Role Of Endothelin-1 and Nitric Oxide Bioavailability in Transplant-Related Vascular Injury

Comparative Effects of Rapamycin and Cyclosporine

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Background—Cyclosporine (CyA) is associated with many side effects, including endothelial dysfunction and transplant vasculopathy (TxV). We previously demonstrated that CyA results in impairment of nitric oxide bioavailability and enhanced sensitivity to endothelin-1 (ET-1). In this study, we evaluated rapamycin (SRL) for its effects on the endothelium.

Methods and Results—Lewis rats (n = 8) were injected with SRL (1.5 mg/kg), CyA (5 mg/Kg), or saline (Con) intraperitoneally daily for 2-weeks. Thoracic aortic segments were assessed for endothelial-dependent (Edep) and independent (Eind) relaxation after exposure to acetylcholine and sodium nitroprusside by deriving the percent maximum relaxation (Emax). ET-1 plasma levels were also measured. Thoracic aortic expression of endothelial nitric oxide synthase (eNOS), ETα and ETβ receptors (Rc), were determined. Oxidative injury was assessed by changes in 8-isoprostane levels. CyA exposure resulted in lower Edep vasorelaxation compared with control and SRL (Emax: SRL, 58±4%; CyA, 24±7%; Con, 52±8%; \(P=0.001\)). No differences in Eind vasorelaxation were seen. CyA exposure also increased sensitivity to ET-1 (% maximum contraction [Cmax]: Con, 211±8%; SRL, 230±5%; CyA, 259±3%; \(P=0.04\)). Only SRL treatment reduced ET-1 plasma levels. CyA reduced eNOS expression by 30% and increased ETA Rc expression by 34% compared with both Con and SRL (\(P=0.02\)). CyA resulted in higher 8-isoprostane levels (CyA, 50±2%; SRL, 3±3%; Con, 2±5%; \(P=0.02\)).

Conclusions—CyA results in vascular dysfunction characterized by impairment of Edep vasorelaxation and enhanced sensitivity to vasospasm. SRL did not impair Edep vasorelaxation or increase sensitivity to vasospasm while lowering ET-1 levels and preserving eNOS protein expression. We conclude that SRL is less deleterious to the vasculature than CyA and may prevent TxV by these mechanisms. (Circulation. 2006;114[suppl I]:I-214–I-219.)

Key Words: cyclosporine ■ endothelial function ■ nitric oxide ■ rapamycin

Cyclosporine (CyA) was the first anti-rejection drug that impacted the results of clinical organ transplantation by reducing the incidence and severity of rejection and remains an important component of modern therapy. Unfortunately, CyA is associated with many side effects, such as nephrotoxicity, hepatotoxicity, neurotoxicity, and hypertension.1,2 CyA has also been implicated in the development of endothelial dysfunction and transplant vasculopathy (TxV).2,3

The mechanisms by which CyA results in endothelial dysfunction are not fully elucidated. However, CyA is known to impair vascular vasodilation4 and may induce vasoconstriction.5 Potential mechanisms resulting in vasospasm include the increased release of vasoconstrictors such as endothelin-1 (ET-1) or increased sensitivity to these vasoconstrictors. Most investigators have found an increase in ET-1 levels after CyA treatment, although this is not a consistent finding.6,7 CyA therapy may alter nitric oxide (NO) regulation, leading to an impaired vasodilatory response.8 Impaired NO homeostasis may be a result of decreases in mRNA or protein expression of endothelial nitric oxide synthase (eNOS) in CyA-treated patients. Several investigators have demonstrated that eNOS mRNA expression is increased after CyA treatment,9 suggesting that impaired NO production is likely due to decreases in eNOS protein synthesis or a shift to free radical production.10 There is also evidence that CyA may generate free radicals.11 These free radicals may result in direct endothelial injury and impaired vasomotor function.

Rapamycin (SRL), a relatively new immunosuppressant, is a macrolide antibiotic. SRL belongs to a class of drugs known as inhibitors of target of rapamycin (TOR). Several studies have demonstrated that SRL has both an anti-proliferative effect and a protective effect against the development of TxV in a rodent model.12,13 Corbin et al have shown that SRL led to vasomotor relaxation of rat aortic rings in a dose-
dependent fashion.\textsuperscript{14} In contrast, Jeanmart et al demonstrated that SRL results in worse endothelial-dependent vasorelaxation than CyA.\textsuperscript{15} However, both of these studies exposed the vasculature to rapamycin ex vivo for a short duration, making in vivo correlation unreliable. In addition the vehicle used for rapamycin in Jeanmart’s study also resulted in endothelial dysfunction.\textsuperscript{14,15} Whether SRL results in endothelial dysfunction or impairs NO–ET-1 homeostasis remains unclear. Clinically, SRL has been demonstrated to reduce the incidence, progression, and severity of TxV.\textsuperscript{16} yet the mechanisms by which SRL leads to endothelial protection and prevention of TxV remain unknown.

Our investigations assess the role of CyA and SRL on the development of endothelial dysfunction in a rodent model of vascular injury. Specifically, we examine the effects of CyA and SRL exposure on NO homeostasis and ET-1 signaling. We chose a nontransplant model for these initial experiments to evaluate the direct effects of CyA and SRL in the absence of either ischemia-reperfusion or immune-mediated injury.

**Materials and Methods**

All authors have read and agree to the manuscript as written. The authors had full access to the data and take full responsibility for their integrity.

Animal care conformed to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals (National Institutes of Health publication 86-23, revised 1996). Male Lewis rats (200 to 300 grams, n = 8 per group) were administered the drug of interest (saline control, CyA [5 mg/kg per day]) or SRL [1.5 mg/kg per day]) via peritoneal injection for a period of 14 days before assessment of endothelial function. On the day of euthanization (day 15), rats were anesthetized using isoflurane. A median sternotomy was performed and the thoracic aorta excised for tissue sampling. Segments of the descending thoracic aorta were procured for assessment of endothelial function. Before tissue excision, 1 mL of blood from the right ventricle was collected for analysis of ET-1 plasma levels. The rats were then exsanguinated under general anesthesia.

**Endothelial Function Assessment**

Endothelial-dependent and independent vascular relaxation was assessed in isolated segments of thoracic aorta after treatment. The aorta was dissected and segments 3 mm in length were used for the assessment of in vitro vascular function using a small vessel myograph for isometric tension recording. After mounting the vessel on a pressure transducer, maximum vasoconstriction was achieved with exposure to phenylephrine (100 nmol/L). After stabilization, endothelial-dependent relaxation was assessed by incremental exposure to acetylcholine (Ach). Endothelial-independent relaxation was assessed using incremental exposure to sodium nitroprusside (SNP).

Complete vasomotor data for all groups are presented in the figures to visualize the dose-dependent effects of each intervention. In addition, Emax\% was calculated by determining the percent maximal relaxation from phenylephrine-induced vasoconstriction. ED\textsubscript{50}, calculated as the concentration required to achieve half-maximum vasorelaxation, was compared between groups. After SNP washout, sensitivity to vasopasm was assessed by incremental exposure to ET-1 and %Cmax calculated as the maximum increase in tension from baseline. Each animal yielded 2 aortic segments. Data were included if the variability between segments was <10% and data were averaged to yield 1 result per animal.

**Plasma Measurements**

Venous blood was aspirated from the right ventricle before exsanguination. Blood samples were then centrifuged (14 000 rpm) to collect the plasma fraction, which was then snap-frozen in liquid nitrogen. Plasma ET-1 was extracted using C\textsuperscript{18} Sep-Pack columns after acidification with 1% trifluoroacetic acid and quantified using a commercial enzyme-linked immunosorbent assay (Biomedica, Vienna, Austria).

**Assessment of Oxidative Injury**

8-isoprostane levels were measured as an indicator of free radical injury.\textsuperscript{17} 8-isoprostane is the stable end product of arachidonic acid oxidation generated by reactive oxygen species injury. Determination of 8-isoprostane levels from thoracic aortic tissue was performed using a commercially available kit (Cayman Chemical Company; Ann Arbor, Mich). Baseline assessments were made on aortic segments harvested from control animals not subjected to intraperitoneal injections and the percent change from these baseline values was calculated to compare differences between groups.

**Western Blot Analysis**

Thoracic aortas were immediately collected after harvesting and snap frozen in liquid nitrogen until analyzed. The collected tissues were homogenized at 4°C and prepared for analysis. Protein determination was determined by the method described by Bradford.\textsuperscript{18}

Western Blot determined protein expression of inducible NOS, and eNOS, with the use of protein specific monoclonal antibodies (Biosciences, Mississauga, Canada), and ET\textsubscript{A} and ET\textsubscript{B} receptor (Rc) with the use of protein specific polyclonal antibodies (Chemicon, Temecula, Calif). Samples were separated using 4% stacking and 10% running tris-glycine SDS-PAGE gels. Gels were then transferred onto polyvinylidine difluoride membranes. Blocking was performed with buffer for 1 hour at room temperature and then monoclonal IgG at a dilution of 1:2500 was reacted with the blots for 12 hours at 4°C. Incubation with secondary antibody was then performed for 1 hour after washing. Comparisons between groups were performed using computerized densitometric analysis with a commercially available software program (BioRad).

**Statistical Analysis**

Statistical analysis was performed with the SAS statistical software program version 8.2 (SAS Institute Inc, Cary, NC). Continuous data were analyzed by analysis of variance (ANOVA) and are expressed as the mean±standard deviation. When the F-statistic of the ANOVA was significant (P<0.05), a Duncan multiple range test was performed to specify differences between groups.

**Results**

All animals survived until day of euthanization with no complications. There was minimal variability from aortic segments within each animal and no animals were excluded from the study. Plasma measurements of each immunosuppressant revealed a mean CyA level of 60±11 ng/mL and an SRL level of 7±1 ng/mL.

**Endothelial Function**

Endothelial-dependent vasorelaxation of the thoracic aorta was impaired after CyA treatment (Figure 1a). CyA resulted in an Emax\% of 24±7%, lower than that of control 52±8% and SRL 58±4% (P=0.001). No significant differences in endothelial-independent vasorelaxation to SNP were seen between groups (Figure 1b). However, when examining the relaxation curves, a lag in vasodilatory response to SNP is observed after CyA therapy. The concentration of SNP necessary to achieve 50% of maximal vasodilatory response demonstrated significant differences between groups (Figure 2). A doubling of the SNP ED\textsubscript{50} was seen after CyA (ED\textsubscript{50} 6.3×10\textsuperscript{-4}±1.2 mol/L) treatment compared with both control (ED\textsubscript{50} 3.2×10\textsuperscript{-8}±1.0 mol/L) and SRL (ED\textsubscript{50} 2.5×10\textsuperscript{-8}±1.1 mol/L) (P=0.01). Differences between SRL and control were not significant.
Sensitivity to ET-1–induced vasospasm revealed significant differences between groups, with %Cmax greater in the CyA-treated group compared with control and SRL (CyA 259±3% versus SRL 230±5% versus control 211±8%; P<0.05). (Figure 1c).

Plasma ET-1 Levels
CyA exposure did not alter plasma ET-1 levels compared with control (CyA 0.9±0.1 fmol/L; control 1.0±0.1 fmol/L) (Figure 3). However, SRL-treated animals demonstrated a significantly lower plasma ET-1 level (0.4±0.1 fmol/L, P<0.05).

Oxidative Injury
Figure 4 demonstrates that CyA treatment resulted in a greater increase in oxidative injury as measured by 8-isoprostane levels compared with control and SRL (CyA 50±2% versus SRL 3±3% versus control 2±5%; P<0.05).

Endothelin Receptor Expression
Thoracic aortic ETA Rc protein expression was significantly increased after CyA exposure compared with both control and SRL (Figure 5a). However, ETB Rc protein expression was not different between groups (Figure 5b) (P=0.29).

Nitric Oxide Synthase Expression
Two-week exposure to CyA resulted in a downregulation of eNOS protein expression compared with both control and SRL (P<0.001) (Figure 5c). However, extremely low inducible NOS expression was observed in all animals, with no difference between groups.

Discussion
Our study confirms that CyA results in endothelial dysfunction. CyA treatment alters normal vascular homeostasis as demonstrated by impaired endothelial dependent vascular
induced vasospasm. CyA also resulted in a higher ED50 to observation that CyA results in greater sensitivity to ET-1–pose vessels to vasoconstriction. This was confirmed by our ET-1 levels seen with CyA treatment may therefore predis-

reduction in ET-1 levels (which was not observed) would be CyA decreased NO levels and therefore a compensatory increased with no concomitant change in ETα Rc protein expression. ETα Rc and ETβ Rc activation on smooth muscle cells results in vasoconstriction; whereas ETβ Rc on endothelial cells results in vasodilation. Therefore, an increased ETα Rc-to-ETβ Rc ratio results in greater vasoconstriction. Although, CyA does not increase ET-1 levels compared with control, a relative increase may exist because CyA decreased NO levels and therefore a compensatory reduction in ET-1 levels (which was not observed) would be required to maintain normal homeostasis. The normal plasma ET-1 levels seen with CyA treatment may therefore predis-

Normal vessel function is maintained by the balance between NO and ET-1. Our study demonstrated that CyA alters both NO and ET-1 regulation. eNOS protein expression was reduced after treatment with CyA. The reduction in eNOS may be a consequence of CyA inhibiting cyclophillin cis-

trans peptidyl-prolyl isomerase function, resulting in im-

paired eNOS folding and therefore increased degradation. Second, although ET-1 levels were not elevated by CyA treatment, ETα Rc protein expression in the thoracic aorta was significantly increased with no concomitant change in ETα Rc protein expression. ETα Rc and ETβ Rc activation on smooth muscle cells results in vasoconstriction, whereas ETβ Rc on endothelial cells results in vasodilation. Therefore, an increased ETα Rc-to-ETβ Rc ratio results in greater vasoconstriction. Although, CyA does not increase ET-1 levels compared with control, a relative increase may exist because CyA decreased NO levels and therefore a compensatory reduction in ET-1 levels (which was not observed) would be required to maintain normal homeostasis. The normal plasma ET-1 levels seen with CyA treatment may therefore predis-

pose vessels to vasoconstriction. This was confirmed by our observation that CyA results in greater sensitivity to ET-1–induced vasospasm. CyA also resulted in a higher ED50 to SNP compared with the other treatment groups indicating impaired cGMP-dependent SMC relaxation. Previous studies have suggested that CyA treatment increases free radical production and our study confirmed that oxidative injury occurred after CyA exposure. We therefore speculate that CyA treatment results in functional uncoupling of the eNOS enzyme producing free radicals instead of NO. Krauskopf et al also showed that CyA can generate superoxide in smooth muscle, which may lead to impaired function. Therefore, oxidative injury may account for the smooth muscle dysfunction observed in our vascular assessments.

SRL therapy did not decrease eNOS protein expression as seen after CyA treatment. As a result, there was no impairment in endothelial-independent or dependent vasodilation. SRL treatment did not result in increased sensitivity to ET-1. In addition, SRL treatment resulted in less oxidative injury compared with CyA. Therefore, SRL may improve endothelial and smooth muscle function by enhancing NO bioavailability and reducing oxidative injury.

The mechanisms by which SRL preserves endothelial function may also be the mechanism by which it attenuates the development of aortic allograft vasculopathy. Simonson et al have demonstrated that ET-1 inhibition attenuates the development of TxV. Therefore, the ability to lower ET-1 levels may be a mechanism by which SRL attenuates allograft vasculopathy in transplant recipients. SRL treatment preserved eNOS expression compared with CyA and represents another mechanism by which SRL protects against vasculopathy. Lee et al have shown that eNOS protects the aortic allograft from the development of transplant atherosclerosis. Oxidative stress is well-known to result in endothelial damage and atherosclerosis and, in these studies, SRL exposure resulted in less free radical injury compared with CyA.

Our findings suggest possible treatment strategies for improving vasomotor function in patients receiving standard immunosuppression. An effective strategy to treat CyA induced vasomotor dysfunction should include ET-1 antagonism in addition to functional coupling of eNOS. This may be achieved by using Bosentan for ET-1 antagonism and tetrahydrobiopterin (BH4), an essential eNOS cofactor, for stabilizing the eNOS complex and reducing free radical production. We have previously demonstrated that the use of BH4 partially attenuated the deleterious effects of CyA. Our findings provide potential mechanisms for the development of CyA-induced hypertension as well as a direct mechanism by which CyA may lead to TxV. SRL may prove to be an alternative therapy to CyA for preserving vasomotor function. Poston et al using a rodent heterotopic heart transplant model demonstrated the ability of rapamycin to reverse chronic graft vascular disease. Their study describes the beneficial effects of rapamycin on vasculopathy but did not evaluate the underlying mechanism of benefit. Their study did reveal that TxV occurs in the absence of myocardial rejection, suggesting that rapamycin’s protective effects are likely not mediated by its immunosuppressive activity. Our studies suggest that alterations in NO–ET-1 homeostasis plays a critical role in the development of TxV. Using the same model, Yamaguchi et al demonstrated that ET-1 blockade reduces TxV, supporting our hypothesis that ET-1 is involved in the pathogenesis of TxV. In this study, ET-1 production was inhibited preoperatively before transplantation with the use of anti-

Figure 4. The 8-isoprostane levels in the thoracic aorta after 2 weeks of treatment. Cyclosporine (CyA) exposure significantly increases oxidant injury compared with rapamycin (SRL) and control (CON).
sense oligodeoxynucleotides, resulting in a 7- to 10-day suppression of ET-1. Our present study demonstrated that ET-1 levels are not elevated by CyA and that ET_A receptor upregulation lasts for at least 2 weeks, indicating that chronic therapy may be required for enhanced protection against CyA induced TxV. Verrier et al demonstrated in a rodent model of orthotopic aortic allograft transplantation that ischemia and reperfusion results in endothelial injury leading to the development of TxV. Therefore, Yamaguchi’s study may in fact demonstrate the protective effect of acute ET-1 blockade in limiting ischemia–reperfusion-induced TxV rather than drug-induced TxV. Both of these studies support our hypothesis that

Figure 5. a, Quantitative Western blot analysis of ET_A receptor (Rc) expression in the thoracic aorta. Cyclosporine (CyA) treatment result in increased ET_A Rc expression compared with rapamycin (SRL) and control (CON). b, Quantitative Western blot analysis of ET_B receptor protein expression in the thoracic aorta. No differences were observed between groups. c, eNOS protein expression. Quantitative Western blot analysis of eNOS protein expression in the thoracic aorta. Cyclosporine (CyA) decreased eNOS expression whereas rapamycin (SRL) demonstrated no reduction in eNOS protein expression compared with control (CON).
Summary of Vascular Effects of Cyclosporine (CyA) and Rapamycin (SRL)

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Ach indicates acetylcholine; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; ET-1Rc, ET-1 receptor (either A or B); ROS, reactive oxygen species.

by maintaining vascular homeostasis rapamycin may prevent TxV and that ET-1 antagonism may provide additional benefit.

Study Limitations

The present study was designed to investigate the direct effect of CyA and SRL on vascular function in the absence of an immune response or a period of ischemia and reperfusion. Clearly, the effects of the latter 2 variables will need further assessment in a heterotopic transplant model similar to that of Poston and Yamaguchi. In addition, we evaluated changes in aortic tissue as opposed to coronary arteries. Although the responses are likely consistent, it is possible that both CyA and SRL exert differential effects on coronary vasculature than seen in thoracic aorta. However, the macrovascular effects of these agents have important clinical implications for the development of postoperative renal insufficiency and hypertension. Lastly, we did not evaluate the effects of combination therapy with both CyA and SRL, a common clinical strategy.

In summary, we have made the following conclusions based on the present data: (1) CyA results in alteration of both NO and ET-1 regulation likely leading to impairment of vasodilation, increased sensitivity to vasopasm, and increased oxidative injury; (2) SRL preserves the eNOS complex and lacks the deleterious effects of CyA on the endothelium; and (3) SRL therapy decreases ET-1 levels.

These findings provide important mechanistic data to explain observed effects in future studies using a heterotopic transplant model. Furthermore, these data strongly suggest a potential clinical role for simultaneous endothelin antagonism and NO augmentation.

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Disclosures

None.

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