± 2</td>
<td>43 ± 2(^b,c)</td>
</tr>
<tr>
<td>Na(^{+},K^{+})-ATPase α1</td>
<td>OM</td>
<td>79 ± 10</td>
<td>66 ± 4(^b)</td>
</tr>
<tr>
<td>TonEBP</td>
<td>Co</td>
<td>94 ± 6</td>
<td>93 ± 3</td>
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<tr>
<td>TonEBP</td>
<td>OM</td>
<td>69 ± 3</td>
<td>56 ± 4(^b)</td>
</tr>
<tr>
<td>TonEBP</td>
<td>IM</td>
<td>66 ± 2(^b)</td>
<td>69 ± 7(^b)</td>
</tr>
<tr>
<td>AQP2</td>
<td>Co</td>
<td>45 ± 5(^b)</td>
<td>36 ± 6(^b)</td>
</tr>
<tr>
<td>AQP2</td>
<td>OM</td>
<td>122 ± 40</td>
<td>40 ± 3(^b,c)</td>
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<tr>
<td>AQP2</td>
<td>IM</td>
<td>96 ± 18</td>
<td>52 ± 9(^b,c)</td>
</tr>
<tr>
<td>UT-A2</td>
<td>OM</td>
<td>71 ± 18</td>
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<td>UT-A1</td>
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</tr>
<tr>
<td>AR</td>
<td>IM</td>
<td>66 ± 13</td>
<td>13 ± 5(^b,c)</td>
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*\(^a\)Rats were treated with vehicle or cyclosporin A (CsA) for 7 (7d CsA) or 28 days (28d CsA) as described in Figure 1. RNA was extracted from cortices (Co), outer medullas (OM), and inner medullas (IM) from the kidneys, and ribonuclease protection assay (RPA) was performed to quantify mRNA. Abundance of mRNA was corrected for loading as described in the text. Percentage of abundance relative to corresponding vehicle-treated sample is shown for 7d CsA and 28d CsA in means ± SEM. n = 3 for 7d CsA; n = 6 for 28d CsA. AQP2, aquaporin 2; AR, aldose reductase; NCC, sodium/chloride transporter; NKCC2, sodium/potassium/chloride transporter type 2; TonEBP, tonicity-responsive enhancer binding protein.

\(^b\)P < 0.05 versus vehicle.

\(^c\)P < 0.05 versus 7d CsA.

**Figure 3.** Effects of CsA on expression of tonicity-responsive binding protein (TonEBP) and its target genes. Immunoblots for TonEBP, aquaporin 2 (AQP2), UT-A2, UT-A1, and aldose reductase (AR) are shown from the samples described in Figure 2. Percentage of abundance in 7d CsA and 28d CsA relative to corresponding VH are shown. IM, inner medulla. *P < 0.05 versus VH; **P < 0.05 versus 7d CsA; n = 6.
ers, and AR (Figures 3 and 4). On day 28, conversely, mRNA levels of AR, UT-A2, and AQP2 were reduced significantly, with the exception of UT-A1 urea transporter (Table 1). The reduction in mRNA levels led to a corresponding decrease in protein abundance, except that AQP2 protein decreased only in the inner medulla (Figures 3 and 4). Whereas the abundance of AQP2 mRNA decreased in the outer and inner medulla in line with the downregulation of TonEBP, AQP2 protein decreased only in the inner medulla. Factors other than tonicity/TonEBP well may be involved in the posttranscriptional compensation of AQP2 in the outer medulla. The reduced expression of UT-A2 and AQP2 are consistent with polyuria and decreased urinary osmolality that are seen in Figure 1.

dDAVP Restores Expression of NKCC2 and TonEBP in the Renal Medulla

In the animal model that was used in this study, we found previously that antidiuretic hormone signaling as measured by cAMP production in the inner medulla was impaired as a result of reduced expression of the stimulatory G protein (Gs), although circulating level of antidiuretic hormone was unaffected (13). We decided to infuse dDAVP in an attempt to correct for the defect. Both the CsA- and vehicle-treated rats responded to dDAVP (Figure 1), although the urinary osmolality was significantly lower and the urinary flow was higher in the CsA-treated rats.

In the vehicle-treated rats, infusion of dDAVP did not affect expression of the sodium transporters except for a moderate, posttranscriptional decrease in the expression of NCC (Figure 5, Table 2). Expression of TonEBP and its target genes did not change significantly (Figure 6) despite a variety of changes in their transcript levels (Table 2). The disconnection between levels of mRNA and proteins illustrates the complexity of regulation in response to long-term dDAVP administration.

In the CsA-treated rats, infusion of dDAVP restored expression of NKCC2 in the outer medulla but not in the cortex (Figure 5). NCC and Na⁺,K⁺-ATPase were not affected by dDAVP. Of interest, there was posttranscriptional correction of TonEBP expression: Increased expression of TonEBP protein (Figure 6) and nuclear localization (Figure 7, D and H) without changes in transcript levels (Table 2). These data suggest that the activity of NKCC2 in the outer medulla regulates TonEBP at the level of protein abundance and nuclear translocation in the renal medulla.

As expected from the restoration of TonEBP expression and nuclear localization, transcript levels of the TonEBP target genes increased significantly (Table 2), including AQP2 in the outer and inner medulla, UT-A1, UT-A2, and AR. Expression of corresponding proteins showed a more complex pattern, indicating multiple layers of posttranscriptional regulation (Figures 6 and 7): AQP2 expression increased in the outer and inner medulla but not in the cortex, UT-A2 expression overshot the control, AR expression did not change, and UT-A1 expression decreased. Increased expression of AQP2 and UT-A2 in the medulla is consistent with the urinary concentration in the rats that were treated long term with CsA.

Discussion

In this study, we find that renal TonEBP is downregulated after 28 d of treatment with CsA. The downregulation involves reduced level of expression, cytoplasmic shift, and reduced activity manifested by decreased expression of target gene mRNA. In disagreement with the previous report that described inhibition of renal TonEBP after 5 d of CsA administration (9), we do not find that TonEBP or its target genes is affected after 7 d of CsA treatment when renal function is normal (1). Our data indicate that CsA does not inhibit TonEBP directly, in agreement with other reports (11,12). The down-
regulation of TonEBP after 28 d of CsA treatment must be due to secondary effects of CsA. The reduced expression of sodium transporters that drive the accumulation of sodium in the medullary interstitium suggests that a decrease in the medullary tonicity leads to the downregulation of TonEBP in the setting of long-term CsA administration. Restoration of the expression and the activity of TonEBP in response to dDAVP in correlation with recovery of NKCC2 in the outer medulla supports this view.

Our data presented here along with some other work (11,12) are in direct contradiction with one study (9), which reported that nuclear translocation of TonEBP was inhibited by CsA and that expression of TonEBP target genes was suppressed dramatically by a 5-d CsA treatment. This report (9) suggested that the effects were due to inhibition of calcineurin. We speculate that differences in the dosage of CsA explain the contradicting results. In cultured cells, 0.1 to 1 μM CsA completely inhibits calcineurin and the nuclear translocation of NFAT. Whereas 1

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**Table 2. Effects of dDAVP in combination with CsA on the renal abundance of mRNA**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Location</th>
<th>VH</th>
<th>VH + dDAVP</th>
<th>CsA</th>
<th>CsA + dDAVP</th>
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<tr>
<td>NKCC2</td>
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<td>79 ± 3</td>
<td>44 ± 8b</td>
<td>48 ± 7b</td>
</tr>
<tr>
<td>NKCC2</td>
<td>OM</td>
<td>100 ± 8</td>
<td>82 ± 4</td>
<td>89 ± 8</td>
<td>95 ± 9</td>
</tr>
<tr>
<td>NCC</td>
<td>Co</td>
<td>100 ± 4</td>
<td>102 ± 7</td>
<td>15 ± 2b</td>
<td>14 ± 3b</td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase α1</td>
<td>Co</td>
<td>100 ± 14</td>
<td>85 ± 3</td>
<td>43 ± 2b</td>
<td>59 ± 3b</td>
</tr>
<tr>
<td>Na⁺,K⁺-ATPase α1</td>
<td>OM</td>
<td>100 ± 4</td>
<td>94 ± 6</td>
<td>66 ± 4b</td>
<td>71 ± 3b</td>
</tr>
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<td>93 ± 3</td>
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<td>AQP2</td>
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<td>AR</td>
<td>IM</td>
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<td>210 ± 16b</td>
<td>13 ± 5b</td>
<td>192 ± 28b,c</td>
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*Rats were treated with vehicle (VH), vehicle with dDAVP (VH + dDAVP), CsA, and CsA in combination with dDAVP (CsA + dDAVP) as described in Figure 5. RNA was extracted from the kidneys, and RPA was performed as in Table 1. Relative abundance is shown as means ± SEM; n = 6.

bP < 0.05 versus VH.

nP < 0.05 versus CsA.

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**Figure 5. Effects of dDAVP in combination with CsA on expression of NKCC2, NCC, and the α1 subunit of Na⁺,K⁺-ATPase. (A) Immunoblotting was performed on Co and OM of kidneys from rats that were treated with vehicle without (VH) or with dDAVP (VH + dDAVP), or CsA without (CsA) or with dDAVP (CsA + dDAVP) as described in Figure 1. Percentage of abundance relative to VH is shown as means ± SEM. *P < 0.05 versus VH; **P < 0.05 versus CsA; n = 6. (B) Immunohistochemistry of NKCC2 on Co (a through d) and OM (e through h) of kidneys from VH (a and e), VH + dDAVP (b and f), CsA (c and g), or CsA + dDAVP (d and h). Magnification, ×400.**
The osmolality of urine (Figure 1) are not affected after 7 d of CsA administration. Consistent with this idea, expression and activity of TonEBP are unchanged, suggesting the lack of changes in the tonicity in the medullary interstitium.

It is surprising that expression of NCC and NKCC2 is decreased in association with reduced transcript levels in the cortex after 7 d of CsA administration. AQP2 mRNA also is reduced without changes in protein. This is because, at this point, renal dysfunction is not detected in terms of fractional excretion of sodium, creatinine clearance, serum creatinine concentration, urine volume and osmolality, renal expression of angiotensin II, and interstitial fibrosis (1,2). However, there is significant infiltration of macrophages and cellular proliferation in the cortex. It seems plausible that cellular proliferation results in decreased transcription of NCC, NKCC2, and AQP2 as a result of the process of dedifferentiation of the tubular epithelia. The decreased transcription is compensated by various mechanisms at this early stage of CsA administration: Reduced expression of NCC and NKCC2 in the cortex is compensated by an increased expression of NKCC2 in the outer medulla, and the level of AQP2 is maintained posttranscriptionally.

Underlying mechanisms look different for the reduced expression of Na\(^+\),K\(^+\)-ATPase and that of NKCC2 in response to CsA treatment. It does not seem that CsA directly influences expression of Na\(^+\),K\(^+\)-ATPase, because there is no change after 7 d of treatment. Reduced transcription and expression are observed after 28 d of CsA treatment. This likely is due to an irreversible change, because dDAVP treatment does not affect it. The action of CsA on NKCC2 expression is far more complicated. In the cortex, CsA inhibits the transcription and expression of NKCC2 (and NCC) in both day 7 and day 28. This seems to be due to direct action of CsA, because the inhibition is seen early (day 7) and not affected by dDAVP treatment. In the outer medulla, a completely different picture has emerged. Whereas transcription of NKCC2 largely is unaffected by the CsA treatment, expression of NKCC2 increases on day 7 but decreases on day 28. It is interesting that expression of NKCC2 is restored on day 28 when treated with dDAVP. Thus, there are multiple posttranscriptional changes in NKCC2 of the outer medulla during the course of CsA and dDAVP treatment.

**Conclusion**

The data presented here demonstrate that in the setting of nephrotoxicity that is induced by long-term CsA administration, TonEBP is inhibited in the renal medulla in the form of reduced protein expression and cytoplasmic shift. This seems to be secondary to the reduced medullary tonicity as a result of downregulation of NKCC2 and Na\(^+\),K\(^+\)-ATPase. The inhibition of TonEBP contributes to the defect in urinary concentration as a result of reduced transcription of its target genes, including AQP2 and UT-A2. It is possible that there are more, yet-to-be-identified, target genes of TonEBP whose downregulation contributes to other aspects of nephrotoxicity that is induced by long-term CsA administration.
Figure 7. Effects of dDAVP in combination with CsA on expression of TonEBP and UT-A2. Immunohistochemistry was performed to visualize expression of TonEBP in OM (A through D) or IM (E through H) and UT-A2 in OM (I through L) from rats that were treated with VH (A, E, and I), VH + dDAVP (B, F, and J), CsA (C, G, and K), or CsA + dDAVP (D, H, and L) as described in Figure 5. Areas indicated by the rectangles in A through H are shown in higher magnification below corresponding panels. Asterisks in C and D denote descending thin limbs. # in G and H denote IM collecting ducts. Magnification: ×600 in A through H; ×400 in I through L.

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Disclosures
None.

References


