Dual immunosuppression enhances vasomotor injury: Interactive effect between endothelin-1 and nitric oxide bioavailability

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Objective: Cyclosporine A and corticosteroids are associated with many side effects, such as endothelial dysfunction and transplant vasculopathy. We examined the effects of cyclosporine A and hydrocortisone exposure on endothelial function of the rat thoracic aorta.

Methods: Lewis rats were injected with cyclosporine A, hydrocortisone, cyclosporine A + hydrocortisone, or intraperitoneal saline daily for 2 weeks. Endothelial-dependent and independent vascular relaxation were assessed in isolated segments of thoracic aorta, as well as endothelin-1–induced vasoreactivity. Protein expression of endothelial nitric oxide synthase, endothelin$_A$, and endothelin$_B$ receptors were also determined in the thoracic aorta.

Results: Exposure to cyclosporine A and cyclosporine A + hydrocortisone resulted in a reduction in endothelial-dependent vasorelaxation compared with control and hydrocortisone ($P = .001$). Cyclosporine A and hydrocortisone-treated rats demonstrated increased vasoreactivity to endothelin-1 compared with control, whereas cyclosporine A + hydrocortisone treatment resulted in a synergistic increase ($P = .04$). All treatment groups displayed a significant reduction in endothelial nitric oxide synthase expression compared with control ($P = .001$). Endothelin$_A$ receptor expression was increased in all treatment groups with a synergistic effect seen after cyclosporine A + hydrocortisone treatment. No differences were seen in endothelin$_B$ receptor expression.

Conclusion: Cyclosporine A and hydrocortisone induce vasomotor dysfunction with a synergistic impairment observed after concomitant exposure. Our findings suggest that the resultant vasomotor dysfunction is the result of alterations in both nitric oxide and endothelin-1 regulation.

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Cyclosporine A (CyA) was the first antirejection drug that affected the results of clinical heart transplantation by reducing the incidence and severity of rejection and remains an important component of modern immunosuppressive therapy. Unfortunately, CyA is associated with many negative side effects, such as nephrotoxicity, hepatotoxicity, neurotoxicity, and hypertension.[1-3] Other adverse effects include endothelial dysfunction and the development of transplant vasculopathy.[1,4-10] CyA can influence transplant vasculopathy by increased plasma lipid concentrations, causing hypertension, or by direct injury to the endothelium.[10-13] It is believed that the endothelial injury induced by CyA results in impaired vascular homeostasis and transplant coronary disease.

The mechanisms by which CyA results in endothelial dysfunction are not fully elucidated. However, CyA is known to impair vasodilation[14-17] and may induce vasoconstriction.[18-20] Potential mechanisms resulting in vasospasm include the increased release of vasoconstrictors or increased sensitivity to these vasoconstrictors. One such vasoconstrictor is endothelin (ET)-1. The effect of CyA on plasma levels of

Abbreviations and Acronyms

CyA = cyclosporine A
eNOS = endothelial nitric oxide synthase
ET = endothelin
Hcort = hydrocortisone
LV = left ventricular
NO = nitric oxide
Rc = receptor
SNP = sodium nitroprusside
TAo = thoracic aorta

ET-1 remains controversial. Most investigators have found an increase in ET-1 levels after CyA treatment, although this is not a consistent finding.[15,19,21-27] Furthermore, altered nitric oxide (NO) homeostasis may result in an impaired vasodilatory response.[15] 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 RNA expression is increased after CyA treatment,[28] suggesting that impaired NO production may be due to decreases in eNOS protein synthesis or a shift to free radical production.[29] There is also evidence that CyA generates free radicals.[4] These free radicals may result in direct endothelial injury and impaired vasomotor function.

Corticosteroids are another commonly used antirejection agent also associated with several side effects. Corticosteroids can inhibit the release of vasodilators, such as histamine and prostacyclin. We hypothesized that the combination of CyA and corticosteroids (commonly used after solid organ transplantation) results in a synergistic impairment of vascular function. Our investigations assessed the role of CyA, and hydrocortisone (Hcort) on the development of endothelial dysfunction in a rodent model of vascular injury. Although these investigations have direct relevance to transplant vasculopathy, we intentionally avoided a transplant model to eliminate the confounding effects of immune-mediated injury. Thus, these studies evaluate the drug-specific changes to vascular function after sustained exposure. Specifically, we examined the effects of CyA and Hcort exposure on NO homeostasis and ET-1 signaling.

Materials and Methods

Animal care conformed to the "Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals" (National Institutes of Health publication no 86-23, revised 1996).

Male Lewis rats (n = 16) (200–300 g) were administered the drug of interest (saline control, CyA (5 mg/kg) or Hcort (20 mg/kg)) via peritoneal injection daily for a period of 2 weeks before assessment of endothelial function. On the day of sacrifice, rats were anesthetized using isoflurane. Median sternotomy was then performed, the heart was excised for myocardial tissue sampling, and segments of aorta were procured for assessment of endothelial function. Before heart 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 were assessed in isolated segments of thoracic aorta (TAo) after treatment. The TAo was dissected, and vascular segments (5 mm in length) were used for the assessment of in vitro vascular function using a small vessel myograft for isometric tension recording. After mounting the vessel on a pressure transducer, maximum vasoconstriction was achieved with exposure to phenylephrine. After stabilization, endothelial-dependent relaxation was assessed by incremental exposure to acetylcholine. Endothelial-independent relaxation was assessed by incremental exposure to sodium nitroprusside (SNP). The maximum relaxation from phenylephrine-induced vasoconstriction (Emax%) was compared between groups. ED_{50}, calculated as the concentration required to achieve half-maximum vasorelaxation, was compared between groups. Sensitivity to vasospasm was assessed in vessels following stabilization after SNP washout. Incremental exposure to ET-1 was performed, and %Cmax was calculated as the maximum increase in tension from baseline. Each animal yielded 2 aortic segments. Data were included if the variability between segments was less than 10%, and data were averaged to yield 1 result per animal.

Plasma Measurements

Venous blood was aspirated from the right ventricle before exsanguination. CyA trough levels were performed on whole blood. For ET-1 levels, blood samples were centrifuged (14,000 rpm) to collect the plasma fraction, which was snap-frozen in liquid nitrogen and stored at −80°C. ET-1 in plasma was extracted using C^{18} Sep-Pack (Waters Corporation, Milford, Mass) columns after acidification with 1% trifluoroacetic acid. Plasma ET-1 levels were measured 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-mediated injury.[30] 8-isoprostane is the stable end product of arachidonic acid oxidation generated by reactive oxygen species injury.[31] Determination of 8-isoprostane levels in left ventricular (LV) myocardial tissue was performed using a commercially available kit (Cayman Chemical Company, Ann Arbor, Mich). The percentage from baseline (LV from animals that received no intraperitoneal injections) was calculated to compare differences between groups.

Western Blot Analysis

LV biopsies and the TAo were immediately collected after harvesting. Biopsy specimens were snap-frozen in liquid nitrogen and stored at −80°C until analyzed. Biopsies were homogenized at 4°C and prepared for analysis. Protein determination was determined by the method described by Bradford.[32]

Western blot determined the protein expression of inducible NOS, eNOS, tumor necrosis factor-α, and transforming growth factor-β with the use of protein-specific monoclonal antibodies (BD Biosciences, Mississauga, Canada) and ET_A and ET_B receptors (Rc's) with the use of protein-specific polyclonal antibodies