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ORIGINAL ARTICLE

Cyclosporin and tacrolimus increase plasma levels of adenosine in kidney transplanted patients

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Summary

The immunosuppressive agents, cyclosporin (CsA) and tacrolimus (FK506), display cardioprotective activities. The mechanism would consist on the inhibition of the enzyme, adenosine kinase (AK), leading to an increase in adenosine (ADO) levels. ADO, inosine (INO) and nucleotide plasma levels were measured in kidney transplant recipients before and 1, 2, 4, 6 and 8 h after the administration of CsA or FK506. After CsA and FK506 administration, ADO plasma levels significantly increased, reaching a peak level after 2 h (483 ± 124 and 429 ± 96 nM, respectively), and then progressively declined. Calculated peak values (t_{max}) of ADO were slightly delayed with respect to those of CsA and FK506. Treatment with rapamycin did not influence the phenomenon. The dynamic profile of plasma changes of ADO, nucleotides and INO were consistent with the inhibition of the enzyme, AK. ADO increase may be clinically relevant in terms of anti-ischaemic, tissue protecting, and immunosuppressive activities as well as in terms of nephrotoxicity.

Introduction

Recent studies provided evidence that the immunosuppressive agents, cyclosporin (CsA) and tacrolimus (FK506), display tissue protective activities in animal models, by reducing ischaemia-dependent, and ischaemia/reperfusion-associated damage in the brain [1], heart [2,3] and liver [4]. Moreover, CsA and FK506 have been shown to mimic ischaemic preconditioning in isolated rabbit [5], and rat hearts [6]. The putative mediator of ischaemic preconditioning is the endogenous nucleoside adenosine (ADO), by the interaction with the A1 receptor coupled to the adenosine triphosphate (ATP)-dependent K^+-channel, and the A3-receptor on cardiomyocytes [7,8]. Indeed, both CsA and FK506 have been recently shown to produce an increase in ADO in plasma and in different cell lines in *ex vivo* and *in vitro* models, which may in turn account for the tissue protective effects observed with these drugs [9–11]. Moreover, since ADO is also provided with immunosuppressive activities [12–19], it has been postulated that ADO increase may contribute to the pharmacological effects of CsA and FK506 on the immune response [9–11]. The mechanism of action of the two drugs leading to the ADO increase is mainly attributed to the inhibition of the activity of the enzyme adenosine kinase (AK) associated with the plasma membrane. As a consequence, AK-mediated ADO dephosphorylation to adenosine monophosphate (AMP) is inhibited, and cell release of ADO is promoted [9–11]. Moreover, an inhibition of ADO cell uptake has also been reported, and CsA has been described to reduce by 67% the number of ADO transporters in the rat brain [20]. The activity of CsA and FK506 on the ADO system was studied *in vitro* in red blood cells [9], endothelial cells [10] and T lymphocytes [11]. In our knowledge, the only *ex vivo* evidence that treatment with CsA or FK506 is accompanied by high mean ADO plasma levels was provided by Guieu *et al.* in a study performed with single ADO measurements on kidney transplant recipients compared with chronic kidney failure patients, and control subjects [9].

The present study was aimed at evaluating the dynamic profile of ADO in plasma from kidney-transplanted patients, following the administration of CsA and FK506.

In order to contribute to clarify the mechanism of action of the drugs on the ADO system, plasma levels of adenine nucleotides and inosine (INO) were also measured.

Finally, as the immunosuppressive agent rapamycin has been shown to significantly decrease ADO uptake into endothelial cells [10], the possible influence of the concomitant treatment with this drug (mean daily dose±SD: 1.57 ± 0.75 mg) on ADO levels was also evaluated.

Materials and methods

Plasma levels of ADO were measured in kidney transplant recipients after obtaining their informed consent in accordance with the Principles of the Declaration of Helsinki, before, and 1, 2, 4, 6 and 8 h after the morning administration of CsA (Sandimmun Neoral, Novartis Farma, Origgio, Varese, Italy; $n = 10$) or FK506 (Prograf, Fujisawa, Milano, Italy; $n = 12$). Plasma concentrations of the drugs and serum creatinine were also measured. Moreover, in five patients from each group, plasma levels of AMP, adenosine diphosphate (ADP), ATP and INO were also measured, and the INO/nucleotide pool (AMP + ADP + ATP), ADO/INO and AMP/ATP ratio were calculated.

Demography and characteristics of patients, as well as concomitant treatment are shown in Table 1.

Adenosine, AMP, ADP, ATP and inosine assay

For ADO, AMP, ADP, ATP and INO determination, blood samples, anticoagulated with heparin (100 IU/ml of blood) were collected in ice-cooled syringes, containing a 'stopping solution' [50 μM dipyridamole, Boehringer, Ingelheim, Germany; 10 μM erythro-6-amino-9-(2-hydroxy-3-nonyl)-purine hydrochloride (EHNA), Wellcome Italia S.p.A., Pomezia, Italy; 79 μM α,β-methylene-adenosine-5′-diphosphate (AOPCP), Sigma Aldrich, Milan, Italy], in order to prevent cellular uptake, enzymatic deamination or production from blood ATP respectively. The blood was centrifuged at 1800 *g* at 4 °C and then the plasma was immediately deproteinized with acetonitrile and the supernatant analysed for ADO concentration by high-performance liquid chromatography (HPLC). The chromatographic method was performed in an isocratic system, the mobile phase of which consisted of 35 μM phosphate buffer, acetonitrile and methanol (95:2.5:2.5, v:v) at the flux rate of 1 ml/min. The wavelength of detection was fixed at 254 nm.

Table 1. Characteristics of patients.

	CsA	FK506	*F*-value	*P*-value
n	10 (8 M/2F)	12 (8 M/4 F)		
Age (years)	43.6 ± 15	47.3 ± 11	0.42	0.52 NS
Days from transplant	11.6 ± 3.1	11.7 ± 3.7	1.02	0.92 NS
Serum creatinine (mg/dl)	1.9 ± 0.9	2.7 ± 0.9	3.76	0.07 NS
Morning dose (mg)	275 ± 75	5 ± 1		
Evening dose (mg)	280 ± 77	5 ± 1		
Concomitant treatment (*n*)				
Glucocorticoids	10	12		
Azathioprine	4	3		
Mycophenolate mofetil	4	4		
Rapamycin	2	5		

Statistical evaluation was performed by the one-way analysis of variance.

CsA, cyclosporin; NS, nonsignificant.

CsA and FK506 assay

Plasma levels of CsA were measured with the fluorescence polarized light immunoassay (FPIA) kit AxSYM System Cyclosporin (Abbott Laboratories, Abbott Park, IL, USA). Plasma levels of FK506 were measured with the immunoenzymatic (MEIA) method IMx System Tacrolimus (Abbott Laboratories).

Calculation of t_{max}

The time to achieve the highest measured concentration of CsA, FK506 and ADO (t_{max}), was calculated with the easy fit computer program by use of a nonlinear least-squares algorithm [21].

Statistical analysis

Statistical evaluation was performed by the one-way ANOVA, and the paired or unpaired Student's *t*-test. The Pearson's correlation coefficient was calculated for comparisons among the various parameters under study. Differences were considered significant at a value of $P < 0.05$.

Results

After CsA administration, ADO plasma levels significantly increased, reaching a peak level after 2 h (from 273 ± 29 to 483 ± 124 nM; Student's *t*-test: $P < 0.001$), and then progressively declined (ANOVA: $F = 14.3$; $P < 0.001$; Fig. 1a). Peak values of ADO coincided with those of CsA (from 322 ± 92 to 1432 ± 453 ng/ml at the second hour; Fig. 1a). Similar results were obtained after FK506 administration: ADO plasma levels increased from 279 ± 39 to

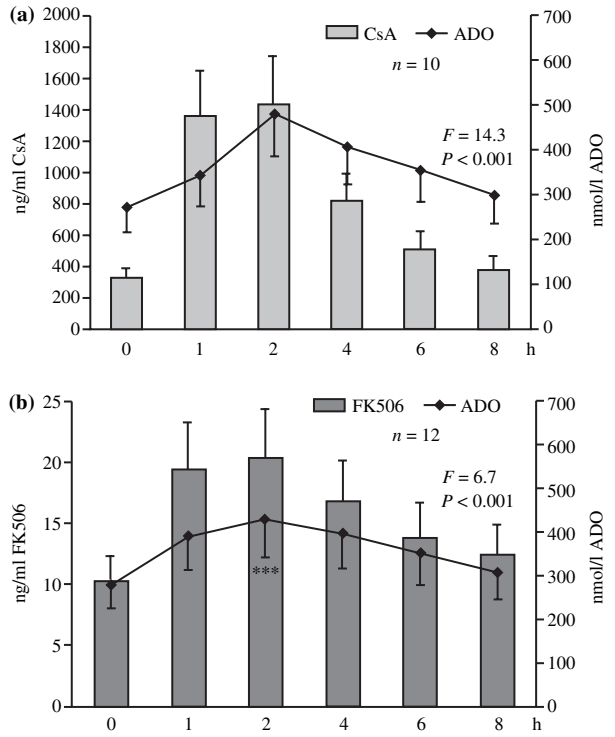

Figure 1 (a) Effect of cyclosporin (CsA) on plasma levels of adenosine (ADO; $n = 10$). (b) Effect of FK506 on plasma levels of adenosine (ADO; $n = 12$). One-way anova and Student's *t*-test for paired data (peak versus baseline). ***$P < 0.001$.

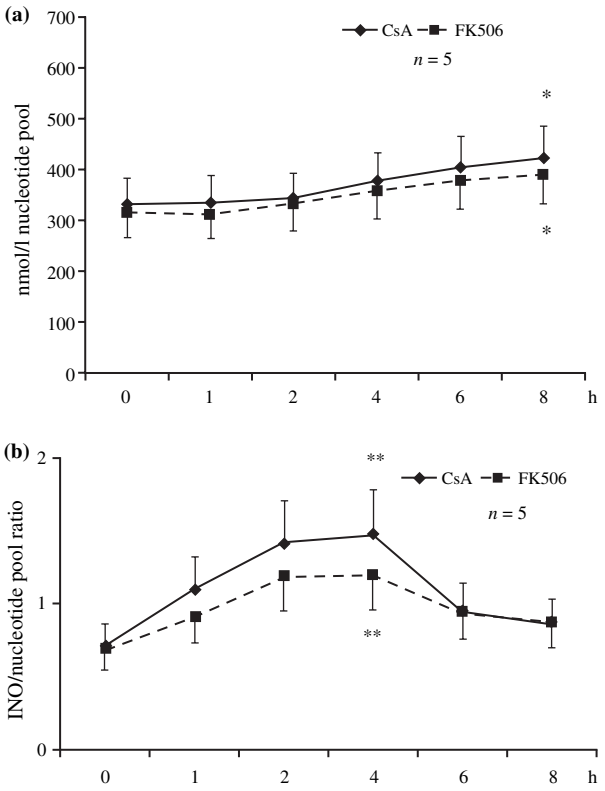

Figure 2 (a) Effect of cyclosporin (CsA) (continuous line) and FK506 (dashed line) on plasma levels of the nucleotide pool (AMP + ADP + ATP). (b) Effect of CsA (continuous line) and FK506 (dashed line) on inosine (INO)/nucleotide pool ratio in plasma. Student's *t*-test for paired data (peak versus baseline). *$P < 0.05$; **$P < 0.01$; $n = 5$.

Table 2. t_{max} (h) of CsA, FK506 and ADO in CsA- and FK506-treated patients.

CsA-treated patients ($n = 10$)		KF506-treated patients ($n = 12$)	
CsA	ADO	FK506	ADO
1.73 ± 0.33	2.66 ± 0.95*	2.00 ± 0.98	2.89 ± 1.06*

CsA, cyclosporin; ADO, adenosine.
Data are expressed as mean ± SD.
Student's *t*-test for paired data (t_{max} of CsA and FK506 versus t_{max} of ADO).
*$P < 0.05$.

429 ± 96 nM after 2 h (Student's *t*-test: $P < 0.001$), and then progressively declined (ANOVA: $F = 6.7$; $P < 0.001$; Fig. 1b). Also in this case, peak values of ADO coincided with those of FK506 (from 10 ± 5 to 20 ± 9 ng/ml at the second hour; Fig. 1b). However, the calculation of t_{max} showed that maximum increase in ADO plasma level was slightly but significantly delayed with respect to that of CsA and FK506 (Table 2).

The CsA and FK506 blood levels correlated with the increase in ADO plasma levels ($r = 0.817$, $P < 0.05$; and $r = 0.968$, $P < 0.01$, respectively). In particular, when considering selectively ADO plasma level and CsA blood concentration after 2 h from drug administration (C2), a correlation was also observed between the two groups of data ($r = 0.639$, $P < 0.05$).

Plasma nucleotide pool (AMP + ADP + ATP) remained unchanged up to the fourth hour after the administration of CsA and FK506, and then progressively increased. Maximal increase was measured after 8 h being 27% and 23.6% for CsA and FK506, respectively (PST: $P < 0.05$; Fig. 2a). Among the components of the nucleotide pool, no significant change was observed in the AMP/ATP ratio (Fig. 2b).

Inosine/nucleotide pool ratio increased from 0.72 to 1.48 and from 0.69 to 1.2 for CsA and FK506, after 4 h from drug administration (PST: $P < 0.01$; Fig. 3a).

Conversely, the ADO/INO ratio did not significantly increased (Fig. 3b).

Treatment with rapamycin did not influence the increase in ADO plasma levels following the administration of CsA (Fig. 4a) and FK506 (Fig. 4b), or it produced any effect on

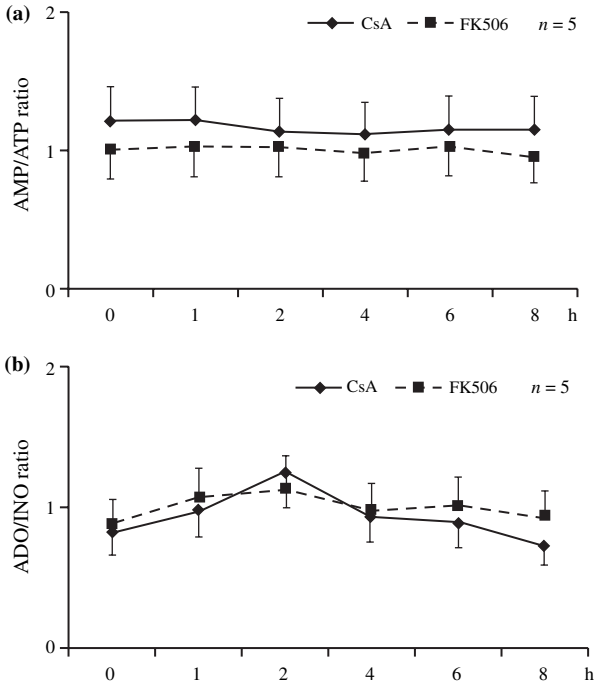

Figure 3 (a) Effect of cyclosporin (CsA) (continuous line) and FK506 (dashed line) on AMP/ATP ratio in plasma. (b) Effect of CsA (continuous line) and FK506 (dashed line) on adenosine (ADO)/inosine (INO) ratio in plasma. Student's *t*-test for paired data (peak versus baseline). Differences were not significant; $n = 5$.

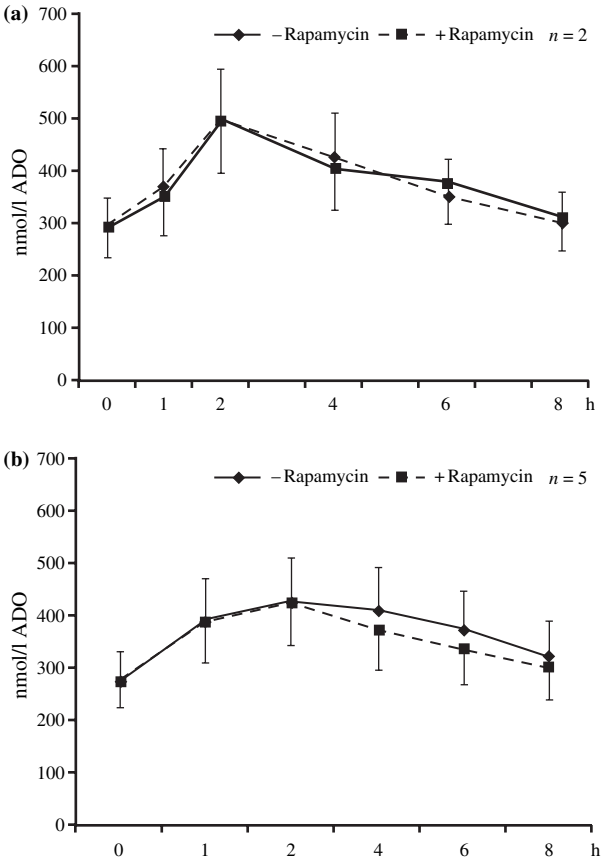

Figure 4 (a) Plasma levels of adenosine (ADO) in cyclosporin-treated kidney transplant recipients with (continuous line) or without (dashed line) the concomitant administration of rapamycin ($n = 2$). (b) Plasma levels of ADO in FK506-treated kidney transplant recipients with (continuous line) or without (dashed line) the concomitant administration of rapamycin ($n = 5$). Student's *t*-test for paired data (rapamycin-treated versus rapamycin-untreated patients). Differences were not significant.

t_{max} of FK506 and ADO (Table 3). Because of the small sample size, the effect of rapamycin on t_{max} in CsA-treated patients was not evaluated.

Discussion

Present data show that CsA and FK506 administration is associated with a significant increase in plasma levels of ADO in kidney transplant patients, also showing for the first time the time-course of this effect.

The ADO plasma levels are dependent on the metabolic activity of endothelial [22,23] and circulating cells, mainly erythrocytes [24] and platelets [25], and of myocytes [22,23]. Intracellular ADO originates from nucleotide dephosphorylation [26] and S-adenosylhomocysteine hydrolysis [27]. Once ADO is formed, it may undergo rephosphorylation to AMP catalysed by the enzyme AK [28], deamination to INO catalysed by the enzyme adenosine deaminase (ADA) [29], or release from the cells [30]. ADO can also be formed in the extracellular compartment by dephosphorylation of AMP by ecto-5′-nucleotidase [23,31]. Once in the extracellular space, ADO may exert its biological functions before undergoing cell reuptake and degradation. In physiological conditions, phosphorylation to AMP by the membrane-associated enzyme

Table 3. Effect of concomitant therapy with rapamycin on t_{max} (h) of FK506 and ADO in FK506-treated patients.

FK506		ADO	
–rapamycin ($n = 7$)	+rapamycin ($n = 5$)	–rapamycin ($n = 7$)	+rapamycin ($n = 5$)
2.07 ± 1.16	1.90 ± 0.78 NS	2.83 ± 1.16	2.98 ± 1.04 NS

ADO, adenosine.
Data are expressed as mean ± SD.
Student's *t*-test for unpaired data (rapamycin-treated versus -untreated patients).

AK is the main route of ADO degradation, accounting for about 70–80% of the catabolism of the nucleoside [19,32], thus predominating over ADA-catalysed deamination to INO. Blocking of AK activity would lead to an

augmented availability of ADO with a net increase in the cell release of the nucleoside [33].

Previous studies showed that CsA and FK506 produce an increase in ADO levels by inhibition of the enzyme AK associated to the plasma membrane, in different cell types [10,11]. Moreover, an inhibition of cell uptake of the nucleoside has been described, and CsA has been shown to reduce by 67% the number of ADO transmembrane transporters in rat brain [20]. The mechanisms are closely related, as it has been demonstrated that it is unlikely that AK blocking may take place without the inhibition of cell uptake of ADO [33,34].

In our study, the evaluation of the mode of action of the compounds is not directly addressed. However, some indirect evidence seems to be consistent with the possibility of an inhibition of the AK activity, as elsewhere reported [10,11].

The finding that CsA or FK506 treatment is associated with an increase in the INO/nucleotide pool ratio is consistent with the possibility that the drugs may inhibit the enzyme AK, thus shifting ADO catabolism towards ADA-mediated INO generation rather than AK-catalysed nucleotide formation [32]. Further, evidence of the possible occurrence of this effect is also provided by the observation that ADO and INO increase occurs promptly, whereas the increase in plasma levels of the nucleotide pool begins at least after 4 h from drug administration. In fact, this phenomenon could be the expression of the CsA- and FK506-dependent inhibition of the AK-catalysed nucleotide formation.

The increase in ADO plasma levels due to the treatment with nucleoside transport inhibitors, in the presence of a normal AK activity, is characterized by a marked increase in ADO/INO ratio, at least in ischaemic conditions [35]. The finding that in our experimental conditions ADO/INO ratio does not significantly increase may further support the view of a blocking in the AK-dependent metabolism of ADO leading to emphasize the ADA-dependent catabolic pathway of the nucleoside.

The possibility that CsA and FK506 may affect the activity of 5′-ecto-nucleotidase thereby contributing to the increase in ADO plasma levels would conflict with the observation that the AMP/ATP ratio does not increase in our study [32]. This conclusion is in agreement with previous observations ruling out an interference of CsA and FK506 with 5′-ecto-nucleotidase [10,11].

It has been previously shown that azathioprine and prednisone do not affect cell release of ADO [9], thereby not influencing plasma levels of the nucleoside in kidney transplant recipients. Conversely, in an *in vitro* study, rapamycin have been shown to significantly decrease ADO uptake into endothelial cell in a concentration-dependent manner [10]. Thus, the possibility that concomitant treatment with rapamycin might influence CsA- and FK506-associated increase in ADO plasma levels in kidney transplant recipients was also evaluated in a subgroup of subjects. In our experimental conditions, plasma levels of ADO in rapamycin-treated patients were not significantly different from those observed in rapamycin-untreated patients. Indeed, the fact that rapamycin trough levels were not measured may represent a limitation of this part of the study. The effect of rapamycin alone on plasma levels of ADO was not evaluated in our study. As a consequence, on the basis of our observations we can only rule out a possible effect of rapamycin at the mean dose of 1.57 ± 0.75 mg on ADO metabolism additive to that of CsA and FK506.

The ADO increase may be of pharmacological interest since following CsA and FK506 administration, ADO reaches values high enough to be of physiological relevance [36,37]. The biological significance of extracellular ADO increase is related to its vasodilating and tissue protecting properties during ischaemia and inflammatory reactions [36–43], mediated by the interaction with four specific membrane receptors, termed A1, A2A, A2B and A3 [18,44,45]. Moreover, ADO is also provided with natural immunosuppressive activities including inhibition of cytokine release, namely tumor necrosis factor (TNF)-α, interleukin (IL)-2 and IL-6 [14,15,17,18,46,47]. Thus, the presently reported CsA and FK506 enhancing effect on ADO plasma levels may be of peculiar interest in the light of a possible contribution to their immunosuppressive activity, also producing a possible explanation for the observed tissue protecting effect towards ischaemia displayed by the two drugs in experimental models, which can mainly be attributed to the activation of the A2A-receptor on inflammatory and immune cells [48]. On the other side, it should be noted that ADO increase might contribute to CsA and FK506 nephrotoxicity [9,49]. In fact, since ADO A1-receptor stimulation mediates renal vasoconstriction, an increase in ADO plasma levels may be detrimental to the renal function, particularly in kidney transplant recipients [9].

In conclusions, CsA and FK506 treatment is associated with an increase in plasma levels of ADO in kidney transplant recipients. The dynamic profile of plasma changes of ADO, nucleotides and INO is consistent with the inhibition of the enzyme AK. Concomitant treatment with rapamycin, does not seem to add any further contribute to the phenomenon. ADO increase may be clinically relevant in terms of anti-ischaemic, tissue protecting, and immunosuppressive activities as well as in terms of nephrotoxicity.

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