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
isoforms. CsA had no effect on plasma 6-keto-PGF$_{1α}$, whole-blood COX-2 activity or PGI-M urine excretion; after rapamycin intake, the former two increased. Plasma TXB$_2$ did not change after drug intake. TXB$_2$ in the COX-1 assay and urine excretion of TxB$_2$ was significantly lower in tacrolimus- and rapamycin-treated patients compared to the CsA group. Platelet aggregation was increased significantly in the CsA group.

**Conclusions.** Although CsA suppressed COX-2 in cultured vascular smooth muscle cells, systemic prostacyclin was not suppressed by either CsA or tacrolimus in vivo. Rapamycin and tacrolimus may actively suppress platelet and renal thromboxane formation. Differential changes in prostanooids may have implications for long-term cardiovascular hazard in patients treated with immunosuppressives.

**Keywords:** cyclooxygenase-2; calcineurin inhibitor; prostacyclin; rapamycin; thromboxane

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**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 (PGI$_2$) release from vascular rings [7] and cultured human endothelium [8]. PGI$_2$ infused to rats attenuates CsA-mediated renal hypoperfusion [9]. PGI$_2$ is synthesized predominantly by cyclooxygenase-2 (COX-2) in healthy humans [10]. Selective suppression of PGI$_2$ 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 PGI$_2$. Taken together, suppression of vascular COX-2 and systemic PGI$_2$ after CNI treatment may afford a causal explanation for some common CNI side effects, e.g. systemic hypertension, renovascular impairment and thromboembolic events. Because PGI$_2$ signalling imposes tonically a constraint on platelet activation, suppression of COX-2 predicts an augmented production of thromboxane (TxA$_2$) 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 PGI$_2$ 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 PGI$_2$ metabolite, 6-keto-PGF$_{1α}$, was subsequently measured in the medium by ELISA NS-398 (Cayman Chemicals, no. 515217).

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>
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<tr>
<td>Number</td>
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<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>
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<tr>
<td>Male (%)</td>
<td>64</td>
<td>91</td>
<td>75</td>
<td>78</td>
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<tr>
<td>Diabetes (%)</td>
<td>36</td>
<td>25</td>
<td>0</td>
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</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>
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<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></td>
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<tr>
<td>Prednisolone</td>
<td>0</td>
<td>0</td>
<td>7</td>
<td></td>
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<tr>
<td>Ca antagonist</td>
<td>10</td>
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<td></td>
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<td>ACE inhibitor</td>
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<td>7</td>
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<tr>
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<td>2</td>
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<td>4</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 GTGTTTCTCAGT-GCTCAGA, antisense: CCTCGAAGCTAGTGATCT, covering bases 1631–1820 nm_000944; Aβ sense: AACCATACTGCCCAGTGA, antisense: GGTCTCAGACAATG, covering bases 1621–1798, Genebank acc no. bc035464; Aγ: sense TTCAGCACAAGATCGGGA, antisense: ACCAGCTGTATGCTATC, 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 prostanoid analysis preceded each blood sampling.

Determination of eicosanoids in plasma and urine

The PGF1α 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 PGF2β 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\textsubscript{1α} added to plasma was close to 100%. The TxA\textsubscript{2} hydrolysis product TxB\textsubscript{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\textsubscript{1α} 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\textsubscript{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\textsuperscript{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 and as a measure 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\textsubscript{2} metabolite 6-keto PGF\textsubscript{1α} 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\textsubscript{1α} concentration. CsA abolished and prednisolone attenuated significantly the effect of FCS on 6-keto-PGF\textsubscript{1α} production (Figure 1B).

Immunofluorescence 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 immunofluorescence. 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 (CnA\textsubscript{α}, CnA\textsubscript{β} and CnA \textsubscript{γ}) were detected by PCR amplification of human cerebral cortex cDNA (Figure 2A). Serum-deprived human aortic VSMC expressed CnA\textsubscript{β} and CnA\textsubscript{γ} isoforms (Figure 2B). CnA\textsubscript{α} was not consistently detected. CnA\textsubscript{β} and CnA\textsubscript{γ} but not CnA\textsubscript{α} 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
Fig. 1. (A) COX-2 mRNA abundance in hVSMC incubated without (−) and with (+) fetal calf serum (FCS, 2 h) and vehicle (control), cyclosporine A (CsA 100 nmol/L), tacrolimus (Tc 100 nmol/L), rapamycin (Rp 100 nmol/L) and prednisolone (Pd 10 µmol/L); n = 4–5/group; *P < 0.05 compared with FCS without drug addition. (B) 6-Keto-PGF1α 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).

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 time = 0, whereas rapamycin remained significantly above the value at t = 0 (Figure 3A). Plasma concentrations of 6-keto-PGF1α 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-PGF1α increased significantly (at 6 h and at 4 and 6 h, respectively, Figure 3B). When the average 6-keto-PGF1α 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

Fig. 2. (A) Amplification of catalytic calcineurin (Cn) subunit Aα, Aβ and Aγ with template cDNA from human cerebral cortex. Negative control (−) is omission of cDNA. Molecular size marker (Mw) is φX174DNA/HaeIII digest. (B) Amplification of CnAβ (178 bp) and Aγ (108 bp) from cultured, quiescent human aortic smooth muscle cells. Negative control is omission of cDNA (−cDNA) and reverse transcriptase (−RT). Molecular size marker (Mw) is φX174DNA/HaeIII digest. (C) PCR amplification of cDNA from three separate preparations of human renal arteries for COX-2 (270 bp), CnAβ (178 bp) and CnAγ (108 bp). Negative control (−) is omission of cDNA and positive control (Pc) is amplification of kidney RNA. Molecular size marker (Mw) is φX174DNA/HaeIII digest.
**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. \( *P < 0.05 \) 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. \( *P < 0.05 \) by ANOVA followed by Dunnett’s post hoc test. Healthy control group \( n = 11 \), numbers in other groups as in (A).

Four groups examined (not shown). When urinary excretion was compared between groups at the peak plasma drug concentration of drugs (2 h) there were no significant differences (not shown) nor when all values were pooled and mean values compared between controls and drug-treated patients (control: 749 pg/min ± 42, CsA: 771 pg/min ± 57, tacrolimus: 592 pg/min ± 50, rapamycin: 740 pg/min ± 63).

**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, not shown).
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).

Effect of immunosuppressive drugs on platelet aggregation

Platelet aggregation was not significantly altered at the time of peak plasma concentration of immunosuppressive drugs (t = 2 h) compared to nadir (t = 0) within each of the patient groups or in controls (data not shown). When data from determinations within each group were pooled, platelet aggregation was significantly higher in CsA-treated patients than in all other groups (Figure 8). Aggregation was not correlated to the plasma concentration of CsA or to age, estimated GFR, time after transplantation or TxB₂: no difference in aggregation was found between patients receiving an angiotensin II antagonist or an ACE inhibitor and other subjects.

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.
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 groups.

**Fig. 6.** Effect of immunosuppressives on urine excretion of thromboxane A_2 derived systemic PGI_2 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 PGI_2 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 PGI_2 since systemic PGI_2 was increased by cyclosporine despite significant suppression of kidney COX-2 and urinary PGI_2 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 PGI_2. Both drugs belong to the macrolide 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-PGF_1α 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 PGI_2 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 properties.

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**Fig. 6.** Effect of immunosuppressives on urine excretion of thromboxane A_2 (A) Excretion of TxB_2 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 TxB_2 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 < 0.05 CsA versus all other groups. *P < 0.05 compared to control. ANOVA followed by Dunnett’s post hoc test.**
Fig. 7. Effect of immunosuppressives on COX-1 activity in the whole-blood assay with samples from renal transplant patients and controls. (A) One millilitre whole blood taken before (t = 0) and after (2, 4 and 6 h) intake of prescribed daily medication incubated 1 h at 37 °C in glass vials. Serum was separated and analysed for thromboxane hydrolysis derivative, TxB2: control (n = 11), cyclosporine A (n = 11), tacrolimus (n = 8) and rapamycin (n = 9). Bars indicate mean. There were no significant differences with time in each group. (B) Left: serum concentration of TxB2 in each group of patients and controls as determined by the whole blood COX-1 activity assay. TxB2 serum concentration values determined in samples taken at 0, 2, 4 and 6 h were pooled in each group and values are mean ± SEM. *P < 0.05 compared to control. The Kruskal–Wallis test followed by Dunn’s multiple comparison test. Right: thrombocyte count in each of the four study groups. Bars show mean.

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 rapamycine-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 PGIL2 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 prostanoid 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 ciclosporin (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 first year after transplantation.

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 post-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 post-opera