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ORIGINAL ARTICLE

The effects of calcineurin inhibitors on prostanoid synthesis: a randomized cross-over study in healthy humans

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Summary

The calcineurin inhibitors (CNIs) cyclosporine (CsA) and tacrolimus (Tac) are implicated in post-transplant complications such as cardiovascular morbidity. Prostanoids are fatty acid-derived compounds essential for controlling cardiovascular homeostasis. We tested the hypothesis that CNIs suppress cyclooxygenase (COX)-2-derived prostacyclin (PGI) and increase thromboxane synthesis in humans. Ten healthy men underwent 5-h infusions of CsA, Tac, and saline in a randomized, double-blind, cross-over study. Blood and urine samples were collected before and after the infusion of each drug/saline, to measure PGI and thromboxane metabolites. CsA decreased whole-blood COX-2 activity by 39% ($P = 0.05$) and basal plasma 6-keto-$PGF_{1\alpha}$ levels by 31%, only nonsignificantly. Urine excretion of PGI-M and TxB_2 did not change significantly after CsA infusion. Tac decreased TxB_2 in the COX-1 *ex vivo* assay by 30% ($P = 0.03$), while no changes were seen in urinary levels of PGI-M or TxB_2. Urinary TxB_2 excretion was 15% lower after saline infusion ($P = 0.03$). These within-treatment differences in prostanoid synthesis did not differ significantly between the treatments (ANOVA). Mean blood levels of CNIs were 486 μg/l for CsA and 12.8 μg/l for Tac. Clinically relevant doses of CsA and Tac induce acute differential changes in prostanoid levels in healthy human subjects. CsA suppresses COX-2 activity, while Tac decreases platelet activity.

Introduction

The calcineurin inhibitors (CNI), cyclosporine (CsA), and tacrolimus (Tac) are immunosuppressive drugs indicated for prophylaxis and treatment of graft rejection in organ transplantation. Introducing the CNIs into the field of transplantation has markedly improved the outcome of solid organ transplants, however, their therapeutic use has proven to be a double-edged sword on account of diverse organ side-effects, such as hypertension, artherosclerosis, and thromboembolic events. These complications may be contributors to the increased cardiovascular morbidity seen in transplant recipients [1–5].

Prostanoids are involved in the regulation of vascular tone and platelet aggregation. Prostacyclin (PGI_2) is continuously released from endothelial cells and exhibits vasodilatory as well as antithrombotic effects. Its more stable metabolic product 6-keto-$PGF_{1\alpha}$ has similar effects, only to a lesser extent. Thromboxane A2 (TxA_2) is produced by activated thrombocytes and opposite to PGI_2, it promotes vasoconstriction and platelet aggregation [6]. Cyclooxygenases (COX)-1 and -2 constitute a necessary step in prostanoid synthesis. They catalyze the conversion of arachidonic acid to prostaglandin H_2, which is converted to the different prostanoids by specific prostaglandin synthases. COX-1 is constitutively expressed in most cells, whereas COX-2 is inducible by inflammatory or stimulatory events in tissues [7]. Current data indicate that COX-2 is mainly responsible for systemic cardioprotective PGI_2 synthesis and selective inhibition of COX-2 is thought to be the underlying cause of the increased cardiovascular morbidity observed in patients treated with selective COX-2 inhibitors and nonselective NSAIDs with COX-2 profiles [8]. Previous *in vitro* and animal studies have shown that CsA and Tac can suppress

COX-2 mRNA and protein expression [9–11], and a few studies have reported increased platelet aggregation in relation to treatment with CNIs [12,13]. It is therefore an open question whether CNIs exert cardiovascular adverse effects through suppression of COX-2 and systemic PGI levels. Previous studies in kidney transplant patients suggested a more favorable profile of Tac compared with CsA with respect to thromboxane formation and platelet aggregation, but many confounding factors in these multi-drug treated patients could have contributed to the observations [9,12]. In the present study designed to address glucose metabolism, it was in addition possible to test the hypothesis that CNIs suppress systemic PGI in healthy volunteers. We herein present the first sequence randomized, double-blind cross-over study comparing the effects of clinically relevant concentrations of CsA and Tac on prostanoid synthesis in healthy human subjects.

Methods

The studies were conducted in accordance with the Helsinki Declaration and following the approval by the local ethics committee (M-20080060), the Danish Medicines Agency, and the Good Clinical Practice (GCP) unit of Aarhus University Hospital. According to the International Committee of Medical Journal Editors, the protocol was registered at Clinicaltrials.gov (identification study1: NCT00766909) before the onset of enrollment. Prior to entering the study, written consent was obtained after receipt of written and oral information.

Study subjects

Ten healthy men with a median age of 27 (21–52) years and mean body mass index of 24.7 ± 0.5 kg/m^2 volunteered in this study. All had a normal physical examination and hematological and renal functions assessed by biochemical screening. Mean systolic and diastolic blood pressures were 126 ± 3 and 74 ± 2 mmHg, respectively. Two had a family history of diabetes mellitus and two were smokers. None of the participants were taking medication regularly during the study period. Women were excluded from the study based on the possible influence of ovarian hormones and oral contraceptives on glucose metabolism.

Study design

This study was designed as a double-blind, placebo-controlled, sequence randomized crossover trial. Upon inclusion sequence randomization, preparation of study medication and blinding was performed and ensured by the hospital pharmacy at Aarhus University Hospital. Each participant underwent three investigations on three separate days 4–6 weeks apart, receiving CsA, Tac, or saline infusions. On each investigational day the participants arrived at the research unit at 8:00 AM after a 10-h overnight fast. They remained at rest in a supine position throughout the entire study procedure. Cannulas were inserted in the antecubital veins on each side for infusion and sampling purposes. At t = 0 min, bolus doses of CsA (0.34 mg/kg), Tac (0.0024 mg/kg), or saline were commenced and infused over a 20-min interval, followed by maintenance doses of CsA (0.155 mg/kg/h), Tac (0.0012 mg/kg/h), or saline until t = 285 min. Blood drug concentrations were measured at t = 0, 120, 165, and 285 min (Fig. 1). Doses and administration modes were based on a targeted area under the curve (AUC) AUC_{0-12} of 8000 μg/l*h for CsA and 200 μg/l*h for Tac, and aiming towards constant levels throughout the experiment [14,15]. These targeted drug levels are comparable with respect to immunosuppressive efficacy in clinical practice. Doses were estimated from previous results at our own laboratory [16]. The subjects emptied their bladder before and after administration of study medication. Blood and urine samples were obtained as outlined in Fig. 1 and following analyses were carried out:

Determination of prostanoids in plasma and urine

Basal PGI_2 levels in EDTA-treated and separated plasma was determined by measuring the PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ by EIA (Cayman chemicals, MI, USA) and basal urinary PGI-M levels were determined by measuring the metabolites 6-keto-$PGF_{1\alpha}$ and 2,3-dinor-6 keto-$PGF_{1\alpha}$ by EIA (Enzo Life Sciences, Postfach, Switzerland) as previously described [9]. Basal levels of thromboxane in the urine were determined by measuring the TxA_2 metabolite TxB_2 by EIA (Enzo Life Sciences). Metabolite concentration in spot urine samples were normalized to urine creatinine

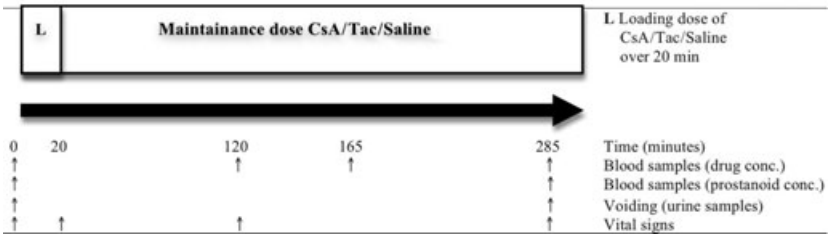

Figure 1 Study design. Please refer to *Methods* for further detail.

levels measured by ABX Pentra Creatinine 120 CP (Horiba ABX SAS, Montpellier, France) according to the manufacturer's instructions.

Cyclooxygenase whole-blood assays

The *ex vivo* experiments were performed as previously described [17].

In brief, to determine maximal COX-2 activity 1 ml heparinized whole-blood samples were incubated with 10 μg/ml lipopolysaccharide (LPS, *E. coli*, 026:B6; Sigma, St. Louis, USA) for 24 h at 37 °C. Accumulated 6-keto $PGF_{1\alpha}$ was measured in the plasma samples by EIA (Enzo Life Sciences) as an indicator for COX-2 activity, and this assay was recently validated by our group [9]. To determine maximal platelet COX-1 activity *ex vivo*, we allowed 1 ml whole-blood collected in glass vials to coagulate by placing the samples immediately at 37 °C for 1 h. The amount of accumulated TxB_2 was measured in serum by EIA (Enzo Life Sciences) as an indicator for COX-1 activity.

Other measures

This study was primarily designed to study the effects of CNIs on glucose metabolism. During the 5-h infusion of study medication, all participants underwent the same series of measures for insulin secretion and insulin sensitivity as previously described [18].

Safety

Adverse events, blood pressure, and other vital signs were monitored throughout the study procedure. Blood pressure was measured by auscultatory technique using approved equipment. Biochemistry including blood hemoglobin, plasma levels of electrolytes, creatinine levels, liver enzymes, urine dipstick, and ECG were monitored in between study days.

Assays

All biochemical analyses were performed in duplicate. Blood Tac and CsA concentrations (μg/l) were determined using Waters Micromass HPLC-MS/MS system.

Statistics

A paired *t*-test was used to compare before and after values of prostanoid levels for each treatment. ANOVA repeated measures were used to test for differences in prostanoid levels and the interaction between time and treatment was considered the term of interest. Adjustments for randomization order and treatment period were included in the model. One-way ANOVA was used to test for differences in diuresis and blood pressure changes between treatments. Results are expressed as mean ± SEM for normally distributed data. To obtain normal distribution, data were transformed when necessary using natural logarithm, otherwise nonparametric tests were used. Statistical analysis was performed using STATA 11.0 software.

Results

Effect of calcineurin inhibitors on prostacyclin synthesis

After a 5-h i.v. infusion protocol, basal plasma 6-keto-$PGF_{1\alpha}$ levels tended to decrease, but this did not reach significance in any of the groups (31% by CsA and 24% by Tac, Table 1 and Fig. 2a). Some hemolysis was observed in these plasma samples. Urinary levels of PGI-M were not significantly altered (Table 1 and Fig. 2b). In whole-blood COX-2 *ex vivo* activity assay, CsA infusion suppressed COX-2 activity by 39% ($P = 0.05$), while Tac and saline infusions showed no significant impact on COX-2 activity (Table 1 and Fig. 2c). The within-treatment differences in COX-2 activity did not differ significantly between the treatments (ANOVA).

Effect of calcineurin inhibitors on thromboxane synthesis

Urinary TxB_2 levels did not change significantly after CsA and Tac exposure, however, after saline infusion, urine TxB_2 excretion decreased marginally (15%, $P = 0.03$, Table 1 and Fig. 3a). In the whole-blood *ex vivo* COX-1 assay, Tac suppressed platelet COX-1 activity by 30% ($P = 0.03$), while no changes were seen in COX-1 activity after CsA or saline infusions (Table 1 and Fig. 3b). These within-treatment differences in COX-1 activity and urine TxB_2 did not differ significantly between the treatments (ANOVA).

Systemic effects

Systolic and diastolic blood pressures did not change significantly during saline and Tac infusions, while a significant increase of 6 mmHg in systolic blood pressure was observed after CsA treatment ($P = 0.05$) (Table 1). There were no significant changes in diuresis during the experiments.

Immunosuppression

Mean CsA and Tac concentrations during the 5-h infusion on study days are given in Table 1 and mean values at each time point during infusion are illustrated in Fig. 4. Mean CsA and Tac concentrations during the 5-h infusion on study days have previously been published [18]. Drug levels were stable after 120 min and throughout the remaining

Table 1. Prostanoid synthesis and clinical characteristics before and after saline, tacrolimus, and cyclosporine infusion.

	Saline		Tacrolimus		Cyclosporine		*P* value paired *t*-test	*P* value ANOVA
	Before	After	Before	After	Before	After		
Basal plasma prostacyclin metabolite pg/ml	72.6 ± 10.6	79.7 ± 12.9	89.8 ± 16.5	68.1 ± 14	95.2 ± 13.1	65.3 ± 11.6	NS	0.47
COX-2 activity (plasma prostacyclin metabolite pg/ml)	1133.1 ± 171.4	935 ± 114	861 ± 122.5	765 ± 110	1118.2 ± 141	*679 ± 95.5	*0.05	0.32
COX-1 activity (serum thromboxane metabolite pg/ml)	271 800 ± 45774	319 000 ± 61 626	335 000 ± 35 533	*235 400 ± 17 398	242 700 ± 40 507	290 800 ± 23 923	*0.03	0.14
Urine thromboxaneme tabolite/urine creatinine	77.8 ± 8.6	*66.5 ± 14.8	133.9 ± 41.2	234.3 ± 99	218.8 ± 21.2	209.6 ± 100.6	*0.03	0.17
Urine prostacyclin metabolite/urine creatinine	79.4 ± 5	109.4 ± 19.7	148.5 ± 64.8	134.9 ± 13	218.8 ± 90.9	170.1 ± 31.3	NS	0.93
Diuresis (ml/min)		1.29 ± 0.2		1.08 ± 0.2		1.17 ± 0.2	NA	† 0.66
Blood pressure systolic mmHg	122 ± 2	121 ± 3	122 ± 3	126 ± 3	124 ± 3	*130 ± 2	*0.05	†0.17
Blood pressure diastolic mmHg	72 ± 3	76 ± 3	72 ± 3	76 ± 2	75 ± 4	78 ± 2	NS	0.91
Mean drug concentration (t120–285) mg/l				12.8 ± 0.5		486.9 ± 23.5	NA	NA

Data are mean ± SEM.

NS, not significant; NA, not applicable.

*Statistically significant differences are marked for comparisons between before and after values within each treatment group.

†One-way ANOVA was used to test for differences in diuresis and blood pressure changes between treatments.

ANOVA repeated measures was used to test for differences in prostanoid levels, the interaction between time and treatment was considered the term of interest (see Statistics).

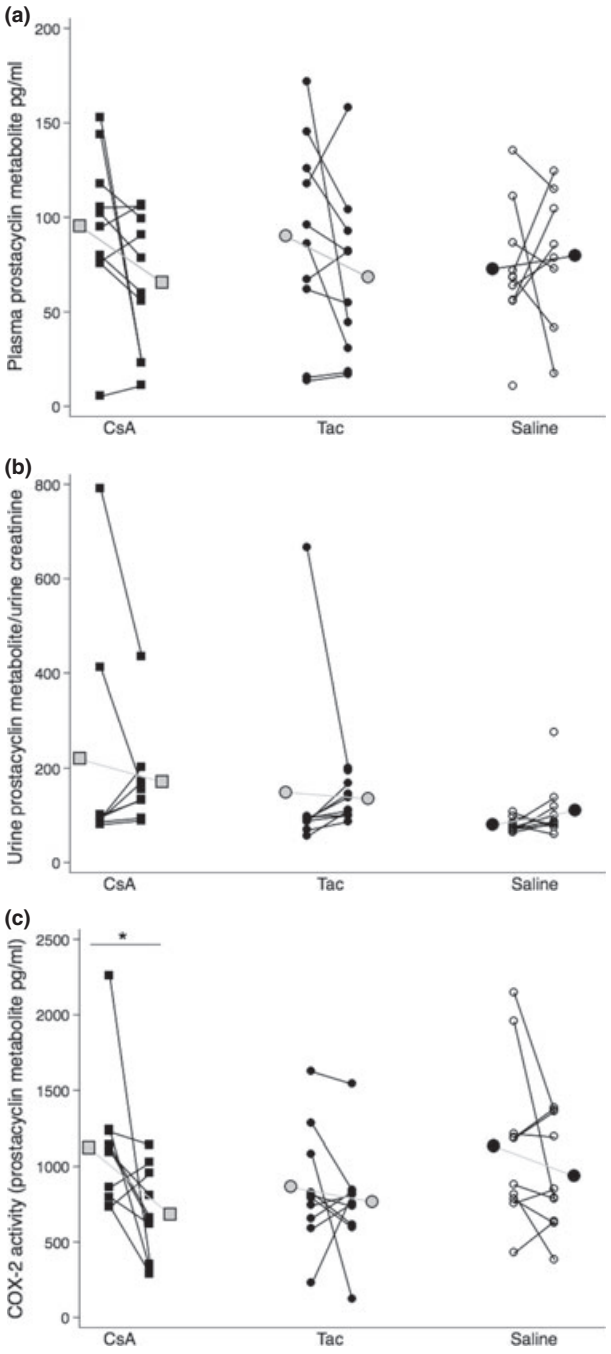

Figure 2 (a) Plasma concentration of prostacyclin metabolite, 6-keto-$PGF_{1\alpha}$ before (t = 0 min) and after (t = 285 min) infusion of study medication in 10 healthy men. (b) Urine concentration of prostacyclin metabolite 6-keto-$PGF_{1\alpha}$ before (t = 0 min) and after (t = 285 min) infusion of study medication. (c) Plasma concentration of prostacyclin metabolite, 6-keto-$PGF_{1\alpha}$ as determined by the whole-blood COX-2 activity assay before (t = 0 min) and after (t = 285 min) infusion of study medication. Cyclosporine (CsA) (■closed squares), tacrolimus (Tac) (●closed circles), and saline (○open circles). Mean values are depicted with enlarged symbols for each treatment. *P = 0.05 (see Table 1).

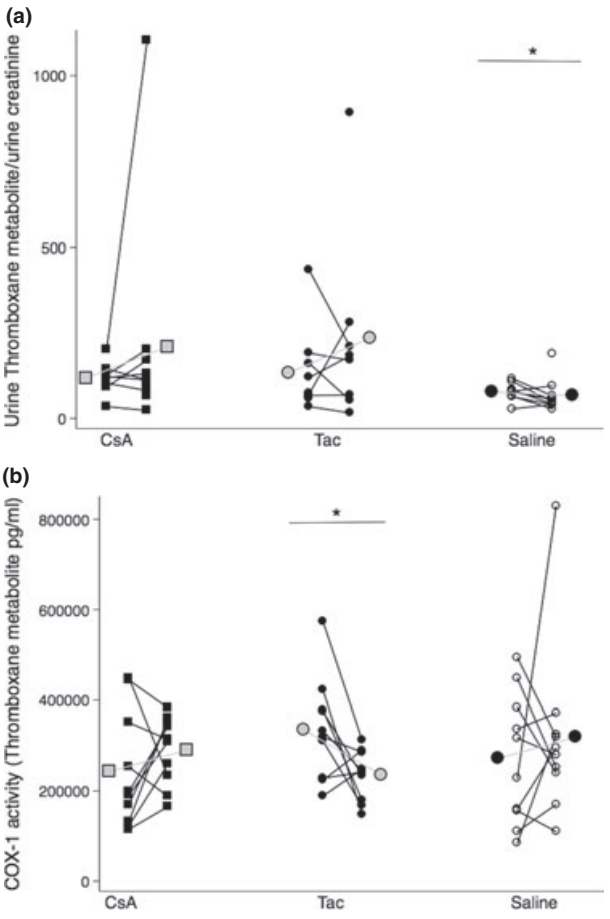

Figure 3 (a) Urine concentration of thromboxane metabolite TxB_2 before (t = 0 min) and after (t = 285 min) infusion of study medication in 10 healthy men. *P = 0.03 (see Table 1). (b) Serum concentration of thromboxane metabolite, TxB_2 as determined by the whole-blood COX-1 activity assay before (t = 0 min) and after (t = 285 min) infusion of study medication. CsA (■closed squares), Tac (●closed circles), and saline (○open circles). Mean values are depicted with enlarged symbols for each treatment. *P = 0.03 (see Table 1).

experimental period. The achieved mean of 12.8 μg/l for Tac corresponds to trough level of about 8 μg/l, and the mean of 487 μg/l for CsA corresponds to a trough level of 172 μg/l or a C2 of 1020 μg/l. These concentrations correspond to desired levels early after transplantation in clinical practice today.

Discussion

The present randomized, cross-over study shows by an interventional approach that over a 5-h acute infusion protocol, the CNIs CsA and Tac differentially affect PGI and thromboxane indices. CsA decreases COX-2 activity significantly in an *ex vivo* assay and Tac suppresses COX-1-derived thromboxane release from activated platelets without affecting PGI. Thus, from these data and the significant

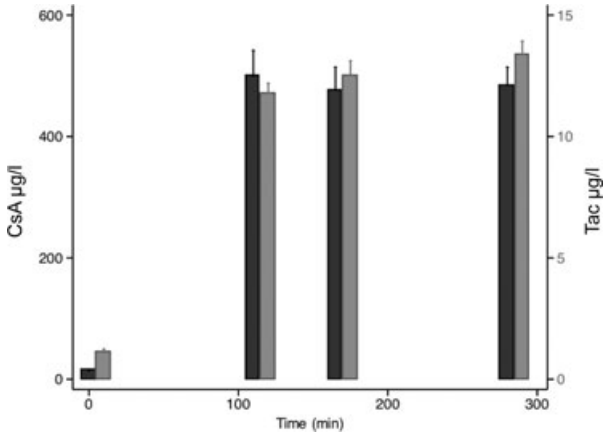

Figure 4 Blood drug concentrations (µg/l) of CsA (black bars) and Tac (gray bars) during the study days. Data are presented as mean ± SEM.

increase in blood pressure only by CsA, the 2 CNIs exert differential cardiovascular effects.

The observations are compatible with previous studies from kidney transplant patients where no change in systemic PGI after CNIs were recorded but selective suppression of thromboxane formation after Tac was found. Thus, platelet aggregation, thromboxane formation by activated platelets, and renal thromboxane excretion were significantly lower in patients treated with Tac and rapamycin as compared to CsA [9]. The present data extend these observations to allow the conclusion that they are not related to the confounding chronic multi-medication and altered kidney function in the transplant patients but rather a phenomenon associated with normal physiological conditions. As with the study of Graff *et al.* [12] the present study may indicate that CsA is more liable to facilitate cardiovascular adverse effects than Tac.

The lack of effect of CsA on basal PGI parameters could be caused by simple lack of statistical power. More importantly, the predicted effect of CsA on COX-2 is on gene expression level and not enzyme activity. Therefore, the acute 5-h protocol might not suffice to detect changes in activity secondary to suppression of COX-2 expression levels, especially in urine samples. The 5-h collection period includes urine before plasma drug levels were in the therapeutic range. Indeed, the suppressive effect of CsA on monocyte PGI observed in blood samples taken after 5-h CsA infusion and 20-h incubation *ex vivo* with LPS is compatible with this view (COX-2 *ex vivo* assay). In transplanted patients and in rats with chronic CsA treatment, basal PGI was not decreased by CsA, which also supports the present observation [9,11]. A marginal decrease (15%) in urinary thromboxane was detected during the experimental period with saline infusion alone (Table 1 and Fig. 3a). Overall, the urinary values for thromboxane and PGI varied considerably between the study days. Several factors not controlled for in the present study, such as sodium and potassium balance or even seasonal fluctuations may have affected urinary excretion of prostanoids [19–21]. In particular, a difference in plasma sodium concentration after saline infusion might explain the differences in urinary thromboxane excretion, as the study by Agnoli *et al.* showed that saline-induced sodium retention (high plasma sodium concentrations) resulted in lower urinary thromboxane excretion [20]. No such changes were observed for urinary PGI-M, which is in line with our results.

In addition to the CNI-induced differential changes in PGI and thromboxane indices, we also observed a significant increase in systolic blood pressure after CsA treatment (Table 1), while no such effect was detected after Tac or saline infusions. Diastolic blood pressures and diuresis were unaltered during all the experiments. From the current study and blood pressure changes alone, not much can be inferred for long-term cardiovascular risk. However, vast literature supports that CsA increases blood pressure, while variable findings are reported regarding the effect of Tac. Thus, our findings of differential effects on prostanoids together with a CsA-induced increase in systolic blood pressure may indicate that CsA is more liable to facilitate cardiovascular adverse effects than Tac.

A limitation in our study is that the individuals underwent tests to study glucose metabolism [18] and hence received insulin and glucose infusions that could potentially affect the prostanoid production, although these effects are disputable [21–23]. However, all subjects underwent the same procedure at each visit, using standard doses of insulin (1 mU/kg/min) and keeping plasma glucose at 5 mmol/l from steady state to the end of the clamp, at which time prostanoid synthesis and excretion was measured. As our findings also support previous observations in transplant recipients [9], we do not expect that insulin or glucose affected prostanoid production significantly in our studies, yet find it more likely that the observed effects on COX activity are caused by Tac and CsA. Furthermore, this study was not powered to detect overall differences in prostanoid synthesis, which might explain why we only find significant differences within each treatment and not between the treatments (Table 1). In conclusion, acute effects of clinically relevant doses of CsA and Tac in healthy human subjects affect systemic prostanoid synthesis. In whole-blood, CsA suppresses COX-2 activity next to an increase in systolic blood pressures, while Tac decreases COX-1 activity and thromboxane release by activated platelets. Thus, these findings imply a more favorable cardiovascular profile of Tac compared with CsA.

Authorship

LAØ: designed the study, collected and analyzed data, wrote the paper. JS: analyzed data, wrote the paper. BJ: designed the study, wrote the paper. BLJ: designed the study, contributed important reagents, wrote the paper.

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