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SHORT COMMUNICATION

The Cyclosporine A-Induced Decrease in Rat Renal Calbindin-D28kDa Protein as a Consequence of a Decrease in its mRNA

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ABSTRACT. Cyclosporine A (CsA) is a potent immunosuppressant with the drawback of renal side-effects. We recently reported that relatively high doses of CsA markedly decreased the calcium-binding protein calbindin-D28kDa in kidneys of male Wistar rats, and showed that this decrease could be associated with some of the drug-induced adverse renal effects. To investigate the events leading to this decrease, the calbindin-D28kDa mRNA level in kidneys of rats treated with 15 or 50 mg/kg/day CsA for 12 days was analysed by reverse transcription followed by polymerase chain reaction. At both doses, a marked dose-dependent decrease in the calbindin-D28kDa mRNA level was found, one very similar to the decrease measured in the calbindin-D28kDa protein abundance. Thus, the CsA-mediated down-regulation of the renal calbindin-D28kDa protein is most likely the result of a decrease in the calbindin-D28kDa mRNA level. BIOCHEM PHARMACOL **55**;7:1131–1133, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. calcium-binding protein calbindin-D28kDa; cyclosporine A; RT-PCR; ELISA; rat kidney

The immunosuppressant CsA† has been widely used for over a decade for treatment of allograft rejection and graft-versus-host disease [1, 2]. One action of CsA which is 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 [4], among which renal impairment is the most important [5]. 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 [4]. 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 analysed kidney homogenates of CsA-treated rats by two-dimensional protein gel electrophoresis (2-DE) and discovered that administration of 50 mg/kg/day CsA for 28 days leads to a more than 95% decrease in the level of the calcium-binding calbindin-D28kDa [6]. Calbindin-D28kDa is postulated to function as a calcium transport molecule that facilitates the diffusion of calcium through the cell and serves as an intracellular calcium buffer maintaining the ionized calcium below toxic levels [7]. In the kidney, transcription of the calbindin-D28kDa gene is activated by 1,25 dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) [8]. More recently, we showed that the CsA-mediated decrease in renal calbindin-D28kDa is linked to increased calcium urine excretion and to intratubular kidney calcification [9], suggesting a CsA-induced dysregulation of calcium metabolism as described previously [10].

In the present study, the question was addressed as to whether the drug-related decrease in calbindin-D28kDa protein abundance is due to the impairment of pre- and/or posttranslational events. Therefore, we analysed calbindin-D28kDa mRNA and protein levels in kidney homogenates of CsA-treated rats. The renal calbindin-D28kDa mRNA level was measured by RT-PCR, and from the same kidney samples the renal calbindin-D28kDa level was measured by ELISA. It was found that both mRNA and protein levels of calbindin-D28kDa were similarly decreased. To our knowledge, this is the first report which describes a CsA-mediated decrease in the renal calbindin-D28kDa mRNA level.

MATERIALS AND METHODS

Animal Treatment Protocol and Sample Preparation

HanIbM: male Wistar rats (Biological Research Labs), 8 weeks of age and weighing 225–280 g, were used. Three groups of 10 rats were treated by oral gavage for 12 days each with either CsA (CsA 100 mg/mL of Sandimmun Placebo A, diluted 1:10 in olive oil), 15 mg/kg/day (low dose), 50 mg/kg/day (high dose), or with the vehicle solution (Sandimmun Placebo A diluted 1:10 in olive oil) (control group). Two animals in group A died before the

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† *Abbreviations:* CsA, cyclosporine A; RT-PCR, reverse transcription-polymerase chain reaction; TAE, tris acetate EDTA buffer.

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end of the experiment. The other animals were killed by decapitation on the day following the final treatment.

RNA Isolation and RT-PCR

Total RNA was isolated using the Qiagen Rneasy Midi Kit (Qiagen, Hilden, Germany). The RNA was quantified by the absorbance at $\lambda = 260$ nm (A_{260nm}), and the purity was estimated by the ratio A_{260nm}/A_{280nm}. RT-PCR experiments were performed for calbindin-D28kDa and β-actin mRNA. For the calbindin-D28kDa gene, the primer sequences were 5′-TTCATGAAGACTTGGAGAAAG TATG-3′, and 5′-CAAGATCTGTTCGGTAAAGCTT CCCTCCATCCGA-3′ (predicted product length: 501 bp). The nucleotide sequences of the β-actin primers were 5′-CAACCGTGAAAAGATGACCC-3′ and 5′-GAAG GAAGGCTGGAAGAGAG-3′ (predicted product length: 457 bp). 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 of deoxynucleotide triphosphates, 1 mM of $MgSO_4$, 0.5 U of AMV reverse transcriptase and 0.5 U of *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. Under no circumstances did PCR product bands appear in these controls. For each sample, an aliquot of the calbindin-D28kDa (10 μL) and of the β-actin (5 μL) RT-PCR mixture were then subjected to agarose gel electrophoresis. After ethidium bromide staining, the gels were scanned and videoprints were prepared using the GelPrint documentation system (MWG-Biotech). Band intensities from the acquired images were then analysed by densitometry using the ONE-Dscan software package (Scanalytics). The individual calbindin mRNA data were calculated by relating, for each sample, the intensity of the calbindin-D28kDa RT-PCR product ($I_{calbindin\text{-}D28kDa}$) to the intensity of the β-actin RT-PCR product ($I_{\beta\text{-}actin}$): $I_{calbindin\text{-}D28kDa}/I_{\beta\text{-}actin}$. The data of the treatment groups were then expressed as a percentage of the mean value of the control group which was set as 100%.

ELISA for Calbindin-D28kDa

Calbindin-D28kDa levels were quantified in kidney homogenates using ELISA techniques as previously described [6]. The protein content of the kidney samples was measured by a modified Bradford assay described by Ramagali and Rodriguez [11]. The data of the treatment groups were then expressed as a percentage of the mean value of the control group which was set as 100%.

Statistical Analysis

Significance was assessed using the two-sample equal variance, two-tailed distribution Student's *t*-test (homoscedastic Student's *t*-test) followed by the Holm-Bonferoni's procedure. Coefficients of determination with a P-value < 0.01 were considered to be statistically significant. The standard deviation was calculated from the average intensity value for each group and was displayed as error bars on the graphics.

RESULTS AND DISCUSSION

The RT-PCR data for the calbindin-D28kDa gene are shown in Fig. 1 (A and C). In kidneys of CsA-treated rats, a statistically significant dose-dependent decrease in the calbindin-D28kDa mRNA level was observed. After twelve days of CsA treatment, the renal calbindin-D28kDa mRNA levels in the low dose group (15 mg/kg/day) and in the high dose group (50 mg/kg/day) were reduced by 82% and 93% respectively as compared to the controls. The data suggest that CsA may influence the transcription of the calbindin-D28kDa gene. However, a possible CsA mediated effect on the half-life of the messenger can presently not be excluded. No change in the β-actin mRNA content was observed as a result of CsA treatment (Fig. 1B), and the decrease in the calbindin-D28kDa mRNA level is therefore considered as a specific response to CsA treatment.

The ELISA data showed a dose-dependent decrease in the calbindin-D28kDa level (Fig. 1C). After twelve days of treatment, the renal calbindin-D28kDa level in the low dose group was reduced by 77%, where in the high dose group the decrease reached 85% as compared to the controls. The results for the high dose treatment were in agreement with data previously reported from our laboratory [6]. These present data showed that not only the high dose of 50 mg/kg/day but also the lower dose of 15 mg/kg/day had a similar effect on the renal calbindin-D28kDa abundance in rat. The average of total kidney protein was 10.69 mg protein/mg tissue in the control group, 10.76 mg protein/mg tissue in the low dose group, and 9.48 mg protein/mg tissue in the high dose group. This data suggested a minor, statistically not significant effect of CsA on protein synthesis following treatment with 50 mg/kg/day.

As emphasised in Fig. 1C, CsA treatment induces a similar reduction in both mRNA and protein levels of the renal calbindin-D28kDa. Nevertheless, a slight but consistent difference was observed in the decrease of calbindin-D28kDa and its mRNA counterpart. In both treatment groups, the calbindin-D28kDa was slightly more abundant than its mRNA. This difference was already noted at the low dose, and became statistically significant ($P < 0.005$) at the high dose. It is presently not clear whether this

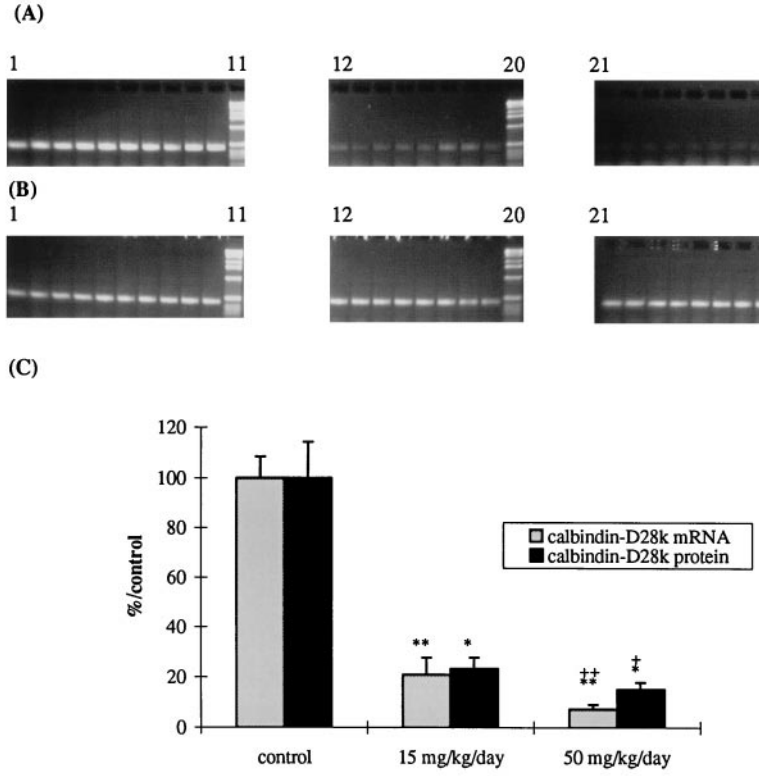

FIG. 1. Calbindin-D28kDa mRNA and protein levels in kidneys of CsA-treated rats. Calbindin-D28kDa (A) and β-actin (B) RT-PCR products separated in a 2.5% agarose gel and stained with ethidium bromide. Lanes 1 to 10: control rat kidneys; lanes 12 to 19: rats treated with 15 mg/kg/day CsA; lanes 21 to 30: rats treated with 50 mg/kg/day CsA; lanes 11, 20 and 31: markers (Kb ladder; Life Technologies). (C) The ELISA and the RT-PCR data from treated rats are presented as percent of control rats. The bars represent the means ± SD of 8–10 rats. Significantly different treated vs control: *$P < 0.005$, Student's *t*-test; **$P < 0.001$. Significantly different 15 mg/kg/day vs 50 mg/kg/day: $^{+}P < 0.005$, Student's *t*-test; $^{++}P < 0.001$.

difference is biologically relevant or whether it is due to differences between the two methods of measurement. It may be interesting to evaluate the calbindin-D28kDa protein and its mRNA abundance at time points earlier than 24 hr after the final administration of the drug.

In this study, we showed that the CsA-mediated decrease in renal calbindin-D28kDa abundance is most likely the result of a decrease in the calbindin-D28kDa mRNA level. It needs to be elucidated whether the decrease in the mRNA is due to a direct CsA-mediated effect on the transcription of the calbindin-D28kDa gene. If this is the case, the compelling question would arise as to whether the drug may impair the vitamin D pathway known to be involved in the activation of the calbindin-D28kDa gene. The possible interaction of CsA with the vitamin D pathway may then lead to a down-regulation of other 1,25 dihydroxyvitamin D_3-induced genes, which may result in a more complete understanding of the CsA-mediated renal adverse side-effects.

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