REGULAR ARTICLE

The Peripheral Serotonergic System and Platelet Aggregation in Cyclosporin A-Induced Hypertensive Rats

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(Received 26 February 1999 by Editor H. Arnesen; revised/accepted 18 June 1999)

Abstract

Cyclosporin A plays an important role in preventing rejection in allograft transplant recipients. However, the therapeutic use of cyclosporin A is associated with increased incidence of thromboembolic complications and drug-related hypertension. In order to study the mechanisms by which cyclosporin A induces these abnormal pathophysiological situations, we have assessed the platelet serotonin contents and whole blood platelet aggregation in control rats as well as in rats treated (orally) with 30 and 5 mg/kg/day of cyclosporin A, after 2 and 7 weeks of treatment. These doses correspond respectively to CsA “peak” and “trough” concentrations achieved in human blood in clinical practice (immediately following an intake of a daily dose of CsA and when the blood concentration stabilizes, respectively). Both trough and peak doses caused an increase in blood pressure after 2 and 7 weeks. Platelet serotonin content decreased in the cyclosporin-treated groups, in contrast with the control. Collagen-induced whole blood platelet aggregation increased drastically for the peak concentration-treated group, while adenosine 5’-diphosphate-induced platelet aggregation did not reach statistical significance. Finally, in vitro platelet thromboxane A₂ generation increased in cyclosporin A concentrations when platelets were stimulated with either collagen or adenosine 5’-diphosphate. In conclusion, both tested cyclosporin A concentrations induced important changes in platelet serotonin and thromboxane content and aggregation, factors which may play a decisive role in the development and/or maintenance of hypertension and thrombotic complications.

Key Words: Cyclosporin A; Hypertension; Thromboembolic complications; Serotonin; Aggregation

Abbreviations: CsA, cyclosporin A; 5-HT, 5-hydroxytryptamine; TXA₂, thromboxane A₂; PRP, platelet-rich plasma; ADP, adenosine 5’-diphosphate; PPP, platelet-poor plasma; TXB₂, thromboxane B₂; SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure.

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Cyclosporin A (CsA) is a potent and clinically relevant immunosuppressive agent and is widely used in organ transplantation [1,2] and also in the treatment of autoimmune diseases [3]. However, despite its effectiveness, CsA therapy has been associated with drug-related hypertension [4–7] and increased risk of thromboembolic complications [8–10], which may be partly due to the direct effects of CsA on platelets [11–14].

It is well known that platelets may play an important role in the pathophysiology of hypertension and also in the pathogenesis of thrombosis. Activated platelets are a source of the most potent,
naturally found vasoconstrictors, such as thromboxanes and endoperoxides. Many studies have reported cyclosporin A-induced changes in the thromboxane/endoperoxides balance [13,15,16], in enhanced hemostasis and reduced thrombolysis [17,18], in increased platelet aggregability [12–14,19], as well as in altered peripheral serotonergic mechanisms [14,20–22]. Serotonin (5-hydroxytryptamine, 5-HT), mainly present in platelets, has been linked with thrombotic complication and increase in blood pressure [23,24]. When activated, platelets adhere and aggregate to the damaged endothelium, releasing (among other compounds) serotonin and thromboxane A2 (TXA2), which may act as vasoconstrictors and also extend aggregation to other agonists, thus promoting thrombus formation.

Therefore, the purpose of this study was to determine the effects of CsA on the peripheral serotonergic system, platelet aggregation, and TXA2 generation.

1. Materials and Methods

1.1. Animals and Diets

Male Wistar rats (Charles River Laboratories Inc., Barcelona, Spain), ~300 g, were maintained in an air-conditioned room, subjected to 12-hour dark/light cycles and given standard laboratory rat chow and free access to tap water. Animal experiments were conducted according to the European Convention on Animal Care, and the whole research project was approved by the Portuguese National Foundation for Science and Technology. The rats were divided into three groups: one group received orange juice only (control) and the others received 5 and 30 mg/kg/day of CsA (Sandimmun Neoral, Sandoz Pharma, Basel, Switzerland) dissolved in orange juice for 7 weeks. Blood pressure values (systolic, diastolic, and mean) were measured using a tail-cuff sphygmomanometer. Thromboxane in vitro studies were carried out through the incubation of platelets with CsA (dissolved in dimethyl sulfoxide) at 0.1 and 1.0 μmol/L concentrations. These concentrations were calculated to match the real blood concentrations of patients taking CsA: 1.0 μmol/L immediately after an intake of a daily dose of CsA (“peak”) and 0.1 μmol/L, when the blood concentration stabilizes (“trough”). These concentrations also corresponded to the 30 and 5 mg/kg/day of CsA administered to the rats.

1.2. Collecting the Blood and Preparing the Platelets

Following intraperitoneal ketamine anesthesia, blood was withdrawn by venipuncture from the jugular vein and added to an anticoagulant solution (0.1 mL/mL blood) containing (in mmol/L) citric acid (71), sodium citrate (85), and d-glucose (111). The blood was centrifuged (160×g for 10 minutes at 20°C) to obtain platelet-rich plasma (PRP) and the platelets were then recovered by recentrifugation at 730×g for 10 minutes at 20°C.

1.3. Platelet Serotonin Contents

The platelet pellet was resuspended in 1 mL of a buffer solution (pH 7.4) containing (in mmol/L): NaCl (145), KCl (5), MgSO4 (1), CaCl2 (1), d-glucose (10), and 120 μL of perchloric acid (70%) was added. Following 15 minutes at ice temperature, the suspension was finally centrifuged at 730×g for 10 minutes at 20°C and the supernatant containing the released serotonin was collected for quantification. Platelet 5-HT was determined by high pressure liquid chromatography with electrochemical detection. The chromatographic system consisted of a Gilson Applied Chromatographic System (Middleton, WI, USA) with a 305 model pump and a 231 injection valve model, with a 50-μL loop. A Biophase ODS Reverse Phase 18 analytical column (250×4.6, Ω=5 μ, Bioanalytical Systems Inc., West Lafayette, IN, USA) was used and separation was made possible by using an isocratic solvent system consisting of an acetate-citrate buffer (sodium acetate 0.1 mol/L, citric acid 0.1 mol/L) containing sodium octane sulphonate (0.5 mmol/L), ethylenedinitrilo-tetraacetic acid (0.15 mmol/L), dibutyramin (1 mmol/L), and 10% methanol. A flow rate of 1 mL/minute was maintained and detection of the chromatographed serotonin was achieved by using a 1411 Gilson electrochemical detector model (650 mV).

1.4. Whole Blood Platelet Aggregation

Whole blood platelet aggregation was monitored by measuring electric impedance, using a Chrono-
log aggregometer (Chrono Log, Havertown, PA, USA). This technique is based on the detection of changes in electrical resistance between two electrodes submerged in the sample. Fresh heparinized (0.5 mL) whole blood and 0.9% NaCl (0.5 mL) were mixed using a magnetic stirrer and allowed to balance at 37°C for 5 minutes before adding the agonists adenosine 5'-diphosphate (1.0 μmol/L) and collagen (5 μg/mL). Platelet count and mean platelet volume were measured with a Coulter counter.

1.5. Platelet Thromboxane B₂ Generation

The blood was collected into plastic syringes containing 1 vol of 3.8% trisodium citrate for every 9 vol of blood. Aliquots were incubated for 3 hours at 37°C with either solvent (control) or CsA in final concentrations of 0.1 and 1.0 μmol/L. The remaining blood was divided into aliquots containing CsA or solvent to measure the potentially maximum amount of releasable thromboxane. After incubation, whole blood samples were centrifuged as described previously to obtain PRP and platelet-poor plasma (PPP). Platelets were counted using the Coulter counter (350,000±50,000 platelets/μL). They were subsequently activated with 1.0 μmol/L of ADP and 5 μg/mL of collagen. Following aggregation, the supernatants were assessed to determine the amount of thromboxane B₂ ([TXB₂], the stable end product of TXA₂] generated. Briefly, the contents of the cuvette were transferred to a microcentrifuge tube containing 10 μmol/L etylene-dinitrilo-tetraacetic acid and 10 μmol/L indomethacin and centrifuged at 12,000×g for 4 minutes. The resulting supernatant was frozen at −70°C to later analyse the TXB₂. A sample of PRP merely spun without the addition of ADP or collagen was also assessed to serve as a control for any spontaneous TXB₂ generation. To determine the total amount of releasable TXB₂ in both the presence and absence of CsA, 20 μmol/L calcium chloride was added to the initial aliquots, and the citrated blood was allowed to clot at 37°C for 30 minutes. Following centrifugation, the serum was separated and stored at −70°C until analyzed for the total amount of TXB₂ generated. The amount of TXB₂ released in both PRP and serum preparations was assessed by radioimmunoassay (R&D Systems, Abingdon, UK), measured by calculating the percentage of iodinated TXB₂ bound to a protein precipitate, and read from a standard curve. All assays were performed in duplicate.

1.6. Chemicals

Cyclosporin A (Sandimmun Neoral) was supplied by Novartis Farma, Lisbon, Portugal. ADP and collagen were obtained at Chrono-log Corp., Havertown, PA, USA. A thromboxane B₂ radioimmunoassay kit was purchased from R&D Systems, Abingdon, UK. All the other chemicals were of the highest analytical grade and were obtained from Sigma, St. Louis, MO, USA.

1.7. Statistical Analysis

Data are expressed as means±SEM of n experiments. Groups were tested for differences by using ANOVA and Student’s t test.

2. Results

2.1. Blood Pressures

Systolic blood pressure (SBP), diastolic blood pressure (DBP), and mean blood pressure (MBP) measurements were performed in all rats of the control and CsA-treated groups, before (0 week) and 2 and 7 weeks after initiating the CsA administrations. The DBP increased significantly after 2 and 7 weeks of treatment in the CsA-treated groups, especially in the CsA(5) group (week 2: 96±1, 119±2, and 114±1 mm Hg; week 7: 103±1, 114±2, and 112±1 mm Hg) (p<0.05) for control, CsA(5), and CsA(30), respectively (Table 1). Similar changes were recorded for the SBP. After 2 weeks, an increase in the SBP was registered in the CsA-treated groups (190±2 mm Hg for CsA5 and 179±1 mm Hg for CsA30), contrasting with the control (163±1 mm Hg; p<0.05). In week 7, the figures were approximately the same as those in week 2 (Table 1). The MBP also increased for the two CsA groups after 2 and 7 weeks, compared with the control (Table 1).

2.2. Platelet Serotonin Contents

The platelet serotonin measurements in the control and CsA-treated groups (5 and 30 mg/kg/day) were
Table 1. The effect of CsA on blood pressure after 2 and 7 weeks of treatment

<table>
<thead>
<tr>
<th>Blood pressure (mm Hg)</th>
<th>Treatment (weeks)</th>
<th>Control (untreated)</th>
<th>Treated with CsA(5)</th>
<th>Treated with CsA(30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP</td>
<td>0</td>
<td>165±1</td>
<td>165±3</td>
<td>161±1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>163±1</td>
<td>190±2*</td>
<td>179±1*</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>164±1</td>
<td>189±3*</td>
<td>178±1+</td>
</tr>
<tr>
<td>DBP</td>
<td>0</td>
<td>100±2</td>
<td>89±5</td>
<td>100±2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>96±1</td>
<td>119±2*</td>
<td>114±1*</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>103±1</td>
<td>114±2*</td>
<td>112±1*</td>
</tr>
<tr>
<td>MBP</td>
<td>0</td>
<td>121±2</td>
<td>112±4</td>
<td>120±1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>118±1</td>
<td>142±2*</td>
<td>136±1*</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>120±3</td>
<td>136±4*</td>
<td>134±1*</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEM of 20 separate values. SBP, systolic blood pressure; DBP, diastolic blood pressure; MBP, mean blood pressure. CsA (5), cyclosporin A (5 mg/kg/day); CsA(30), cyclosporin A (30 mg/kg/day).

*p<0.05 between the control group and each of CsA-treated groups.

performed in week 0 (before starting the administrations) and in weeks 2 and 7. In week 0, the platelet 5-HT concentration of the control group was similar to that of the CsA-treated groups (Figure 1). In week 2, there was a decrease in the platelet serotonin contents of the CsA-treated groups in contrasting to the control group [in ng/mL: 1105±41 for control, 899±25 for CsA(5) and 698±21 for CsA(30); p<0.05]. Similar results were obtained in week 7: 999±24 for control, 877±17 for CsA(5), and 672±19 ng/mL for CsA(30), p<0.05 (Figure 1).

2.3. Whole Blood Platelet Aggregation

We tested the effects of cyclosporin A on whole blood platelet aggregation induced by collagen (5 μg/mL) and ADP (1.0 μmol/L), after 2 and 7 weeks of treatment. Before the start of CsA administrations, similar values were obtained for platelet aggregation in the control and the CsA-treated groups. Thus, only one mean is presented in week 0, which expresses values from the three groups (Table 2). After 2 weeks of treatment, no statistically significant increases in ADP-induced whole blood platelet aggregation were identified. In the CsA-treated groups, values for collagen-induced aggregation were significantly increased after the second week of treatment compared to those of the control group. These changes were most noticeable in the group that received 30 mg/kg/day of CsA. The results after 7 weeks of administration more or less confirmed those obtained after the second week (Table 2).

2.4. Platelet Thromboxane B₂ Generation

Baseline supernatant TXB₂ levels were 251.8±7.9 ng/mL in control platelets compared to 315.6±8.3 ng/mL and 304.5±8.1 ng/mL (p<0.05) in CsA-treated platelets, trough and peak, respectively (Figure 2). In ADP-stimulated platelets, TXB₂ release increased in the 0.1 and 1.0 μmol/L CsA-treated platelets compared to control. When platelets were stimulated with collagen, there was a sig-
Table 2. The effects of CsA on whole blood platelet aggregation and platelet count and volume after 2 and 7 weeks of treatment

<table>
<thead>
<tr>
<th>Treatment (week)</th>
<th>Rat group</th>
<th>Aggregation (Ohms)</th>
<th>Platelet (×10^9/L)</th>
<th>MPV (fL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>ADP (1.0 μmol/L)</td>
<td>Collagen (5 μg/mL)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>Control</td>
<td>16.0±0.5</td>
<td>10.6±0.6</td>
<td>611±16</td>
</tr>
<tr>
<td></td>
<td>CsA(5)</td>
<td>17.1±1.1</td>
<td>13.4±1.0*</td>
<td>611±18</td>
</tr>
<tr>
<td></td>
<td>CsA(30)</td>
<td>15.9±0.7</td>
<td>16.5±1.1*</td>
<td>609±27</td>
</tr>
<tr>
<td>7</td>
<td>Control</td>
<td>15.0±0.6</td>
<td>12.0±1.5</td>
<td>598±32</td>
</tr>
<tr>
<td></td>
<td>CsA(5)</td>
<td>15.8±0.9</td>
<td>13.3±0.8*</td>
<td>573±24</td>
</tr>
<tr>
<td></td>
<td>CsA(30)</td>
<td>15.4±0.5</td>
<td>17.3±0.9*</td>
<td>603±21</td>
</tr>
</tbody>
</table>

Data are expressed as means±SEM of 10 separate values. ADP, adenosine-5′-diphosphate; MPV, mean platelet volume; CsA(5), cyclosporin A (5 mg/kg/day); CsA(30), cyclosporin A (30 mg/kg/day).

*p<0.05 between the control group and each of CsA-treated group.

2.5. Platelet Count and Mean Platelet Volume

As control parameters, we also counted the platelets and measured the mean platelet volume. No significant differences were obtained between the three groups of rats at any stage of the study (Table 2).

3. Discussion

The present investigation was aimed at studying the peripheral serotonergic system, platelet aggregation, and blood pressure of rats treated with cyclosporin A at 5 and 30 mg/kg/day after 2 and 7 weeks of administration. These CsA concentrations were chosen because they corresponded to different stages of the CsA absorption in clinical practice. Therefore, 30 mg/kg/day of CsA administered to a rat represents the peak value of CsA achieved in human blood immediately following an intake of a daily dose, whereas 5 mg/kg/day in the rat corresponds to the trough concentration after blood concentration stabilization. The choice of the 2- and 7-week time periods was based on the fact that 2 weeks could correspond to an acute situation and 7 weeks to a chronic situation. The blood pressures rose significantly in the rats of the CsA-treated groups (5 and 30 mg/kg/day) after both 2 and 7 weeks of administration. So, as we initially expected, both trough and peak concentrations were really effective in increasing blood pressures.

Serotonin, due to its proaggregatory and also vasoconstrictor properties, may play an important role in the thromboembolic and hypertensive effects of CsA. Platelets are the main storage site of 5-HT and changes in their contents may reflect important physiological alterations, especially re-
garding thrombotic and hypertensive effects. In this study we have identified a decrease in platelet 5-HT contents in both CsA-treated groups. Similar results were already obtained by others in a CsA treatment patient situation [14] and also in other hypertensive situations [25-27]. Low platelet serotonin concentration may be due to decreased uptake or increased release, as was documented in other studies, both in CsA [14] and non-CsA induced hypertension [25,28]. If serotonin is abnormally released by platelets, an increase in the plasma concentration of this amine could be expected and consequently an increased deposition on collagen fibres of the vessel wall, thus promoting direct vasoconstriction and platelet aggregation amplification. In agreement with our hypothesis, several papers have already reported that the increase in whole blood and plasma 5-HT is intensified during CsA treatment [14,20,22] and in other hypertensive situations as well [25,27]. On the other hand, cyclosporin A induces endothelial damage [29,30] that may contribute to increased vasoconstrictor response and platelet aggregation [31].

Thus, considering the above-mentioned information, an increase in platelet aggregation with CsA treatment is expected. In our study, whole blood platelet aggregation induced by collagen, but not by ADP, was increased in the rats of the CsA-treated groups when compared with control, as also was shown in other studies carried out by other investigators. In kidney transplant recipients, Malyszko et al. [14] found that CsA increased collagen- and ADP-induced whole blood and PRP aggregation when compared with healthy volunteers. However, by ADP stimulation whole blood and PRP aggregations were not statistically significant, as was also confirmed by Taylor et al. [32]. Mysliwiec et al. [21] indicated a rise in ADP-induced and 5-HT-augmented platelet aggregation in uremic rats, injected subcutaneously with 5 mg/kg/day of CsA after 2 and 4 weeks of administration, but did not find statistical significance in nonuremic rats treated with CsA when compared with the control. Grace et al. [12] demonstrated that platelet aggregation was increased in response to subthreshold doses of several agonists, such as ADP and epinephrine, in in vitro studies with cyclosporin A concentrations reflecting levels achieved in clinical practice [33,34]. They also found that the intake of a single dose of CsA in normal volunteers caused maximal platelet activation after 2 hours but reverted to normal after 4 hours, thus suggesting that sustained exposure is needed to activate platelets consistently. In fact, they did not observe the same reversion in patients taking cyclosporin A: platelet activation at trough levels increased, but underwent a further increase at peak concentrations. Their results are in agreement with those obtained by Vanreunterghem et al. [8], who point out a positive link between CsA levels and enhanced aggregation. Our results confirmed the nonsignificant increases in ADP-induced aggregation and the significant increases in aggregatory response to collagen, already obtained by Malyszko et al. [14] and Taylor et al. [32], and the positive link between CsA levels (trough and peak) and increases in collagen-induced aggregation, extending the results obtained by Grace et al. [12] and Vanreunterghem et al. [8].

Confirming a platelet state of hyperactivity, we have obtained significant increases in platelet TXA2 release at baseline and by platelet stimulation with both ADP and collagen. Peak-trough differences were observed by collagen stimulation in agreement with aggregation results and also with platelet 5-HT content results, thus suggesting a proportional relationship between CsA levels, thromboxane, and 5-HT release and platelet aggregation.

In conclusion, this study points out that both CsA concentrations that we tested influence blood pressure, platelet serotonin, and thromboxane contents and aggregation. Increased platelet aggregation, together with increased proaggregating and vasoconstricting agents concentration (at least 5-HT and TXA2), near a damaged and/or dysfunctional vessel wall might provide the conditions to intensify vasoconstriction and platelet thrombus formation, which may certainly contribute to the development and/or maintenance of hypertension and increased risk of thromboembolic complications.

References

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