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

Comparative Effects of Rapamycin and Cyclosporine

Danny Ramzy, MD; Vivek Rao, MD, PhD; Laura C. Tumiati BSc; Ning Xu, MSc; Santiago Miriuka, MD; Diego Delgado, MD; Heather J. Ross, MD, MSc

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), ETA and ETB 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 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
dependent fashion. In contrast, Jeanmart et al demonstrated that SRL results in worse endothelial-dependent vasorelaxation than CyA. 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. 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 TxE yet the mechanisms by which SRL leads to endothelial protection and prevention of TxE 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 5 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. Because the mechanical properties of the aortic media can vary among species, animals, and 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.

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 Em% 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).

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 C18 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. 8-isoprostane is the stable end product of arachidonic acid oxidation generated by reactive oxygen species injury.

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.
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 = 0.04) (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 Re protein expression was significantly (P = 0.004) increased after CyA exposure compared with both control and SRL (Figure 5a). However, ETB Re 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–
reduction in ET-1 levels (which was not observed) would be 
compensatory effects of SRL treatment, which maintains ET-1-NO 
mechanism of CyA-induced endothelial injury and the bene-

treatment, ETA Rc protein expression in the thoracic aorta 
Normal vessel function is maintained by the balance between 
NO and ET-1. Our study demonstrated that CyA alters both 

dilation. The novel aspects of our study relate to the 
mechanism of CyA-induced endothelial injury and the benefi-
cyA impaired eNOS protein expression. 
4. CyA exposure led to greater oxidative injury as mea-
sured by 8-isoprostane levels compared with both SRL 
and control. 

1. SRL exposure reduced plasma ET-1 levels. 
2. CyA exposure significantly increased ETα Rc expression whereas SRL had no effect on ETα Rc expression. 
3. CyA impaired eNOS protein expression. 
4. CyA exposure led to greater oxidative injury as measured by 8-isoprostane levels after 2 weeks of treatment. Cyclosporine (CyA) exposure significantly increases oxidant injury compared with rapamycin (SRL) and control (CON).

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 ETA 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 ETA receptor (Rc) expression in the thoracic aorta. Cyclosporine (CyA) treatment result in increased ETA Rc expression compared with rapamycin (SRL) and control (CON). b, Quantitative Western blot analysis of ETB 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.\(^23,24\) 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 vaso vaspain, 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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