Differential effects of immunosuppressive drugs on COX-2 activity in vitro and in kidney transplant patients in vivo

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Abstract

Background. It was hypothesized that calcineurin inhibitors suppress vascular cyclooxygenase (COX)-2 and exert a reciprocal influence on in vivo prostacyclin and thromboxane. This could contribute to cardiovascular morbidity in transplanted patients.

Methods. The ability of immunosuppressive drugs to suppress vascular COX-2 expression in vitro was studied in cultured human vascular smooth muscle cells. Blood and urine samples were collected from 28 renal transplant patients before and 2, 4 and 6 h after intake of immunosuppressives and from 11 controls. ELISA was used to measure (1) plasma 6-keto-PGF1α and TxB2; (2) urine excretion of PGI-M and TxB2; (3) 6-keto-PGF1α in the whole-blood COX-2 assay; and (4) TxB2 in the whole-blood COX-1 assay. Platelet aggregation was measured optically.

Results. COX-2 in cultured vascular smooth muscle cells was suppressed by cyclosporine A (CsA); tacrolimus and rapamycin had no effect. Human renal arteries and vascular smooth muscle expressed calcineurin Aβ and Aγ
Immunosuppressives and COX-2

Introduction

Premature cardiovascular morbidity is the leading cause of death in transplanted patients [1,2]. Immunosuppressive therapy is thought to contribute to cardiovascular disease [3]. The calcineurin inhibitors (CNI), cyclosporine A (CsA) and tacrolimus, raise the blood pressure [4] and are believed to promote atherosclerosis, microangiopathy and thromboembolic events [5]. The mechanisms underlying these side effects are not clear. CsA impairs endothelium-dependent vasorelaxation [6] and decreases prostacyclin (PGI2) release from vascular rings [7] and cultured human endothelium [8]. PGI2 infused to rats attenuates CsA-mediated renal hypoperfusion [9]. PGI2 is synthesized predominantly by cyclooxygenase-2 (COX-2) in healthy humans [10]. Selective suppression of PGI2 is thought to be responsible for the increased cardiovascular morbidity and mortality seen in patients treated with selective COX-2 inhibitors [11]. Furthermore, CsA suppresses the expression of COX-2 in cultured cells induced by vascular endothelial growth factor (VEGF) [12] and both CsA and tacrolimus suppressed constitutive COX-2 expression in rat kidney [13]. COX-2 is expressed physiologically in human vasculature [14,15] and may be the source for systemic vasodilatory and antithrombotic PGI2. Taken together, suppression of vascular COX-2 and systemic PGI2 after CNI treatment may afford a causal explanation for some common CNI side effects, e.g. systemic hypertension, renovascular impairment and thromboembolic events. Because PGI2 signalling imposes tonically a constraint on platelet activation, suppression of COX-2 predicts an augmented production of thromboxane (TxA2) and platelet aggregation. Increased platelet aggregation has been reported in relation to CNIs [16–19]. Immunosuppressives that target molecules other than calcineurin, e.g. mTOR inhibitors, may not display these effects. In the present study, we hypothesized that the CNIs CsA and tacrolimus, but not the mTOR inhibitor rapamycin, suppress vascular COX-2 expression and systemic PGI2 production and augment platelet thromboxane synthesis and aggregation.

The hypothesis was explored in vitro with cultured human vascular smooth muscle cells (VSMCs) and in vivo in renal transplant patients with stable graft function routinely treated with CsA, tacrolimus or rapamycin. Four consecutive blood and urine samples were taken from each individual under similar conditions from nadir to peak in plasma immunosuppressive drug concentrations and indices of systemic COX activity were analysed. Because several factors were not controlled for [NaCl intake, age and gender, glomerular filtration rate (GFR), medication], the full capacity of COX-1 and -2 activities was determined also ex vivo in standardized whole-blood assays with four consecutive samples [20].

Methods

In vitro studies

Human renal arteries were taken from patients, who underwent radical unilateral nephrectomy for renal cancer. All patients gave their informed written consent to participate in the study (VF 20010035). None of the cancer patients had received chemotherapy or radiation therapy before nephrectomy. Kidneys were extirpated and immediately transported to the Institute of Pathology. Sections of the renal artery were dissected and immediately frozen. Total RNA was isolated by the acid-guanidinium-thiocyanate method [14]. Total RNA from the human cerebral cortex was obtained from Ambion (Applied Biosystems, Naerum, Denmark). Human aortic vascular smooth muscle cells (hVSMCs) were cultured according to the instructions (ATCC: LSG Standards AB, Boras, Sweden). In all series, subconfluent cells were deprived of serum for 24 h before the experiments and supplied with 0.5% bovine serum albumin.

Series 1. Cells were exposed to fetal calf serum (FCS) 10% for variable times and RNA was isolated by means of RNAeasy mini columns (Qiagen, Albertslund, Denmark).

Series 2. Cells were exposed to FCS 10% for 2 h with and without CsA, tacrolimus, rapamycin and prednisolone. Next, total RNA was harvested and cDNA was synthesized. COX isoform and β-actin mRNA abundance were determined as in series 1.

Series 3. Cells grown on coverglass were exposed to FCS 10% for 2 h. Then, cells were fixed and permeabilized cells were labelled for COX-2 by immunofluorescence as described [14].

Series 4. hVSMCs were exposed to FCS (20%, 20 h) with or without immunosuppressive drugs (CsA, tacrolimus, rapamycin, prednisolone; Sigma Aldrich, Denmark) and the COX-2 inhibitor, NS-398 (Cayman Chemicals, Michigan, USA). Then, the cell-conditioned medium was collected and the PGI2 metabolite, 6-keto-PGF1α, was subsequently measured in the medium by ELISA NS-398 (Cayman Chemicals, no. 515211).

Synthesis of cDNA was performed with 1 µg total RNA (iScript cDNA synthesis kit, BioRad). For Q-PCR, 50 ng cDNA in duplicate was used as template and mixed with the respective primers and iQ-SYBR Green Supermix (BioRad) was added in a final volume of 25 µL. The mixture was denatured for 3 min at 95°C and 44 cycles
**Table 1.** Baseline characteristics of renal transplant patients receiving cyclosporine A (CsA), tacrolimus (Tac) and rapamycin (Rap) and healthy control subjects

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>CsA</th>
<th>Tac</th>
<th>Rap</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
<td>11</td>
<td>11</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>53 (29–78)</td>
<td>49 (19–63)</td>
<td>49 (39–62)</td>
<td>47 (26–70)</td>
</tr>
<tr>
<td>Male (%)</td>
<td>64</td>
<td>91</td>
<td>75</td>
<td>78</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>36</td>
<td>25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>GFR (mL/min/1.73 m²)</td>
<td>77 (64–112)</td>
<td>72 (35–152)</td>
<td>65 (53–96)</td>
<td>39*** (18–74)</td>
</tr>
<tr>
<td>Mean arterial BP (mmHg)</td>
<td>99 (87–112)</td>
<td>108 (97–120)</td>
<td>105 (84–123)</td>
<td>107 (89–126)</td>
</tr>
<tr>
<td>C-reactive protein (CRP) (mg/L)</td>
<td>5 (1–12)</td>
<td>5 (0–12)</td>
<td>27 (2–148)</td>
<td>17 (4–99)</td>
</tr>
<tr>
<td>Time since tx (days)</td>
<td>814 (21–4320)</td>
<td>525 (34–1932)</td>
<td>1931 (176–4768)</td>
<td>1931 (176–4768)</td>
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<tr>
<td>MMF</td>
<td>11</td>
<td>8</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>Ca antagonist</td>
<td>10</td>
<td>4</td>
<td>5</td>
<td>5</td>
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<tr>
<td>ACE inhibitor</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>7</td>
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<td>AT1 antagonist</td>
<td>2</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>β-blocker</td>
<td>6</td>
<td>3</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>α-blocker</td>
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<td>4</td>
<td>6</td>
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</table>

Mean and range are given. GFR is estimated from the Cockcroft–Gault equation. Comparison with the control group: ***P < 0.01.

MMF: mycophenolate mofetil.

were run on an iQ-Thermocycler (BioRad): denaturation 30 s 95°C, annealing and extension 45 s at 60°C. Emitted fluorescence was detected during the annealing/extension step in each cycle. The standard curve was constructed by plotting threshold cycle (Ct-values) against serial dilutions of plasmid template or PCR product. Negative controls with no cDNA and positive controls with kidney cDNA were run in each plate. Specificity was established post-run for each plate setup by melting curve analysis. COX-1, COX-2 and beta-actin primers were as described [14]. Calcineurin primers were CnAα: sense GTGTTTCTCAGTGCTCAGA, antisense: CCTCGAAGCTAGTGATCT, covering bases 1631–1820 nm_000944; Aβ sense: AACCATACTGCCCAGTGA, antisense: GGTCTCAAGAAGCACAATG, covering bases 1621–1798, Genebank acc no. bc035464; Aγ: sense TTCAGCACAAGATCGGGA, antisense: ACCAGCGTGTATGCTATC, covering bases 1681–1788 acc no. nm_005605 (MWG Ebersberg, Germany).

**Human subjects**

The study was approved by the regional ethical committee (VF 20040144) and all subjects gave their written informed consent. Patients were recruited from the outpatient clinic or when hospitalized after renal transplantation. Twenty-eight renal transplant patients with stable graft function were studied (Table 1). All patients were on prescribed treatment that was controlled independent of the present study. As additional immunosuppression, all patients except one in the rapamycin group received mycophenolate mofetil, and seven of nine patients in the rapamycin group received glucocorticoids. Most patients received antihypertensive drugs and some were taking diuretics (Table 1). Acetyl salicylic acid or NSAID/selective COX-2 inhibitor intake ceased 7 days before the study day. Age- and sex-matched control persons received no medication; their urine was screened for glucose and protein, and the blood for haematological parameters, electrolytes, C-reactive protein, alanine aminotransferase and alkaline phosphatase.

**In vivo protocol**

The subjects emptied their bladder at 07.00 a.m. and drank 200 ml of fluid every 30 min throughout the study period. After being placed in a seated position at 08.30, an intravenous cubital line was placed for blood sampling, and at 09.00 the first sample was taken followed by urination. The patients then ingested their usual immunosuppressive medication followed by blood and urine sampling at 2, 4 and 6 h. The subjects were allowed to eat lightly except for 30 min before blood collection during which period they were seated, and the blood pressure was measured after 20 min in the seated position. Blood pressure was measured with an automatic device using the oscillometric method (UA787, A&D Company LDL, 1-243, Saitama, Japan), and the average of three measurements was used. Blood was taken and immediately used for the platelet aggregation study (at 0 h and 2 h) and plasma was separated for analysis of prostanoids and immunosuppressive drug concentrations (at 0, 2, 4 and 6 h). Urine collection periods for prostanooid analysis preceded each blood sampling.

**Determination of eicosanoids in plasma and urine**

The PGI2 metabolites, 6-keto-PGF1α and 2,3-dinor-6-keto-PGF1α (PGI-M), were measured in urine by ELISA (Assay Designs, Ann Arbor, Michigan). The PGI2 metabolite 6-keto-PGF1α was measured in plasma by ELISA (Cayman Chemicals, Michigan USA). The performance of the assays was tested (recovery, accuracy and precision). Without extraction, the assay was accurate as judged from serial dilutions of pooled human plasma (variance 11%). The
recovery of pure 6-keto-PGF$_{1a}$ added to plasma was close to 100%. The TxA$_2$ hydrolysis product TxB$_2$ was measured in both plasma and urine by ELISA (Assay Designs, Ann Arbor, Michigan). Plasma samples (250 μL) were acidified with 18 μL 50% formic acid and extracted with 1 mL ethylacetate/hexane (70/30) by vortexing and centrifugation. The supernatants were vacuum dried and dissolved in a 250 μL assay buffer before ELISA.

Cyclooxygenase ex vivo whole-blood assays

As an index of the ability to activate maximally COX-2 in preferential monocytes/macrophages, the method of Patrignani et al. [20] was used: 1 mL aliquots of whole blood containing 10 IU of heparin was incubated with or without lipopolysaccharide (LPS, *E. coli*, 026:B6, Sigma, 10 μg/mL at 37°C for 24 h). Accumulated 6-keto-PGF$_{1a}$ was measured in plasma. Pilot experiments were performed to validate the assay (LPS concentration-response, sensitivity to COX-2 inhibitor, NS-398 and dexamethasone). As a measure of the capacity of platelets to metabolize arachidonic acid through the COX-1 pathway, aliquots of non-anticoagulated whole blood (1 mL) were incubated for 1 h at 37°C in glass vials. Serum was then separated by centrifugation at 1500 g for 10 min and TxB$_2$ was determined by ELISA (Assay Design 901-002).

Platelet aggregation

Venous blood was collected into vacutainer tubes containing 0.129 mol/L sodium citrate. Platelet aggregation was assessed within 1 h of sampling by means of optical platelet aggregometry and was performed in a lumi-aggregometer [Platelet Aggregation Profiler (PAP-4), BIO/DATA Corporation, Horsham, PA, USA]. Platelet-rich plasma was prepared by centrifugation at 180 g for 10 min at room temperature. The residual blood was centrifuged at 1500 g for 10 min to obtain platelet-poor plasma. The platelet count in platelet-rich plasma was measured, and the platelet-poor plasma was used to standardize the platelet count to 250 × 10$^9$/L. After 1 min of incubation at 37°C, platelet aggregation was induced by the addition of adenosine diphosphate (ADP) (1.0 μmol/L). Change in turbidity because of aggregation was recorded and given in % point. Maximal aggregation was measured in duplicate within 6 min of addition of ADP.

Plasma concentration of immunosuppressives and estimation of renal function

CsA, tacrolimus and rapamycin plasma concentrations were measured with standard techniques [Cyclosporine TDx Monoclonal Whole Blood, IMx Tacrolimus II Enzyme Immunoassay IL (Abbott, IL, USA), rapamycin by LCMSMS]. Glomerular filtration rate (GFR) was estimated from creatinine values measured in the plasma of blood samples taken before drug intake, and age, weight and gender of the subject according to the Cockcroft–Gault equation [21].

Statistics

For the cell culture studies, data were evaluated for significance by one-way analysis of variance followed by Dunnett’s post hoc test. Data sets from the human study were tested for normal distribution. If data were normally distributed, the test of significance was done by ANOVA, followed by Dunnett’s multiple comparison post hoc test (control t = 0) or unpaired t-tests where relevant. $P < 0.05$ was considered statistically significant. All calculations were performed with GraphPad Prism software.

Results

Effect of immunosuppressives on COX-2 in human vascular smooth muscle cells

COX-2 was expressed at a low level in cultures of serum-deprived hVSMC (Figure 1A). FCS increased the COX-2 mRNA level maximally at 2 h after which the abundance declined (not shown n = 3). The FCS-induced increase in COX-2 mRNA at 2 h was attenuated significantly by CsA ($P < 0.05$) but not altered by tacrolimus, rapamycin or prednisolone (Figure 1A). FCS, with or without immunosuppressants, had no effect on COX-1 mRNA (not shown, n = 5). FCS increased significantly the accumulation of PGI$_2$ metabolite 6-keto PGF$_{1a}$ in a cell-conditioned medium, and the increase was abolished by the COX-2 selective blocker, NS-398 (Figure 1B). Tacrolimus and rapamycin had no significant effect on the 6-keto-PGF$_{1a}$ concentration. CsA abolished and prednisolone attenuated significantly the effect of FCS on 6-keto-PGF$_{1a}$ production (Figure 1B). Immunochemistry labelling showed that COX-2 immunoreactive protein was at the detection limit in serum-deprived (24 h) hVSMC (Figure 1C). Exposure to FCS (2 h) led to a significant rise in COX-2 immunochemistry. COX-2 was associated with the majority of FCS-exposed cells and the signal was attenuated significantly by concomitant exposure to CsA (Figure 1C). No signal was detected in the absence of primary COX-2 antibody (Figure 1C).

Expression of catalytic calcineurin A isoforms in human vasculature

All three known catalytic calcineurin A isoforms (CnAα, CnAβ and CnAγ) were detected by PCR amplification of human cerebral cortex cDNA (Figure 2A). Serum-deprived human aortic VSMC expressed CnAβ and CnAγ isoforms (Figure 2B). CnAα was not consistently detected. CnAβ and CnAγ but not CnAα were detected in three preparations of human renal artery cDNA (Figure 2C). COX-2 mRNA was detected in human renal arteries (Figure 2C). PCR products were obtained only in the presence of reverse transcriptase and cDNA.

Patient characteristics

There were no differences between the patient groups and the controls regarding age (Table 1). The estimated GFR
was not statistically significantly different between patients on CsA and tacrolimus and controls (72, 65 and 77 mL/min/1.73 m², means, respectively). GFR was lower in patients on rapamycin (39 mL/min/1.73 m², \( P < 0.01 \)) than in controls (Table 1). There were no significant differences in the time elapsed after transplantation between experimental groups (Table 1). The mean arterial blood pressure tended to be higher in the transplanted patients than in controls but no significant difference between groups was found (Table 1). Blood pressure did not change significantly during the study day in any of the groups (data not shown). As shown in Table 1, the use of blood pressure lowering agents, diuretics and prednisolone, and the occurrence of diabetes differed between patient groups. C-reactive protein did not differ between groups (Table 1).

Effect of immunosuppressive drugs on prostacyclin metabolites in the plasma and urine of patients with kidney grafts

From the trough level before intake (\( t = 0 \)), drug concentrations peaked 2 h after intake, where CsA increased significantly, approximately five times, and tacrolimus and rapamycin approximately two times (Figure 3A). At 6 h, CsA and tacrolimus plasma concentrations were not different from the trough level at \( t = 0 \), whereas rapamycin remained significantly above the value at \( t = 0 \) (Figure 3A). Plasma concentrations of 6-keto-PGF\(_{1\alpha}\) did not change significantly in control persons and CsA-treated patients between drug nadir and peak over the 6 h collection period (Figure 3B). In tacrolimus and rapamycin-treated patients, the plasma concentration of 6-keto-PGF\(_{1\alpha}\) increased significantly (at 6 h and at 4 and 6 h, respectively, Figure 3B). When the average 6-keto-PGF\(_{1\alpha}\) plasma concentration at the peak plasma drug concentration (\( t = 2 \) h) was compared between groups, there was no significant difference (not shown). The urinary excretion rate of PGI-M did not change significantly over the 6 h period within any of the

Cox-2 and cyclooxygenase-2 inhibitors were tested on human vascular smooth muscle cells (hVSMC) in serum and drug free conditions. COX-2 mRNA abundance was not significantly different between controls and patients treated with CsA, tacrolimus or rapamycin. 6-Keto-PGF\(_{1\alpha}\) concentration in the cell-conditioned medium from hVSMC incubated without and with FCS for 20 h with vehicle (ethanol), COX-2 inhibitor NS-398 (NS), cyclosporine A (CsA), tacrolimus (Tc), prednisolone (Pd) and rapamycin (Rp). Values are mean ± SE. * \( P < 0.05 \) compared with FCS without drug addition. (C) COX-2 immunofluorescence labelling of hVSMC cultures without and with FCS and addition of cyclosporine A (CsA 1 µmol/L). Negative control is without primary antibody (Ab).
**Fig. 3.** (A) Plasma concentration of immunosuppressive drugs in the patient groups before \((t = 0)\) and after (2, 4 and 6 h) intake. Values are average ± SE. \(\ast P < 0.05\) by ANOVA followed by Dunnett’s post hoc test. Cyclosporine A \((n = 11)\), tacrolimus \((n = 8)\) and rapamycin \((n = 9)\). (B) Plasma concentration of prostacyclin metabolite, 6-keto-PGF\(_{1\alpha}\) before \((t = 0)\) and after (2, 4 and 6 h) intake of prescribed daily medication in the three patient groups: bars show mean values. \(\ast P < 0.05\) by ANOVA followed by Dunnett’s post hoc test. Healthy control group \(n = 11\), numbers in other groups as in (A).

Effect of immunosuppressive drugs on COX-2 activity in the whole-blood ex vivo assay

Addition of LPS to whole blood from control persons led to a significant, concentration-dependent accumulation of 6-keto-PGF\(_{1\alpha}\) as compared to samples incubated with no LPS (Figure 4A, \(n = 4\)). The COX-2 selective inhibitor, NS-398 (0.1–10 \(\mu\)mol/L, Figure 4A), and dexamethasone (1 nmol/L–1 \(\mu\)mol/L) (DEXA, Figure 4B) inhibited in a concentration-dependent way the LPS-induced accumulation of 6-keto-PGF\(_{1\alpha}\). There were no significant differences in the accumulation of 6-keto-PGF\(_{1\alpha}\) in response to LPS between \(t = 0\) and \(t = 6\) h within control, CsA and tacrolimus groups (Figure 5A). At 6 h after rapamycin intake, the concentration of 6-keto-PGF\(_{1\alpha}\) was significantly enhanced compared to 0 h (Figure 5A). At the peak plasma drug concentration \((t = 2\) h), formation of 6-keto-PGF\(_{1\alpha}\) was significantly higher in the CsA group than in all other groups (Figure 5B,
Effect of immunosuppressives on thromboxane metabolites in plasma and urine

TxB₂ plasma concentrations did not change between time 0 and 6 h in each of the four groups (not shown). There was no difference between mean plasma concentration values from each group (not shown). There were no significant changes in the urinary excretion rate of TxB₂ in the 6 h collection period within any of the four groups (Figure 6A). When values in each group were pooled and the mean values were compared, TxB₂ excretion was significantly higher in CsA-treated patients than in all other groups (Figure 6B). TxB₂ excretion was significantly lower in the tacrolimus- and rapamycin-treated patients compared to controls (Figure 6B).

Effect of immunosuppressive drugs on COX-1 activity in the whole-blood ex vivo assay

The TxB₂ concentration was 1000- to 10 000-fold higher in serum from coagulated blood samples compared to plasma from the same individual. COX-1 activity in whole blood did not change significantly during the 6 h observational period within any of the four groups (Figure 7A). Values from each group were pooled and the group means were compared. The patients who received tacrolimus and rapamycin exhibited significantly lower COX-1 activity compared to control and CsA-treated patients (Figure 7B, left). Platelet counts were not significantly different between groups (Figure 7B, right).

Discussion

The present study shows that immunosuppressive drugs display differential effects on COX-2 in vitro: Cyclosporin A (CsA) inhibits FCS-stimulated COX-2 activity and expression in cultured human vascular smooth muscle whereas a different CNI, tacrolimus and the mTOR inhibitor rapamycin have no such effect. In renal transplant patients treated with CNIs, basal and stimulated indices of COX-2 activity were not suppressed. Tacrolimus and rapamycin-treated patients displayed significantly lower platelet aggregation, thrombocyte COX-1 activity and thromboxane urinary excretion, compared to CsA-treated patients.
Fig. 5. Effect of immunosuppressives on COX-2 activity in the whole-blood assay with samples from renal transplant patients and controls. (A) One millilitre heparinized whole blood taken before (t = 0) and after (2, 4 and 6 h) intake of immunosuppressive drugs incubated 20 h at 37°C with LPS (10 µg/mL, E. coli 026:B6). Plasma was separated and analysed for the prostacyclin metabolite 6-keto-PGF₁α: control (n = 11), cyclosporine A (n = 11), tacrolimus (n = 8) and rapamycin (n = 9). Bars indicate average. *P < 0.05. ANOVA followed by Dunnett’s post hoc test. (B) Left: mean ± SEM plasma concentration of 6-keto-PGF₁α as determined by the whole-blood COX-2 assay in each study group at the peak plasma drug concentration (2 h). Right: whole-blood COX-2 assay with blood from control persons. Cyclosporine A was added directly to blood samples from control persons in a range of concentrations (0.1–100 µmol/L) with LPS (10 µg/mL). Values are average ± SEM, n = 10.

We observed that the PGI₂ metabolite, 6-keto-PGF₁α, was a valid measure of COX-2 activity, both in vascular smooth muscle and in the whole-blood ex vivo assay. In patient plasma, CsA reached concentrations well above those that suppressed COX-2 in cultured hVSMCs but indices of systemic COX-2 activity (plasma concentration and urine excretion of 6-keto-PGF₁α) were not inversely related to the CsA plasma concentration. The concentration of CsA in cells differs significantly from values in plasma [22] depending on the cell type [23] and condition of the cell [22]. CsA acts primarily through inhibition of gene expression and therefore a certain delay would be expected, but cellular inhibition of calcineurin followed plasma concentrations of CsA [24]. Since the plasma concentration and urine excretion of 6-keto-PGF₁α did not differ between the CsA and control group, chronic suppression of COX-2/PGI₂ is not likely to have blurred the results in the kinetic study. In contrast to the prediction of the hypothesis, we observed in the ex vivo whole-blood assay that COX-2 activity was significantly elevated at the plasma peak of CsA (Figure 5B). This finding is similar to recent data from rats, where plasma PGI₂ was significantly elevated by CsA.
The sample size in each group is similar to figures above. Groups investigated: control, cyclosporine A, tacrolimus and rapamycin. Pooled from the values determined at 0, 2, 4 and 6 h in each of the four settings.

Fig. 6. Effect of immunosuppressives on urine excretion of thromboxane A2 metabolite, TxB2. (A) Excretion of TxB2 before (t = 0) and after (2, 4 and 6 h) intake of immunosuppressives in the three patient groups and in controls receiving no medicine. Bars show mean. Sample sizes were as in Figure 5. No statistically significant differences were detected by ANOVA. (B) Columns depict the mean urine excretion rate of TxB2 pooled from the values determined at 0, 2, 4 and 6 h in each of the four groups investigated: control, cyclosporine A, tacrolimus and rapamycin. The sample size in each group is similar to figures above. **P < 0.01 CsA versus all other groups. *P < 0.05 compared to control. ANOVA followed by Dunnett’s post hoc test.

Several interpretations can explain the disagreement between in vitro and in vivo observations on PG12. CsA suppresses consistently induced and spontaneous production of PG12 from vascular in vitro preparations [6,7,26]. COX-2-derived systemic PG12 is synthesized primarily through shear stress stimulation of the endothelium in vivo [27].

The present data were obtained with hVSMCs and the calcineurin activity could be less important for COX-2 induction in endothelium in vivo. Even if calcineurin and COX-2 are co-localized in endothelium, the expression of COX-2 in vivo could be supported by pathways independent of calcineurin. Thus, COX-2 activity was induced by LPS independent of calcineurin in the whole-blood assay (present study) and endogenous fatty acids engage this pathway to activate COX-2 [28,29]. Moreover, in the in vivo setting, the COX-prostanoid system is redundant since COX-1 is expressed in human endothelium in some vascular areas and could supply PG12 to the systemic circulation even with suppressed COX-2.

Our results indicate that the CNI’s CsA and tacrolimus exhibit differential effects on COX-2 activity in vitro and on indirect indices of COX-2 activity in vivo. The reason for this is not clear from our results. The two CNI’s display different potencies, they are chemically different and inhibit calcineurin through binding to two different proteins, cyclophilin A and FK-binding protein. Thus, if binding proteins are differentially expressed in vascular tissue this could contribute to different sensitivities to the drugs. There are no data available on the effect of CNIs on vascular COX-2 expression in vivo. To avoid human allograft rejection, plasma CsA has to be in molar concentrations that are about ten times higher than tacrolimus. Hocherl et al. demonstrated inhibition of COX-2 in the rat kidney cortex by both CsA and tacrolimus [13]. They used doses of tacrolimus that were relatively high compared to CsA. Later studies by Hocherl et al. show that the kidneys are not the source for systemic PG12 since systemic PG12 was increased by cyclosporine despite significant suppression of kidney COX-2 and urinary PG12 excretion [25]. Our results point to certain similarities between the CNI tacrolimus and the mTOR inhibitor rapamycin to suppress thromboxane release and promote, not inhibit, systemic PG12. Both drugs belong to the macroside family and although the mechanisms are not clear from the present data, it is interesting that conventional macrolides are known to display anti-inflammatory properties [30]. The plasma concentration of 6-keto-PGF1α and whole-blood COX-2 activity increased significantly, parallel with the plasma rapamycin concentration in patients, but rapamycin had no significant effect on COX-2 in cultured smooth muscle. Most rapamycin-treated patients also received glucocorticoids that would be expected to suppress COX-2 activity. Our observations are in agreement with the finding that rapamycin stimulates the release of PG12 from cultured endothelial cells in vitro [31] and a direct effect of rapamycin on COX-2 activity in vivo cannot be excluded. Further studies should be undertaken to elucidate whether rapamycin enhances cardioprotective
PGI2 release by the endothelium. Such an effect would also be in agreement with the observed suppression of platelet activity and renal TxA2 excretion in rapamycin-treated patients.

Earlier observations indicate that CsA enhances platelet aggregation, ADP-induced platelet TxA2 release in vitro [32] and renal TxA2 synthesis [33]. The present study confirms these findings. Urinary excretion of TxB2 reflects predominantly intrarenal and not systemic TxA2 synthesis [34]. Since no major changes in systemic PGI2 were detected, the increased platelet aggregation and TxA2 release in CsA-treated patients is likely not caused by chronically reduced PGI2. The lower level of TxA2 in rapamycin-treated patients is in accordance with recent in vitro data that show that rapamycin inhibits de novo synthesis of COX-1 in mature platelets [35].

It is interesting that the immunosuppressives display effects compatible with differential interaction with eicosanoid formation in platelets, smooth muscle, kidneys and the endothelium. The results of the present study should be evaluated taking into account the limited number of patients and the differences in the patient groups regarding the occurrence of diabetes, antihypertensive treatment regimens and use of glucocorticoids, making the within-group changes demonstrated of higher interest than between-group changes. As with the study of Graff et al. [17], the
present study may indicate that CsA is more liable to facilitate thrombosis than tacrolimus and rapamycin, although, to our knowledge, no differences regarding frequency of cardiovascular thrombotic events between patients on systemic CsA, tacrolimus and rapamycin have been demonstrated in the randomized studies available [36–38]. Studies powered to elucidate differences in the cardioprotective potential of tacrolimus, and rapamycin in contrast to CsA would be of interest. In summary, the present data show that CsA, but not tacrolimus or rapamycin, inhibits COX-2 in human VSMCs. In an in vivo setting, systemic and renal indices of COX-2 activity and PGI2 formation were not suppressed in renal transplant patients treated with CNIs. Platelet aggregation, thromboxane formation by activated platelets and renal thromboxane excretion were significantly lower in patients treated with tacrolimus and rapamycin as compared to CsA. Differential effects on prostanooid synthesis by immunosuppressives may have implications for long-term cardiovascular hazards in patients treated with immunosuppressive drugs.

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References


Chronic kidney disease after heart transplantation

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Abstract

Background. Chronic kidney disease (CKD) is a complication of heart transplantation related to calcineurin inhibitor nephrotoxicity. However, it is unclear whether early cyclosporin (CsA) exposure influences CKD in the long term.

Methods. We analysed risk factors for CKD in 352 patients who underwent orthotopic heart transplantation (1995–2005). In 2000, we reduced our target CsA levels in the postoperative period.

Results. Actuarial patient survival was 79% at 1 year and 62% at 10 years. Estimated median glomerular filtration rate (eGFR) by the four-variable Modification of Diet in Renal Disease formula was 64 ml/min/1.73 m² before transplantation, inter-quartile range (IQR) 54–78. After transplantation, the eGFR was 48 (IQR 37–61) at Year 1, and 41(35–57) at Year 10. The cumulative probability of eGFR <45 ml/min/1.73 m² was 45% at Year 1, 71% at Year 5 and 83% at Year 10. A multivariable logistic regression model was constructed for the development of eGFR <45 ml/min/1.73 m² by 3 years. The risk factors were postoperative renal replacement therapy for acute renal failure (ARF), P < 0.001; pretransplant diabetes, P = 0.005; increasing recipient age, P < 0.001; female recipient, P = 0.029; female donor, P = 0.04, but not CsA regimen. The cumulative probability of developing stage 5 CKD (eGFR <15) was 3% at Year 5 and 12% at Year 10. Although lower cyclosporin initial levels were associated with less renal nephrotoxicity, the cumulative probability of developing stage 5 CKD was not significantly different between CsA and tacrolimus.

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