Acute Exposure to Cyclosporine Does Not Increase Plasma Homocysteine in Rats


ABSTRACT

There is interest in the postulate that cyclosporine (CsA) contributes to the elevated homocysteine levels seen in organ transplant recipients, as hyperhomocysteinemia is now considered an independent risk factor for cardiovascular disease (CVD) and may partially explain the increased prevalence of CVD in this population. The main purpose of this investigation was to determine the effect of CsA administration on plasma homocysteine. Eighteen female Sprague Dawley rats (4 months old) were randomly assigned to either a treatment or a control group. For 18 days the treatment group received of CsA (25 mg/kg/d) while the control group received the same volume of the vehicle. Blood samples obtained following sacrifice to measure CsA, total homocysteine, and plasma creatinine.

There were no significant differences in plasma homocysteine (mean values ± SD: treatment = 4.79 ± 0.63 μmol/L, control = 4.46 ± 0.75 μmol/L; P = .37). Homocysteine was not significantly correlated with final CsA concentrations (r = .17; P = .69). There was a significant difference in plasma creatinine values between the two groups (treatment = 60.44 ± 7.68 μmol/L, control = 46.33 ± 1.66 μmol/L; P < .001). Furthermore, plasma homocysteine and creatinine were positively correlated with the treatment group (r = .73; P < .05) but not the controls (r = -.10; P = .81). In conclusion, CsA does not influence plasma homocysteine concentrations in rats.

Atherosclerotic vascular events resulting in myocardial infarction and stroke are the leading cause of mortality in organ transplant recipients.1–3 Traditional cardiovascular disease (CVD) risk factors, such as smoking, hypertension, and dyslipidemia fail to predict morbidity and mortality in this population.2 Therefore, other factors need to be identified.

Elevated plasma homocysteine (hyperhomocysteinemia) has been suggested to be an independent risk factor for arterial and venous thromboembolism and atherosclerotic vascular disease of the coronary, cerebral, and peripheral vessels.4–8 Purported mechanisms that may explain this effect include increased endothelial injury, smooth muscle cell proliferation, low-density lipoprotein oxidation, and alters hemostatic balance.9 There is now substantial evidence demonstrating that hyperhomocysteinemia is present in transplant recipients.10–15 Although a definite link between homocys-

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tein and CVD has yet to be established in any population, such an association would provide a plausible explanation for the high prevalence of CVD seen in these patients.

Several case-control and retrospective studies in humans have suggested that cyclosporine (CsA) is a contributing factor to the increased plasma homocysteine seen in recipients.\(^{11,12,16,17}\) CsA-treated renal transplant recipients show significantly elevated plasma homocysteine concentrations compared to non-CsA-treated patients.\(^{11}\) and plasma homocysteine was elevated following transplantation in patients receiving higher doses of CsA.\(^{12}\) Furthermore, other studies have shown that CsA and plasma homocysteine are significantly correlated in renal\(^{18}\) and cardiac\(^{17}\) patients. Indeed, Cole et al.\(^{17}\) reported that a 100 \(\mu\)g/L increase in CsA resulted in a 1.75 \(\mu\)M increase in plasma homocysteine concentrations.

Suspected mechanisms to explain the effect of CsA to elevate homocysteine include interference with the remethylation of homocysteine or CsA-induced nephrotoxicity leading to decreased renal metabolism of the compound.\(^{19}\) The effects of CsA on renal function make it difficult, in clinical studies, to determine actions on homocysteine separate from changes in renal function. Furthermore, differences in confounding variables that influence homocysteine, such as dietary B vitamin intake, other therapeutic treatments and comorbidities, make assessment of CsA on homocysteine difficult in clinical populations.

Therefore, the purpose of this study was to use an animal model to determine the effects of CsA administration on plasma homocysteine. We hypothesized that administration of CsA would have no significant effect on plasma homocysteine based on the work of Bostom et al.\(^{20}\) that renal function was the strongest independent determinant of plasma homocysteine concentration, but that CsA had no influence.

MATERIALS AND METHODS

Animals

Eighteen female Sprague Dawley rats weighing 247 ± 22 g (mean ± SD) were randomly assigned to one of two experimental groups: CsA or control \((n = 9\) per group). Rats fed rat chow and water ad libitum were maintained on a 12-hour light/dark photo-period with a room temperature of 21°C ± 2°C. For approximately 7 days prior to the beginning of the experiments, animals were handled daily by laboratory technicians to reduce contact stress. The treatment group were administered CsA (25 mg/kg dissolved in extra light olive oil and 4% ethanol) daily for 18 days via intraperitoneal injection. The control rats were sham-injected daily with 10 \(\mu\)L of 0.9% sodium chlorohydride (0.212 mol/L) or 10 \(\mu\)L saline for 18 days via intraperitoneal injection. The control rats were sham-injected daily for 18 days with the same volume of vehicle. Animal mass was recorded daily; CsA doses were adjusted to changes in body mass. All injections were performed at approximately the same time of day in both groups. The drug dose, vehicle, administration route, and treatment period were based on a number of previous animal studies investigating the effect of CsA on the liver and kidney.\(^{21–24}\)

Blood Collection

Blood samples were collected approximately 3 hours after the final CsA injection based on the findings of Ibarra et al.\(^{25}\) that CsA concentrations peak 2 to 4 hours following an intraperitoneal injection in rats. Animals were injected with 90 mg/kg sodium pentobarbital intraperitoneally. After a surgical plane of anesthesia was reached, rats were exsanguinated via intracardiac puncture; approximately 5 mL of blood was collected from each rat. From this sample 300 \(\mu\)L of whole blood was transferred to a tube containing ethylene diaminetetraacetic acid to assay peak CsA concentrations within 48 hours of collection. The remaining volume of the original sample was centrifuged (within 15 minutes of collection) at 4000 rpm and 4°C for 5 minutes. Plasma was removed and stored in aliquots at −80°C for biochemical analysis for plasma homocysteine and plasma creatinine.

Biochemical Analyses

**Plasma Homocysteine.** Plasma homocysteine concentrations were determined by high-performance liquid chromatography (HPLC) in the biochemistry laboratory at the Defense Nutrition Research Centre (DNRC; Scottsdale, Tasmania, Australia) using a modification of the method of Ji et al.\(^{26}\) The DNRC is a member of the European Research Network for Evaluation and Improvement of Screening, Diagnosis and Treatment of Inherited Metabolic Disorders external quality assurance program for plasma homocysteine analysis. Sulfide bonds were reduced by mixing 0.1 mL plasma with 10 \(\mu\)L sodium borohydride (0.212 mol/L) prior to incubation at 42°C in a water bath for 10 minutes. Monobromobimane (10 \(\mu\)L; 70.0 mmol/L) was then added to label the plasma thiols with a fluorescent moiety, before incubating at 42°C again. Plasma proteins and excess reducing reagent were then removed by adding 50 \(\mu\)L perchloric acid (1.5 mol/L) and spinning at 12,400g for 10 minutes. Plasma supernatant (20 \(\mu\)L) was transferred to HPLC vials and neutralized by adding 20 \(\mu\)L tris base (2.0 mol/L) to ensure a pH between 3 and 4. The analysis of homocysteine was performed using a Waters HPLC system with fluorescence detection (Waters, Milford, Mass, USA). Homocysteine concentrations were calculated by the use of an internal standards ratio method against homocysteine and cysteamine standards. The coefficient of variation (CV) data from in-house pooled plasma control material (−10 \(\mu\)mol/L), was estimated within- and between-batch CV were less than 4%. Plasma homocysteine concentrations from the rats were normally distributed; parametric statistics were used.

**Plasma Creatinine.** Plasma creatinine was determined using the Jaffe reaction method. Briefly, plasma was combined with picric acid in an alkaline solution forming a bright orange-red complex.\(^{27}\) Absorbance of the complex was measured at 520 nm using the Olympus AU600 analyzer (Olympus Optical, Tokyo, Japan).

**CsA.** Whole blood CsA concentrations were determined at the Hunter Area Pathology Service (Hunter Hospital, NSW, Australia) using the EMIT 2000 Specific Assay (Dade Behring; Syva, Deerfield, Ill, USA). The method is based on a homogenous enzyme immunoassay performed on a Roche Cobas Mira chemistry system (Roche, Basel, Switzerland).\(^{28}\) The Hunter Area Pathology Service is enrolled in the United Kingdom Cyclosporine Quality Assessment Scheme.

Data Analysis

Unpaired two-tailed t-tests were used to determine statistical differences between the treatment and control groups for body weight, plasma homocysteine, and creatinine. Pearson correlation analysis was used to determine the relationships between these variables. SPSS version 10 for Windows (SPSS Inc, Chicago, Ill, USA) was used for statistical calculations. The level of significance was set at \(P < .05\). All data shown are expressed as mean values ± SD.
RESULTS

CsA-treated animals showed a significant decrease in body weight over the trial period (control decreased to 99.8% ± 3.2% vs CsA decreased to 93.2% ± 4.2% original weight; \( P < .05 \)).

Table 1 shows individual and group mean data for plasma homocysteine, creatinine, and peak CsA concentrations. There was no significant difference \( (P > .05) \) in plasma homocysteine between the treatment and control groups. However, CsA treatment caused a significant increase \( (P < .05) \) in plasma creatinine in treated compared to control animals.

Pearson product correlation analysis revealed a significant positive correlation \( (r = .73; P < .05) \) between plasma creatinine and homocysteine in the treatment group (Fig 1) but not in the control group \( (r = -.10; P = .81) \). Among the CsA-treated animals, blood CsA concentration was not correlated with plasma homocysteine \( (r = .17; P = .69) \) nor plasma creatinine \( (r = .09; P = .83) \).

DISCUSSION

This is the first study using an animal model to examine the effect of CsA administration on plasma homocysteine. The major finding of this study was that CsA did not significantly increase plasma homocysteine. Blood concentrations of the two parameters were not significantly correlated. However, CsA increased plasma creatinine, a marker of renal function that correlated with plasma homocysteine. Overall, these data indicate that CsA impairs renal function but does not cause hyperhomocysteinemia.

Although CsA-increased plasma creatinine and homocysteine was significantly related to creatinine, it is surprising that CsA did not affect homocysteine. We speculate that the effect of CsA on renal function is greater than the effect of renal function on homocysteine. Indeed, it is known that homocysteine is metabolized through both intra- and extrarenal pathways. Therefore, although the renal function may be significantly decreased and this should theoretically increase plasma homocysteine concentrations, the extrarenal metabolism of the homocysteine may increase in a compensatory manner to limit significant elevations in plasma homocysteine. This implies that CsA by itself does not impair the metabolism of homocysteine and that changes in homocysteine concentrations among individuals on CsA are due to changes in renal function. Our findings and this postulate agree with Bostom, who stated that elevated homocysteine in transplant recipients may simply be another marker of renal dysfunction.

In agreement with the finding that plasma homocysteine was not significantly elevated in the CsA group, a number of studies in humans have also reported that plasma homocysteine was not significantly different between recipients either receiving or not receiving CsA. Importantly, in the studies by Ducloque et al and Bostom et al the analyses were adjusted for renal function and other determinants of plasma homocysteine such as age, gender, and time since transplantation. Early studies by Arnadottir et al reported a significant increase in plasma homocysteine between CsA users and nonusers did not adjust for renal function indices and other variables affecting plasma homocysteine.

In the present study CsA was not correlated with plasma homocysteine \( (r = .17; P = .69) \), further indicating that CsA...
is not related to plasma homocysteine. Previous studies in humans by DuCloux et al. and Arnadottir et al. also reported that plasma concentrations of CsA and homocysteine were not correlated in recipients. In addition, Arnadottir et al. found no relationship between CsA and posttransplant plasma homocysteine concentrations.

There was a significant difference in plasma creatinine concentrations between the treatment and control groups. Given the toxic effect of CsA on the kidney, it was expected that renal function would decline in the animals treated with CsA. Provoost et al. demonstrated that CsA significantly decreased renal function. Myers et al. compared patients treated with CsA and prednisone versus those treated with azathioprine and prednisone, observing that the CsA group had a significantly lower GFR compared to patients in the azathioprine group. In a second study, the same authors demonstrated that serum creatinine was significantly higher in recipients treated with high-dose CsA (trough CsA = 100 to 300 ng/mL) compared to low-dose CsA (trough CsA = 50 to 100 ng/mL).

While the exact mechanisms of CsA nephrotoxicity are not fully known, there is evidence to suggest that the drug elevates oxidative stress and causes intrarenal vasoconstriction. Cardiac recipients treated with CsA for 1 to 2 years demonstrated significantly reduced renal plasma flow and increased renal vascular resistance compared to azathioprine-treated controls. Similar findings have been demonstrated in rats administered CsA or sham-operated controls. Human studies in cardiac transplant recipients have also produced data supporting the present finding that CsA decreases renal function. Myers et al. compared patients treated with CsA and prednisone versus those treated with azathioprine and prednisone, observing that the CsA group had a significantly lower GFR compared to patients in the azathioprine group. In a second study, the same authors demonstrated that serum creatinine was significantly higher in recipients treated with high-dose CsA (trough CsA = 100 to 300 ng/mL) compared to low-dose CsA (trough CsA = 50 to 100 ng/mL).

The animals in the treatment group showed a significantly greater weight loss than controls. This observation raises the possibility that malnutrition may have been involved in suppressing any effect of CsA on homocysteine. However, although food intake was not monitored in the present study, it has been previously shown that weight loss occurred in CsA-treated rats despite similar food intake to rats not receiving CsA.

In summary, CsA administration for 18 days failed to increase plasma homocysteine; however, plasma creatinine was significantly elevated in CsA-treated animals and CsA concentrations significantly correlated with plasma creatinine values. The clinical implications of these findings are that CsA appears unlikely to directly cause the hyperhomocysteinemia seen in transplant recipients. Elevations of homocysteine in recipients are due to impaired renal function caused by the immunosuppressant. Future research should focus on methods to counteract the reduced renal function secondary to CsA toxicity, such as the use of vasodilators to improve renal plasma flow, thus preventing ischemic injury and loss of nephron mass or antioxidants to counteract the increased oxidative stress. Continued investigations into the mechanisms of CsA-induced nephrotoxicity may also aid in identifying possible strategies to reduce renal failure following organ transplantation.

REFERENCES

10. Arnadottir M, Hultberg B: Transplantation 64:1087, 1997