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SHORT COMMUNICATION

Cyclosporine A-induced Decrease in Calbindin-D 28 kDa in Rat Kidney but not in Cerebral Cortex and Cerebellum

*Maria del Carmen Varela, Agustin Arce, Brigitte Greiner, Marianne Schwald, Lothar Aicher, Daniel Wahl, Olivier Grenet and Sandra Steiner**

PRECLINICAL SAFETY, TOXICOLOGY, NOVARTIS PHARMA AG, BASEL, SWITZERLAND

ABSTRACT. Recently, we reported that in rat, cyclosporine A (CsA) markedly decreases the levels of calbindin-D (CABP-D) 28 kDa in kidney. CABP-D 28 kDa is a calcium-binding protein which is highly expressed in calcium-transporting tissues such as kidney or brain. In this study, we investigated whether, in addition to the kidney, CsA also has an effect on CABP-D 28 kDa in rat brain. Three groups of male Wistar rats received 15 mg/kg/day or 50 mg/kg/day of CsA orally for 12 days, whereas controls received vehicle solution for the same period. CABP-D 28-kDa protein and CsA were quantified in homogenates of kidney, cerebral cortex and cerebellum, and the localization of CABP-D 28 kDa was assessed in the different tissue sections by immunohistochemistry. In kidney, CABP-D 28 kDa was strongly and dose dependently decreased, and was located in tubular epithelial cells. In brain, CABP-D 28 kDa was not changed and was mainly located in pyramidal cells of the cortex and in cerebellum exclusively in Purkinje cells. High CsA concentrations were measured in kidney, more than 17-fold greater than those found in cortex. In cerebellum, CsA was below the limit of detection. These data suggest that at clinically relevant doses, CsA may not affect CABP-D 28-kDa levels in brain. BIOCHEM PHARMACOL **55**;12:2043–2046, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cyclosporine A; calcium-binding protein calbindin-D 28 kDa; rat; kidney; brain; cerebellum

CsA† is a potent immunosuppressant used to prevent organ graft rejection. Its immunosuppressive action is linked to the binding of the drug to the intracellular receptor cyclophilin [1] and the subsequent blockage of the calcium-dependent serine-threonine phosphatase calcineurin by the cyclophilin-drug complex [2]. The inactivation of calcineurin was shown to inhibit the calcineurin-dependent activation of transcription factors which ultimately regulate the transcription of the IL-2 gene [3, 4].

Recently, we reported that CsA induces a marked decrease in rat renal CABP-D 28 kDa protein [5, 6], which is paralleled by an increase in urinary calcium excretion, intratubular calcification and basophilic tubules [7]. CABP-D 28 kDa is described as a cytosolic calcium-binding protein with characteristics of known calcium-sensitive proteins such as troponin C and calmodulin [8]. It has been postulated to function as a calcium transport molecule which facilitates the diffusion of calcium through the cell and to serve as an intracellular calcium buffer, maintaining the ionized calcium below toxic levels during transcellular calcium transport [9]. This protein is found in many mammalian species and in various calcium-transporting tissues with its highest concentration in kidney and brain; in the latter the highest expression is found in cerebellum [10, 11]. Because it is known that at high doses, CsA can cross the BBB [12], we investigated whether CsA also has an effect on the expression of CABP-D 28 kDa in brain. Following treatment of rats with different doses of CsA, the concentration of the drug was determined in kidney, cerebral cortex and cerebellum, and CABP-D 28 kDa levels were quantified in the respective tissues.

MATERIALS AND METHODS

Animal Treatment Protocol

HanIbM: male Wistar rats (Biologic Research Labs), 8 weeks of age and weighing 225–280 g, were used. The animals were fed commercial rodent food (KLIBA 32-343-4, rat/mouse maintenance diet, Klingentalmühle AG) and tap water *ad lib*. Three groups of ten rats each received 15 mg/kg/day or 50 mg/kg/day of CsA orally by gavage (Sandimmun 100 mg/mL diluted 1:10 in olive oil) for 12 days or the vehicle solution (Sandimmun Placebo A diluted 1:10 in olive oil) for an identical period. The animals were sacrificed by decapitation 24 hr after the last treatment.

* Corresponding author: Dr. S. Steiner, Preclinical Safety, Toxicology WS-2881, Novartis Pharma AG, CH-4002 Basel, Switzerland. Tel. +41 (61) 324 10 09; FAX +41 (61) 324 10 27; E-mail: sandra.steiner @pharma.novartis.com.

† *Abbreviations:* BBB, blood-brain barrier; CABP-D, calbindin-D; CsA, cyclosporine A.

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TABLE 1. CABP-D 28 kDa concentration in kidney, cerebral cortex and cerebellum expressed as μg of CABP-D 28 kDa per mg of tissue

	Control	CsA 15 mg	CsA 50 mg
Kidney	3.4 ± 0.49	0.8 ± 0.1*	0.5 ± 0.1*†
Cerebral cortex	0.7 ± 0.08	0.7 ± 0.13	0.7 ± 0.09
Cerebellum	11.4 ± 1.18	12.1 ± 1.12	11.8 ± 2.6

Values represent the means ± SEM of 8–10 rats.
*Significantly different from control with $P < 0.01$.
†Significantly different from CsA 15 mg/kg/day with $P < 0.01$.

Sample Preparation

Renal cortex (150–200 mg) from the upper pole of the right kidney, prefrontal cortex from the right hemisphere of the brain (150–200 mg), or the right half of the cerebellum (150–200 mg) was homogenized in eight volumes of 9 M of urea, 4% Nonidet P-40, 1% dithiothreitol (DTT), and 2% carrier ampholytes pH 8–10.5 (Pharmacia) using a 1-mL Wheaton glass homogenizer (B. Braun). The homogenates were centrifuged at 420,000 *g* at 18° for 12 min (TL100 ultracentrifuge, TLA 100.3 rotor, 100,000 rpm, Beckman Instruments). The supernatant was removed, divided into four aliquots, and stored at −80° until analysis.

Quantification of CABP-D 28 kDa

Tissue CABP-D 28 kDa was quantified by ELISA as described previously for renal CABP-D 28 kDa [6, 7]. Samples from the different tissues and from control and treated animals were diluted in PBS to parallel the concentrations of the standard curve. The following dilutions were used: with kidney samples 1:250 in group A, 1:50 in group B and 1:25 in group C; cerebral cortex samples from all groups were diluted 1:25 and all cerebellum samples were diluted 1:400. CABP-D 28-kDa tissue protein levels were calculated from the standard curve obtained with concentrations of 2 to 128 ng of reference CABP-D 28 kDa (Swant). The protein content of the samples was measured by a modified Bradford assay as described previously [13].

Quantification of CsA Levels

In the tissue homogenate, the CsA concentrations were measured by RIA using a Sandimmun-Kit (Sandoz Pharma AG).

Histological Techniques

The center part from the right kidney, the left hemisphere of the brain, and the left part of the cerebellum were collected at necropsy and fixed in 10% buffered formalin. Cuts of the organs were embedded in paraplast. The sections were deparaffinized in xylol, alcohol gradient (100%, 95%, 80%, 50%) and water (5 min each), and then put into a solution of hydrogen peroxide in methanol for 10 min to block endogenous peroxidases. The slices were then washed twice for 5 min in PBS, and incubated for 10 min at room temperature (RT) in a humidity chamber with normal horse serum (Elite Vectastain ABC-kit; Vector Laboratories) to block background reactions. After the incubation, excess serum was removed with tissue paper, and sections were incubated overnight with monoclonal anti-CABP-D 28 kDa (mouse ascites fluid; Sigma) diluted 1:1000 in PBS at 4°. The slices were then washed with PBS and incubated at room temperature for 30 min with biotinylated goat anti-mouse IgG (Elite Vectastain ABC-kit). After incubation, the sections were washed with PBS and incubated for 30 min with a horseradish peroxidase-conjugated avidin-biotin complex (Elite Vectastain ABC-kit). Following another PBS-washing step, the slices were incubated with aminoethyl carbazole (AEC) until a red-brown color appeared in the calbindin-positive controls. The slices were washed again and counterstained with hematoxylin. Finally, the slices were treated with crystal mount, dried at 52°, and examined under the light microscope.

Statistical Analysis

Statistical analysis was performed using the Microsoft Excel 5.0 software package (Frontline Systems Inc). Significance was assessed using the two-sample unequal variance Student's *t*-test. Coefficients of determination with a *P*-value < 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

In kidney of CsA-treated rats, a marked decrease in CABP-D 28 kDa was observed in both dose groups. The CsA-related decrease in calbindin was highly statistically significant ($P < 0.01$, Student's *t*-test) vs the control group and was dose-dependent. Calbindin levels in the group treated with 15 mg/kg/day showed a $P < 0.01$ difference from the levels of the group treated with 50 mg/kg/day (Table 1). Immunostaining for CABP-D 28 kDa in kidney sections showed specific staining for tubular epithelium cells. In all treated animals, less and fainter staining was found, the effect being most prominent in the high dose group. In the kidneys of treated rats, a dose-dependent increase in CsA levels was found (3.93 ± 1.37 and 15.26 ±

3.26 μg of CsA/mg of tissue for 15 and 50 mg/kg/day of CsA, respectively).

In control rats, CABP-D 28 kDa in the cerebral cortex levels were approximately 5 times lower than in the kidney. No changes in cerebral cortex CABP-D 28 kDa were found after treatment with 15 mg/kg/day or 50 mg/kg/day of CsA for 12 days (Table 1). Immunoreaction for CABP-D 28 kDa in cerebral cortex sections showed immunostaining in scattered, mainly pyramidal, cells. In cerebral cortex, low concentrations of CsA could be detected at 50 mg/kg/day (0.86 ± 0.16 μg of CsA/mg of tissue), whereas at 15 mg/kg/day, CsA concentrations were below the limit of detection of 8 ng/mg of tissue.

In control rats, CABP-D 28 kDa levels in the cerebellum were found to be approximately three-fold those detected in kidney (Table 1). Immunostaining for CABP-D 28 kDa in cerebellum sections showed specific staining for the Purkinje cells. In the cerebellum, CsA concentrations were below the limit of detection of 8 ng/mg of tissue for both the high and low doses.

In kidney, a marked decrease in calbindin levels was found, which is consistent with earlier data from our laboratory [5–8]. Immunostaining identified the calbindin-positive cells as tubular epithelium cells. With the current material, it was not possible to conclude whether the cells were proximal or distal tubular cells. According to the literature, calbindin is described as being expressed in the distal tubular cells of kidney [14, 15].

In cerebral cortex and cerebellum, no effect of CsA on CABP-D 28-kDa levels was detected. This result can be explained by the finding that only low concentrations of CsA reached the brain tissue. It is known that CsA, despite its highly lipophilic nature, is not well transported through the BBB [16–18], which was thought to be due to the extensive blood binding of the drug [19] and its high molecular weight [20]. More recently, it was shown that CsA is transported by the P-glycoprotein [21–25], a transmembrane efflux transporter which is found on the luminal side of capillary endothelial cells that comprise the BBB. P-glycoprotein is the protein involved in the transport of antitumour agents in multidrug-resistant tumour cells [23, 26]. The brain penetration of CsA is reported to be dose-dependent [12], which is consistent with the detection of the low amount of CsA in cerebral cortex in high-dosed but not low-dosed rats.

Similarly, these data show that in rats treated with relatively high doses of CsA (50 mg/kg/day), only low concentrations of the drug reached the brain and that no effects on cerebellar or cerebral cortex CABP-D 28 kDa levels were found. It is assumed that for clinically relevant doses, under the assumption that the transmembrane efflux transporter is as effective in humans as in rodents [27], it is very unlikely that CsA has an effect on brain CABP-D 28 kDa.

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References

1. Handschumacher RE, Harding MW, Rice J and Drugge RJ, Cyclophilin, a specific cytosolic binding protein for cyclosporin A. *Science* **226:** 544–547, 1984.
2. Liu J, Farmer J, Lane W, Friedman J, Weisman I and Schreiber SL, Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66:** 807–815, 1991.
3. Fruman DA, Klee CB, Bierer BE and Burakoff SJ, Calcineurin phosphatase activity in T lymphocytes is inhibited by FK506 and cyclosporin A. *Proc Nat Acad Sci USA* **89:** 3686–3690, 1992.
4. O'Keefe SJ, Tamura J, Kincaid RL, Tocci MJ and O'Neill EA, FK506 and cyclosporine, a sensitive activation of the IL-2 promoter by calcineurin. *Nature* **357:** 692–694, 1992.
5. Benito B, Wahl D, Steudel N, Cordier A and Steiner S, Effects of cyclosporine A on the rat liver and kidney protein pattern and the influence of vitamin E and C coadministration, *Electrophoresis* **16:** 1273–1283, 1995.
6. Steiner S, Aicher L, Raymackers J, Meheus L, Esquer-Blasco R, Anderson NL and Cordier A, Cyclosporine A decreases the protein level of the calcium binding protein calbindin-D 28 kDa in rat kidney. *Biochem Pharmacol* **51:** 253–258, 1996.
7. Aicher L, Meier M, Norcross AJ, Jakubowski J, Varela MC, Cordier A and Steiner S, Decrease in kidney calbindin-D 28 kDa as a possible mechanism mediating cyclosporine A- and FK 506-induced calciuria and tubular mineralization. *Biochem Pharmacol* **53:** 723–731, 1997.
8. Bredderman PJ and Wasserman RH, Chemical composition, affinity for calcium, and some related properties of the vitamin D-dependent calcium-binding protein. *Biochemistry* **13:** 1687–1694, 1974.
9. Feher JJ, Facilitated calcium diffusion by intestinal calcium-binding protein. *Am J Physiol* **244:** C303–C307, 1983.
10. Sonnenberg J, Pansini AR and Christakos S, Vitamin D-dependent rat renal calcium-binding protein: Development of a radioimmunoassay, tissue distribution, and immunological identification. *Endocrinology* **115:** 640–648, 1984.
11. Thomasset M, Parkes CO and Gleizes-Cuisinier P, Rat calcium-binding proteins: Distribution, development, and vitamin D dependence. *Am J Physiol* **243** (*Endocrinol Metab* **6**)**:** E483–E488, 1982.
12. Lemaire M, Bruelisauer A, Guntz P and Sato H, Dose-dependent brain penetration of SDZ PSC 833, a novel multidrug resistance-reversing cyclosporin, in rats. *Cancer Chemother Pharmacol* **38:** 481–486, 1996.
13. Ramagali LS and Rodriguez LV, Quantification of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* **6:** 559–563, 1985.
14. Rhoten WB and Christakos S, Cellular gene expression for calbindin-D28k in mouse kidney. *Anat Rec* **227**(2)**:** 145–151, 1990.
15. Borke JL, Caride A, Verma AK, Penniston JT and Kumar R, Plasma membrane calcium pump and 28-kDa calcium binding protein in cells of rat kidney distal tubules. *Am J Physiol* **257** (*Renal Fluid Electrolyte Physiol* **26**)**:** F842–F849, 1989.
16. Niederberger W, Lemaire M, Maurer G, Nussbaumer K and Wagner O, Distribution and binding of cyclosporin in blood and tissues. *Transplant Proc* **15:** 2419–2421, 1983.
17. Begley DJ, Squires LK, Zlokovic BV and Mitrovic DM, Permeability of blood-brain barrier to immunosuppressive cyclic peptide cyclosporin A. *A J Neurochem* **55:** 1222–1230, 1990.
18. Cefalu WT and Pardrige WM, Restrictive transport of a lipid soluble peptide (cyclosporin) through the blood-brain barrier. *J Neurochem* **44:** 1954–1956, 1985.
19. Lemaire M, Pardridge WM and Chaudhuri G, Influence of blood components on the tissue uptake indices of cyclosporin in rats. *J Pharmacol Exp Ther* **244:** 740–743, 1988.

20. Partridge WM, Peptide lipidization and liposomes. In: *Peptide Drug Delivery to the Brain* (Ed. Partridge WM), pp. 123–148. Raven, New York, 1991.
21. Sakata A, Tamai I, Kawazu K, Deguchi Y, Ohnishi T, Saheki A and Tsuji A, *In vivo* evidence for ATP-dependent and P-glycoprotein mediated transport of cyclosporin A at the blood-brain barrier. *Biochem Pharmacol* **48:** 1989–1992, 1994.
22. Tsuji A, Tamai I, Sakata A, Tenda, Y and Terasaki T, Restricted transport of cyclosporin A across the blood-brain barrier by a multidrug transporter, P-glycoprotein. *Biochem Pharmacol* **46:** 1096–1099, 1993.
23. Hegmann EJ, Bauer HC and Kerbel RS, Expression and functional activity of P-glycoprotein in cultured cerebral capillary endothelial cells. *Cancer Res* **52:** 6969–6975, 1992.
24. Didier AD and Loor F, Decreased biotolerability for ivermectin and cyclosporin A in mice exposed to potent P-glycoprotein inhibitors. *Int J Cancer* **63:** 263–267, 1995.
25. Schinkel AH, Wagenaar E, Van-Deemter L, Mol CA and Borst P, Absence of the mdr1a P-glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96:** 1698–1705, 1995.
26. Lemaire M and Tillement JP, Role of lipoproteins and erythrocytes in the *in vitro* binding and distribution of cyclosporin A in the blood. *J Pharm Pharmacol* **34:** 715–718, 1982.
27. Takeguchi N, Ichimura K, Kioke M, Matsui W, Kashiwagura T and Kawahara K, Inhibition of the multidrug efflux pump in isolated hepatocyte couplets by immunosuppressants FK506 and cyclosporine. *Transplantation* **55:** 646–650, 1993.