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SHORT COMMUNICATION

Evidence for the Impairment of the Vitamin D Activation Pathway by Cyclosporine A

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ABSTRACT. Cyclosporine A (CsA) is a potent immunosuppressant with the drawback of renal side effects. We reported that CsA markedly decreases calcium-binding protein calbindin-D28k mRNA levels in rat kidneys, and showed that this decrease is associated with its adverse renal effects. The transcription of the calbindin-D28k gene is activated via the vitamin D pathway. In this work, the potential CsA-mediated impairment of the vitamin D pathway was investigated. Wistar rats were treated for 12 days with 50 mg/kg/day CsA or for 20 days with 50 mg/kg/day of the non-immunosuppressant and non-nephrotoxic SDZ PSC 833, which had been previously shown not to affect calbindin-D28k mRNA levels. The expression of the three vitamin D-regulated genes calbindin-D28k, 1,25-dihydroxyvitamin D_3-24-hydroxylase (24-OHase), and vitamin D receptor (VDR) were quantified in rat kidney homogenates by real-time reverse transcription–polymerase chain reaction. Plasma parathyroid hormone (PTH) as well as plasma and kidney 1,25 dihydroxyvitamin D_3 (calcitriol) levels were monitored in all animals. CsA induced a 85% decrease in calbindin-D28k mRNA levels as well as a 40% and 69% decrease in VDR and 24-OHase mRNA levels, respectively. Plasma and kidney 1,25 dihydroxyvitamin D_3 as well as plasma PTH levels were increased by CsA, but not by SDZ PSC 833. The treatment with SDZ PSC 833 did not affect calbindin-D28k or VDR expression, but did cause a 73% decrease in 24-OHase mRNA levels. Taken together, these results indicate an association between CsA-mediated down-regulation of rat renal calbindin-D28k mRNA and the decrease in other 1,25 dihydroxyvitamin D_3-regulated genes, suggesting an impairment of the vitamin D pathway by CsA which may be related to its adverse renal side effects and its immunosuppressive activity. BIOCHEM PHARMACOL **59**;3:267–272, 2000. © 1999 Elsevier Science Inc.

KEY WORDS. vitamin D pathway; cyclosporine A; TaqMan RT–PCR; rat; 1,25 dihydroxyvitamin D_3; immunosuppression

The immunosuppressant CsA† is widely used for treatment of allograft rejection and graft-versus-host disease [1, 2]. An action of CsA central to its immunosuppressive effect is its ability to inhibit interleukin-2 gene transcription [3]. CsA exhibits, however, a variety of "non-immunologic" side effects, among which renal impairment is the most important [4]. Prolonged treatment with CsA appears to result in microcalcification within or adjacent to tubular cells, and upon chronic administration of the drug, irreversible chronic renal failure with tubulo-interstitial fibrosis and focal glomerulosclerosis are described [5]. The entire cascade of cellular and molecular events induced by CsA and leading to its renal side effect is still not fully understood. Recently, we analyzed kidney homogenates of CsA-treated rats by two-dimensional protein gel electrophoresis (2-DE) and discovered that administration of CsA leads to a strong decrease in the level of the calcium-binding calbindin-D28k [6]. Later, we showed by conventional RT–PCR that the CsA-mediated down-regulation of the renal calbindin-D28k protein is the result of a decrease in the calbindin-D28k mRNA level [7]. Calbindin-D28k is postulated to function as a transport molecule that facilitates the passage of calcium through the cell and serves as an intracellular calcium buffer maintaining the ionized calcium below toxic levels [8]. In the kidney, the hormone 1,25 dihydroxyvitamin D_3 (calcitriol) regulates transcription of the calbindin-D28k gene [9]. This hormone is one of the major regulators of calcium metabolism, stimulating intestinal calcium absorption and increasing bone resorption. In earlier reports, it was shown that CsA increases plasma 1,25 dihydroxyvitamin D_3 levels in rodents [10]. 1,25 Dihydroxyvitamin D_3 is known to be an inhibitor of PTH production, both by direct action on the parathyroid glands and indirectly by raising plasma calcium levels. 1,25 Dihydroxyvitamin D_3 production itself is stimulated by PTH, thus providing an effective control loop.

The action of 1,25 dihydroxyvitamin D_3 is mediated by the VDR [11–13] via its heterodimerization with RXR beta. In the present work, the question was therefore addressed as to whether the CsA-induced decrease in calbindin D-28k

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† *Abbreviations:* CsA, cyclosporine A; RT–PCR, reverse transcription–polymerase chain reaction; calcitriol, 1,25 dihydroxyvitamin D_3; 24-OHase, 1,25-dihydroxyvitamin D_3-24-hydroxylase; VDR, vitamin D receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RXR, retinoic X receptor; and PTH, parathyroid hormone.

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TABLE 1. Primer sequences used for the cRNA preparation of target genes

Gene	SP6 primer (5′ > 3′)	T7 primer (5′ > 3′)
CaBP-28kDa	**AACGATTTAGGTGAC** **AC**TATATTCATGAAGA CTTGGAGAAAGTATG	**AATTAATACGACTCACTATA**T TCGGTACAGCTTCCCTCCATCC GA
24-OHase	**CGATTTAGGTGACAC** TATAAGTGAGCTGAA CAAGTGGTC	**ATTAATACGACTCACTATA**GT CTGATTGTCAGGCAGCAC
VDR	**ACGATTTAGGTGACA** **C**AAGCAGCAGCCCCT TGTGGTGGAG	AGC**ATTAATACGACTCACTAT** **A**CTGCTCCTTCAGGGATGGAG GCA
RXR beta	A**ACGATTTAGGTGAC** **A**CTATAAGCAGCCCA AATGACCCAG	A**ATTAATACGACTCACTATA**G AGGATGCCATCTCGGACAT
GAPDH	A**ACGATTTAGGTGAC** ACTATATTGCCGGCCT CGTCTCATAGA	A**ATTAATACGACTCACTATA**T TGGGTAAACTACAATCGCCCT

Bold: SP6 or T7 recognition sequence. CaBP, calbindin.

was associated with a change in other 1,25 dihydroxyvitamin D_3-regulated genes (24-OHase, VDR) or in genes involved in 1,25 dihydroxyvitamin D_3 signal transmission (RXR beta). Male Wistar rats were treated with CsA (50 mg/kg/day) for 12 days. This dose has been shown to provoke the most dramatic decrease in calbindin-D28k mRNA levels. We compared the effects of CsA treatment with those of a non-immunosuppressant and non-nephrotoxic cyclosporine SDZ PSC 833 dosage (50 mg/kg/day) for 20 days. The duration of the treatment by SDZ PSC 833 was longer than with CsA in order to increase the likelihood of the appearance of a nephrotoxic effect. We tried to elucidate whether the impairment of the vitamin D pathway might be related to the immunosuppressive and/or renal toxicity of CsA. The results obtained support the view that renal dysfunction induced by CsA is very likely associated with impairment of the vitamin D pathway in rat.

MATERIALS AND METHODS

Animal Treatment Protocol and Sample Preparation

Animals were administered CsA via oral gavage as previously described [14]. Briefly, HanIbM male Wistar rats (Biological Research Labs), eight weeks of age and weighing 225–280 g, received 50 mg/kg/day CsA for 12 days or 50 mg/kg/day SDZ PSC 833 for 20 days. The animals were killed on the day following the last administration, and 150-mg tissue samples from each kidney pole were collected, frozen immediately in liquid nitrogen, and stored at −80° until analysis.

1,25 Dihydroxyvitamin D_3 Measurement

Plasma and kidney 1,25 dihydroxyvitamin D_3 levels were determined using the human ^{125}I-1,25 dihydroxyvitamin D_3 radioimmunoassay (RIA) kit from Immuno Diagnostic System (Tyne and Wear). Kidneys (300 mg) were homogenized using 500 μL of lysis buffer (1% Triton X-100, 50 mM Tris–HCl pH 8, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 20 mM iodoacetamide, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 5 mM EDTA, 10 mM NaF, 10 mM sodium orthovanadate, 10 mM sodium pyrophosphate). Plasma samples of 250 μL were delipidated, recovered in two aliquots of 100 μL, and immunopurified. The immunoextraction was performed using immunocapsules and after elution, the eluate was incubated overnight at 2–8° with the anti-1,25 dihydroxyvitamin D_3 antibody. ^{125}I-1,25 dihydroxyvitamin D_3 was added as a tracer and the samples incubated for 2 hr ± 15 min at room temperature. The bound fraction of 1,25 dihydroxyvitamin D_3 was separated by centrifugation, the supernatant discarded, and samples counted for ^{125}I radioactivity in a gamma counter.

Parathyroid Hormone Measurement

Plasma PTH was determined using the rat ^{125}I PTH IRMA kit (Nichols Institute Diagnostics). Two aliquots of 200 μL were incubated overnight at room temperature with ^{125}I-labeled anti-rat PTH antibody and with rat PTH-coated beads. After centrifugation, the supernatant was discarded and the sample was washed three times with washing buffer, prior to gamma counting for at least one minute.

Gene Expression Analysis

Rat renal total RNA extraction was performed using RNAzol B (ams Biotechnology) and the Bio101 system for tissue homogenization as described previously [7]. The calbindin D-28k, 24-OHase, VDR, RXR beta, and GAPDH cRNA standards were prepared by *in vitro* transcription of a PCR product containing recognition sequences for SP6 and T7 polymerases (in 5′ and 3′, respectively). The SP6–T7 primer sequences used for the RT–PCR were as shown in

TABLE 2. Primer and probe sequences used for the TaqMan RT–PCR quantification of target genes

Gene	Forward (5′ > 3′)	Reverse (5′ > 3′)	Probe (5′ > 3′)
CaBP-28kDa	AAACAAGACCG TGCATGATACG	TCCCTGGAATTTAA GAAGGAAATTT	AACCTGGCCATC TCTGTCAGCTCC AG
24-OHase	CGGGCTGCTGG GAATATCT	GCTGGCTTGGGACA CCATT	CCCTGCATCGAC AACCGCCTACA
VDR	CCGCATCACCA AGGACAAC	ACTCCTTCATCATG CCGATGT	CAGGCCTGCCGG CTCAAACG
RXR beta	GAAGGACGCAA TGAGGAGCT	TCGTGGAGTGGGC AAAGAG	ACTTTTCCTCCCT ACCTCTGGACGA CCA
GAPDH	AGATGGTGATG GGTTTCCCA	CCTCAACTACATGG TCTACATGTTCC	CCCACGGCAAGT TCAATGGCACA

CaBP, calbindin.

Table 1. The RT–PCR was performed in an automatic DNA thermal cycler Trio-Thermoblock (Biometra) using the Access RT–PCR kit (Promega). The RT–PCR mixture was prepared as follows: 50 ng total RNA template, 50 pmol of each primer, 0.2 mM deoxynucleotide triphosphates, 1 mM $MgSO_4$, 0.5 U AMV reverse transcriptase, and 0.5 U *Tfl* DNA polymerase, PCR buffer, and RNase free water up to a final volume of 50 μL. The reverse transcription was performed at 48° for 45 min, followed by a step at 94° for 2 min. The step cycle program was then performed for a total of 40 cycles in the same tube without further manipulations as follows: 94° for 30 sec, 60° for 1 min, 68° for 2 min, and final extension at 68° for 7 min. Two negative controls were included: one PCR reaction mixture with water in place of the RNA sample and one without the reverse transcriptase. Concentrations of cRNAs were calculated by the absorbance at $\lambda = 260$ nm (A_{260nm}), and the purity was estimated by the ratio A_{260nm}/A_{280nm}. The number of standard molecules was calculated to be $2.2.10^{12}$ molecules/μL for the calbindin-D28k cRNA; 2.10^{12} molecules/μL for 24-OHase; 2.10^{12} molecules/μL for VDR; $2.1.10^{12}$ molecules/μL for RXR beta; and $2.3.10^{12}$ molecules/μL for the GAPDH. Standard curves specific to each quantified gene were generated by serial dilution of each cRNA down to 10^4 molecules/μL.

The primer and TaqMan probe sequences used for the real-time RT–PCR are shown in Table 2. The probes and primers were purchased from Perkin Elmer. The RT–PCR mixture was prepared as follows: 50 ng total RNA template, 400 nM of each primer, 0.2 mM deoxynucleotide triphosphates, 1 mM $MgCl_2$, 0.5 U murine leukemia virus reverse transcriptase, and 0.5 U *Taq* DNA polymerase, Taq PCR buffer and RNase free water up to a final volume of 50 μL. The reverse transcription was performed using the ABI Prism 7700 Sequence detection system (Perkin Elmer) at 48° for 30 min followed by a step at 95° for 10 min. The step-cycle program was performed for a total of 40 cycles in the same tube without further manipulations as follows: 95° for 30 sec, 56° for 1.5 min. Two negative controls were included: one PCR reaction mixture with water in place of the RNA sample and one without reverse transcriptase. Under no circumstances did amplification products appear in these controls. After the RT–PCR reaction, the data were analyzed using the Sequence detector v.1.6 software (Perkin Elmer). Data were expressed relative to the expression of the housekeeping gene GAPDH.

RESULTS AND DISCUSSION

Gene expression profiling by real-time RT–PCR showed the previously reported CsA-mediated decrease in renal calbindin-D28k mRNA levels [7]. The 85% decrease in the calcium-binding protein was associated with a statistically significant decrease after CsA treatment in VDR and 24-OHase mRNA levels of 40% and 69% respectively (Fig. 1). In contrast, SDZ PSC 833 showed no effect on calbindin D-28k and VDR mRNA, but did show a 73% decrease in 24-OHase mRNA levels. The down-regulation of 24-OHase by SDZ PSC 833 is a new observation whose relevance is not clear yet. The down-regulation of VDR, 24-OHase, and calbindin D-28k, all of which are transcriptionally regulated by vitamin D_3, supports the hypothesis of an impairment of the vitamin D activation pathway by CsA.

Following 12 days of treatment with 50 mg/kg/day cyclosporine A, a 3-fold increase in mean plasma 1,25 dihydroxyvitamin D_3 levels was observed when compared with controls (Fig. 2), as reported previously in the rat by others [10, 15]. There was no effect observed with SDZ PSC 833. A similar CsA-mediated increase in 1,25 dihydroxyvitamin D_3 levels was observed in the kidney. From these data, it can be deduced that the CsA-mediated decrease in renal calbindin D-28k mRNA levels was not the consequence of a reduction in plasma or kidney 1,25 dihydroxyvitamin D_3 levels, but rather indicates an impairment in the transduction of the 1,25 dihydroxyvitamin D_3 signal.

PTH has been shown to be a potent down-regulator of VDR mRNA levels [16]. It is likely that the potential up-regulation of VDR mRNA levels by 1,25 dihydroxyvitamin D_3 may be blocked by increased PTH levels. A 41% increase in plasma PTH levels was observed in the group administered 50 mg/kg/day CsA (Fig. 3.). Increased plasma

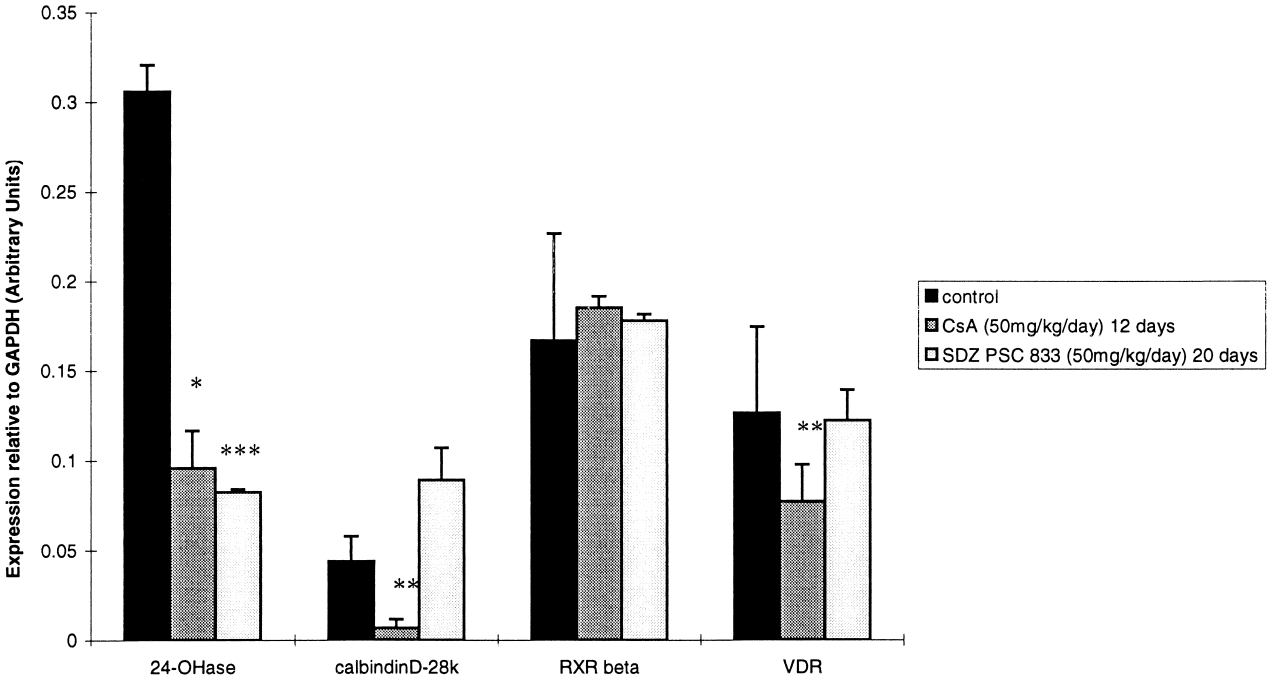

FIG. 1. Gene expression quantification in the rat kidney after treatment with CsA or SDZ PSC 833. Significantly different, treated versus control: *($P < 0.05$, Student's *t*-test); **($P < 0.001$, Student's *t*-test); and *($P < 0.0001$, Student's *t*-test), N = 5.**

PTH levels may play a key role in the loss of sensitivity of 1,25 dihydroxyvitamin D_3-inducible genes. PTH has indeed been shown to inhibit 24-OHase mRNA expression stimulated by 1,25 dihydroxyvitamin D_3 in the rat kidney [17]. These data could be related to the recently reported up-regulation of the parathyroid hormone-related protein (PTHrP) which is associated with chronic CsA-induced nephrotoxicity, suggesting a link between PTH and CsA-injured kidney [18].

Here, we showed for the first time that a drug-induced increase in rat plasma 1,25 dihydroxyvitamin D_3 was paralleled by an increase in kidney 1,25 dihydroxyvitamin D_3 levels. As 1,25 dihydroxyvitamin D_3 is a crucial component in the activation pathway for calbindin D-28k transcription, its increase may be regarded as a positive feedback to the decrease in calbindin D-28k levels. However the CsA-mediated effect on 1,25 dihydroxyvitamin D_3 levels may be counterbalanced by reducing the responsiveness of these genes to 1,25 dihydroxyvitamin D_3 activation, as reported in the case of uremic rats [19, 20]. A recent study indicates that uremic toxins could modify the VDR DNA-binding domain and inhibit the binding of the VDR to the vitamin D responsive elements [21]. Therefore, CsA may act on the vitamin D pathway by blocking the

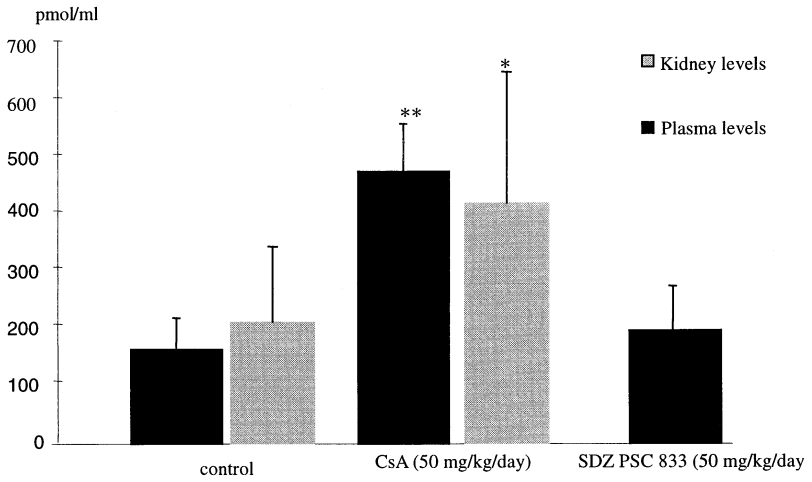

FIG. 2. Plasma and kidney calcitriol levels following 12 days CsA (50 mg/kg/day) or 20 days SDZ PSC 833 (50 mg/kg/day) treatment in rat. Significantly different, treated versus control: *($P < 0.05$, Student's *t*-test) and **($P < 0.001$, Student's *t*-test), N = 5.

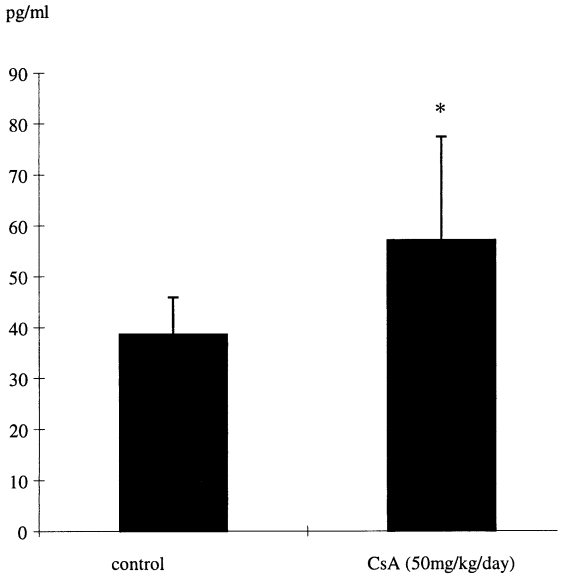

FIG. 3. Plasma PTH levels following 12 days CsA (50 mg/kg/day) treatment in rat. Significantly different: *($P < 0.05$, Student's *t*-test), N = 5.

transcriptional activation mediated by VDR via inhibition of the binding of this transcription factor on VDRE (vitamin D responsive elements). The finding that the non-immunosuppressive and non-nephrotoxic SDZ PSC 833 has no effect on 1,25 dihydroxyvitamin D_3 suggests that the 1,25 dihydroxyvitamin D_3 disregulation found with CsA may be related to its immunosuppressive action and its renal side effects. Interestingly, 1,25 dihydroxyvitamin D_3 was reported to have an intrinsic immunosuppressive activity [22–25] and was found to induce calcification in the kidney [26].

The question could be raised as to whether there is a relationship between the immunosuppressive effect of CsA and its renal side effects in the rat. The link between these two actions may be the vitamin D pathway. The decrease in the vitamin D-regulated gene calbindin-D28k has been shown to be linked to the appearance of CsA-mediated renal calcification [27]. One of the interesting sites of action of vitamin D_3 is the immune system, where VDR is expressed in activated lymphocytes [28]. This led to the hypothesis that the lymphocyte could be a target of vitamin D action. The finding that T-cell-mediated immune responses can be inhibited by an excess of 1,25 dihydroxyvitamin D_3 [29] is certainly a relevant one. Finally, in transplant rejection experiments, it could be shown that 1,25 dihydroxyvitamin D_3 is superior to CsA in preventing rejection of rat embryonic heart transplant.*[1] In the rat, the non-immunosuppressive SDZ PSC 833 did not induce renal side effects and failed to affect either calbindin D-28k or VDR mRNA levels, or plasma or kidney 1,25 dihydroxyvitamin D_3 levels, thus supporting the hypothesis of a link between CsA immunosuppression and its renal side effect.

* Cantorna M, Hullett D, Sollinger H and DeLuca HF, unpublished results.

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