Expression of S100B Protein in Ischemia/Reperfusion-Induced Brain Injury After Cyclosporine Therapy: A Biochemical Serum Marker with Prognostic Value?

Background: Accumulating evidence has indicated that S100B protein may be involved in the pathophysiology of ischemia-reperfusion brain injury. Cyclosporine has been shown to have neuroprotective functions. This study investigated the effect of cyclosporine on S100B serum levels and the severity of brain tissue damage in a rat model of cerebral ischemia-reperfusion (I/R).

Material/Methods: Twelve-week-old Wistar male rats were randomly divided into Control I/R and Cyclosporine I/R groups (n=10 each). Cyclosporine was given orally by gavage for 5 days prior to cerebral I/R, at a total volume of 15 mg/kg/day. The Control group received an equal volume of saline. Body weight was measured and all animals were subjected to 60-min focal ischemia by filament occlusion of the middle cerebral artery. ELISA was used to assess the concentrations of serum S100B and development of brain infarct size and neurological outcomes were determined at 2 and 24 h after occlusion withdrawal.

Results: Cyclosporine improved the neurological deficit score and decreased the cerebral infarct size and body weight. S100B serum levels were significantly elevated in Cyclosporine-treated rats compared with untreated Control rats during the reperfusion phase. Total infarct size was positively associated with S100B serum levels in the Control I/R group, but no significant correlation was observed in the Cyclosporine I/R group.

Conclusions: Cyclosporine seems to affect both ischemia-reperfusion brain tissue damage and S100B protein serum levels. S100B serum level appears to be a state marker for the severity of the cerebral ischemia-reperfusion, rather than a trait marker for Cyclosporine responsiveness.

MeSH Keywords: Brain Infarction • Cyclosporine • Hypoxia-Ischemia, Brain • Reperfusion Injury • S100 Calcium Binding Protein beta Subunit

Abbreviations: I/R – ischemia-reperfusion; MCA – middle cerebral artery; MCAO – middle cerebral artery occlusion; CsA – Cyclosporine A; S100B – calcium-binding protein B; CNS – central nervous system; SEM – standard error of the mean; ICA – internal carotid artery; ECA – external carotid artery; CCA – common carotid artery; ELISA – enzyme-linked immunosorbent assay; OD – optical density; HE – hematoxylin-eosin

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Background

Stroke is a leading cause of acquired disability in adults. If secular trends continue, it is estimated that there will be 23 million first-ever strokes and 7–8 million stroke deaths in 2030 [1]. In the treatment of acute stroke, blood supply restoration can eliminate brain tissue injury by salvaging a reversibly damaged penumbra of tissue [2]. However, in the reperfusion phase, a paradoxical increase in tissue damage is observed, associated with restitution of blood flow to ischemic tissues [3]. Ischemia/reperfusion (I/R) is a complex pathologic process, involving intracellular and extracellular pathways that result in metabolic, thrombotic, and inflammatory changes in the affected tissues [4].

Cyclosporine (CsA) is a powerful immunosuppressive and anti-inflammatory agent [5]. The ability of CsA to reduce immunologic activity within short time spans has also made it a drug of choice for the treatment of various immune-mediated disorders [5]. Some studies have shown that CsA can suppress inflammation, oxidative stress, and cellular apoptosis and thus exhibits neuroprotective functions [6]. However, experimental results on the effects of CsA in focal models of stroke are conflicting.

S100B (calcium-binding protein B) is a low-molecular-weight Ca2+-binding protein, primarily found in astrocytic glial cells of the central nervous system (CNS), such as astrocytes, maturing oligodendrocytes, dendritic cells, and Schwann cells, and represents astrocytic activation [7]. S100B has significant calcium homeostatic functions and is secreted by astrocytes for neuroprotective and neurotrophic cellular functions in the CNS [8]. Recent studies have provided more detailed information about the mechanism(s) of action of S100B as an intracellular regulator and an extracellular signal transducer [9]. S100B is involved in the inflammatory pathological process, which plays a vital role in the pathogenesis of brain I/R. After brain damage and destruction of blood brain barrier, serum levels of S100B were increased in patients [10]. Some published reports [10,11] describe the relationship between serum S100B protein and brain tissue damage following I/R injury. A review of the literature shows a number of conflicting reports with regard to the use of S100B as a marker of brain damage, since several biochemical features of this protein and its physiological and pathological variations are not well characterized.

Although numerous studies have reported neuroprotective and neuro-regenerative activity of CsA, no published studies have directly examined the ability of CsA to alter S100B serum levels in animal models during cerebral I/R injury. The present study focused on the differential effects of CsA on S100B serum levels and clinical outcomes following cerebral I/R injury. Also, we investigated whether S100B serum levels in I/R injury represent a state marker for the current severity of the disorder or a trait marker for cyclosporine responsiveness.

Material and Methods

Animal grouping – study design

Twelve-week-old male Wistar rats were obtained from the Animal Division of the Hellenic Pasteur Institute. The rats had a mean body weight of 390 [5.5] g and were maintained in climate-controlled chambers (temperature 20 [2]°C, humidity 55 [5%] under controlled lighting (12 h light per day) and central ventilation (15 air changes/h) for 15 days in order to adapt to the new environment. The rats were fed a normal chow diet of food pellets containing full nutrient supplementation. All possible precautions were taken to avoid animal suffering at each stage of the experiment. Twenty rats were randomized into 2 experimental groups (n=10 each). The Cyclosporine I/R group received CsA pretreatment at a dose of 15 mg/kg/day by oral gavage for 5 days before cerebral I/R. The Control I/R group received equal volumes of saline. Animals were subjected to 60 min of focal ischemia by filament occlusion of the middle cerebral artery (MCAO) and were sacrificed 24 h after reperfusion. The experimental protocol was approved by the “Scientific Committee for the Approval of Protocols Using Animals for Scientific Purposes” established in the Laboratory for Experimental Surgery and Surgical Research “N.S. Christeas” of Athens Medical School and by the Veterinary Directorate of Attica Region.

Drug delivery

CsA was diluted in saline and was given orally by gavage for 5 days before cerebral I/R at a total volume of 15 mg/kg/day (15 mg/kg was based on published pharmacokinetic studies of CsA). CsA concentrations were determined before cerebral I/R in whole blood by high-performance liquid chromatography. For the Control group, an equal volume of saline was administrated.

Body weight

The body weight of rats was measured at the following times: 5 days prior to surgery, 2 days prior to the ischemic insult, and on the day of surgery.

Surgical procedure

Rats were fasted but were given free access to water 12 h prior to surgery, and neural defect scores were assessed to identify rats with congenital neural dysfunctions. A combination of preanesthetic agents containing ketamine (50–100 mg/Kg)
and xylazine (1–5 mg/Kg) was administrated intraperitoneally to prevent apprehension and pain. Each animal was anesthetized with isoflurane via inhalation at a dose of 2.0–3.0ml/L. Every rat was immobilized in the supine position on a surgical table with a 10-ml tube placed under the neck to facilitate visualization of neck vasculature. All rats were injected subcutaneously with 0.2 ml of 0.5% bupivacaine along the prospective incision site, which was covered with a sterile surgical drape. Focal brain ischemia was induced by transient MCAO, according to Longa method [12]. In brief, the right external, internal, and common carotid arteries were exposed and carefully dissected, avoiding injury to soft tissues and nerves. The internal carotid artery (ICA) was isolated and carefully separated from the adjacent vagus nerve. The external carotid artery (ECA) was ligated as distally as possible with a 6-0 silk suture. Another 6-0 silk suture was placed loosely around the carotid field was exposed. The intraluminal suture was removed at the caudal end of the surgical field. At this point, the intraluminal suture blocked the origin of the middle cerebral artery (MCA) and the MCA territory became ischemic. The occlusion time was recorded with a timer. The wound was covered with sterile gauze pads and the animal was monitored during the 60-min occlusion period.

After 60 min, the gauze pads were removed and the surgical field was exposed. The intraluminal suture was removed and blood perfusion to cerebral tissue was released. From this moment on, the reperfusion phase started. The incision layers were closed and the animals were subcutaneously administered 3 ml of normal saline to prevent dehydration. The reperfusion phase was allowed for 24 h after ischemia. At the end of the reperfusion phase, all surviving rats were euthanized.

Neurologic examination

After full recovery of rats, we performed an infarction-related behavioral test as follows. Neurologic examinations were performed at 2 and 24 h after occlusion withdrawal. Neurological findings were scored on a 5-point scale: a score of 0 indicated no neurologic deficit, a score of 1 (failure to extend left forepaw fully) a mild focal neurologic deficit, a score of 2 (circling to the left) a moderate focal neurologic deficit, and a score of 3 (falling to the left) a severe focal deficit; rats with a score of 4 did not walk spontaneously and had a depressed level of consciousness.

Histopathological examination

Following euthanasia, the brain tissue was immediately fixed in 10% formalin at room temperature for 24 h. After fixation and tissue processing, coronal serial sections of 5 mm thickness were cut in 3 sites (1 mm anterior, 1 mm posterior, and 3 mm posterior to the bregma). The sections were stained with hematoxylin-eosin (HE) and examined for histopathological changes using light microscopy.

Infarct size measurement

The HE-stained sections were assessed for cerebral infarct size. Correlation of macroscopic tissue staining intensity and tissue infarction (pan-necrosis) has been established by a number of investigators for several stains. The evaluation of brain injury severity was estimated by a semi-quantitative method [13]. The HE-stained sections from each brain were examined to confirm infarction in lightly-stained regions (pale pink) and absence of infarction in normally-stained regions. The HE stained sections were digitally photographed and the infarct size was measured using Image J software [14]. The area of tissue necrosis was divided by the total size of the whole-brain coronal section [12]. The mean value for each animal was determined by averaging values from all images taken from that animal.

Blood collection – ELISA measurements

Blood samples were collected from rats before surgery (t0), 60 min after maximal occlusion of the right MCA (t1), and 24 h after occlusion withdrawal (t2). All blood samples were obtained using capillary tubes introduced into the medial retro-orbital venous plexus, under light ether anesthesia. S100B serum levels were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Cat. No E-EL-R0868 and Lot. No AK0017JUN20035) (Intra-assay Precision (CV) <10%) from Wuhan Elabscience Biotechnology Co., Hubei, China. All samples were run in 96-well plates. The optical density (OD) was measured spectrophotometrically at a mean wavelength of 450 [2] nm. The OD value is proportional to the concentration of S100B.

Statistical mode of analysis

Normal distribution was confirmed using histogram plots, box plots, and the Shapiro-Wilk test. Continuous data are presented as means [SEM]. Categorical variables were compared using the two-sided Fischer’s exact test. We employed the two-tailed
t test to compare continuous variables. Correlations were analyzed using Pearson correlation coefficients. A p-value of less than 0.05 was considered statistically significant. Data analyses were performed using SPSS version 17.0.

Results

Body weight

At baseline time (ie, 5 days before cerebral I/R) no significant differences were observed in rat weights between the Control I/R group and the Cyclosporine I/R group (mean weight 394 [6.3] g and 386 [9.2] g, respectively). Two days prior to ischemic insult, both groups exhibited a reduction of body weight, and the mean weight differed significantly between the 2 groups (Control I/R rats 390 [6.1] g and Cyclosporine I/R rats 365 [6.9] g, p=0.013). On the day of surgery, the mean body weight of the Control I/R group was increased to 406 [8.5] g, whereas the body weight continued to reduce to 357 [6.9] g for the Cyclosporine I/R group. The difference in body weight between the study groups was statistically significant at this time-point (p<0.001, Figure 1A). The percentage of change in the body weight was significantly higher in the Cyclosporine I/R group than in the Control I/R group during the study period (Figure 1B).

Neurological deficit score

Occlusion of the right MCA and the ipsilateral CCA caused higher neurological deficit scores in the Control I/R group than in the Cyclosporine I/R group (1.10 [0.23] vs. 0.44 [0.18] and 1.30 [0.21] vs. 0.78 [0.28]), at 2 and 24 h after occlusion withdrawal. The difference between the 2 groups at 2 h after surgery was statistically significant p=0.0416 (<0.05). One rat in the Cyclosporine I/R group died during the ischemic period and was excluded from further analysis.

The neurological deficit score in both groups was increased at 24 h after reperfusion in comparison to the score 2 h after surgery (1.30 [0.21] vs. 1.10 [0.23] for the Control I/R group, respectively) and (0.78 [0.28] vs. 0.44 [0.18] for the Cyclosporine I/R group, respectively) (Figure 2).
Histopathological of brain

The HE-stained micrographs showed neuropathological changes. The classic appearance of acute neuronal degeneration was ‘eosinophilic neurons’ (also termed ‘red’ or ‘red dead’ neurons), which were characterized by cell body shrinkage, darkly stained pyknotic nuclei, and an intensely stained red eosinophilic cytoplasm (Figure 3A–3C).

Infarct size measurement

Infarct size was assessed at 24 h after ischemia. Results are reported as intensity of staining density in both ipsilateral and contralateral hemispheres (Figure 3D). Comparison infarct sizes between the 2 groups showed that the percentage of brain tissue necrosis was significantly lower in Cyclosporine I/R rats compared with Control I/R rats (5.9% [2.3] vs. 13.4% [1.9], p=0.025) at the coronal level 1 mm anterior to the bregma. Likewise, at the coronal level 1 mm posterior to the bregma, the brain infarct size was lower in CsA-treated rats in comparison with the Control I/R rats (1% [0.6] vs. 1.9% [0.5], p=0.27). At the coronal level 3 mm posterior to the bregma, the infarct size of cerebral was higher in the Cyclosporine I/R group than in the Control I/R group (5.1% [1.7] vs. 1.6% [0.8], p=0.08). The total infarct size of each brain was calculated as the sum of the infarct size of 3 brain slices, and was lower in Cyclosporine I/R rats than in Control I/R rats (12% [3.95] vs. 16.9 [2.77], p=0.32 (Figure 4).

S100 calcium-binding protein B

In both groups, serum levels of S100B protein were measured. Compared to the rats in the Cyclosporine I/R group, the Control I/R group had lower S100B levels at baseline and 2 time-points (0.25 [0.01] vs. 0.21 [0.02], p=0.095 and 0.58 [0.10] vs. 0.21 [0.03], p=0.0018, respectively). On the other hand, S100B protein was higher in the Control I/R group than in the Cyclosporine...
I/R group at t1 (0.23 ± 0.04 vs. 0.21 ± 0.02). This difference, however, was not statistically significant (p=0.57) (Figure 5A).

Step-by-step, the percentage of change in the S100B protein was significantly higher in the Cyclosporine I/R group than Control I/R group (140 ± 43.8% vs. 0.66 ± 13.6%) in the period between t1 and t2 (p=0.0069 < 0.05), as well as in the period between t0 and t2 (117 ± 45% vs. 0.69 ± 9.7%, respectively, p=0.022) (Figure 5B).

Additionally, the absolute difference in the S100B protein levels was slightly higher in the Cyclosporine I/R group in comparison with the Control I/R group (–14.7 ± 6.4% vs. 13.6 ± 16.4%, respectively) between t0 and t1, p=0.12 (Figure 5B).

**Correlation analyses of S100B serum levels and brain infarct size**

We studied the potential correlation between S100B protein and total infarct size 24 h after reperfusion at baseline, t1, and t2 time-points. The S100B protein was significantly correlated with the percentage of total infarct size in the Control I/R group before surgery (t0) and 24 h after occlusion withdrawal (t2) (r²=0.49, p=0.02 and r²=0.71, p=0.002, respectively). Larger total infarct size was distinctively associated with higher S100B serum levels. On the other hand, there was no correlation between these 2 values in the Control I/R group (r²=0.14, p=0.29) at the t1 time-point (60 min after maximal occlusion of the right MCA) (Figure 5C). In the Cyclosporine I/R group, we found no significant correlation between S100B protein and total infarct size at t0, t1, and t2 time-points (r²=0.05, p=0.52; r²=0.029, p=0.64; r²=0.18, p=0.23, respectively) (Figure 5D).

**Discussion**

This study investigated the differential effects of CsA on S100B serum levels and clinical outcomes following cerebral I/R injury. Our results suggest that CsA regulates S100B serum levels and exhibits tissue-specific protective activity against cerebral I/R injury.
injury. Moreover, S100B serum level is a state marker for the severity of the cerebral I/R rather than a trait marker for CsA responsiveness.

A major finding of this study is that S100B serum levels increase progressively during brain I/R, consistent with the results of previous studies, which found increased concentrations of S100B in serum of patients after brain damage [10]. Interestingly, S100B is reported to be neuroprotective at low concentrations and neurotoxic at high concentrations [8]. Elevated S100B may be the endpoint of a pathophysiologic cascade that exerts a neurotoxic effect and leads to secondary brain tissue damage.

Since the association between circulating S100B levels in the serum of rats following cerebral I/R with cerebral infarct size and severity of I/R damage remains controversial, our study investigated this topic in more detail. Our results suggest a positive correlation between S100B serum levels and total infarct size for the Control I/R group only, which shows that the larger total infarct size is associated with higher S100B serum levels. This finding is in line with 2 previous stroke studies [11,15], which found similar results in patients and rats. Taking the above into consideration, our study suggests that S100B serum levels may be a potential biomarker for charting brain tissue damage following cerebral I/R injury.

Further, CsA treatment improved the neurological deficit score and decreased the brain infarct size at the beginning of ischemia. This finding confirms previous results by Leger et al. and Paulis et al., who found that CsA has a tendency to protect immature rat brains against mild ischemic injury [6]. It was reported that CsA, at a dose of 15 mg/kg/day, has a high immunosuppressive effect in rats, without acute toxicities [16]. Considering this protective effect of CsA and the positive association of S100B serum levels with brain tissue damage in the Control I/R group, the present study investigated the effect of CsA on S100B serum levels and clinical outcomes following cerebral I/R injury. Firstly, we assumed that the neuroprotective influence of CsA is positively associated with its effect on S100B serum levels in ischemia/reperfusion-induced brain injury. According to this hypothesis, S100B serum levels would be lower in the Cyclosporine I/R group compared with those in the Control I/R group. Conversely, our study found that S100B serum levels increased rapidly and were significantly higher with CsA pretreatment compared with untreated Control rats during the reperfusion phase. This finding may seem paradoxical, since the clinical outcome was better for the CsA-treated rats. To further elucidate elevated S100B serum levels in the Cyclosporine I/R group, we next examined whether S100B serum levels are associated with more severe brain tissue damage after CsA treatment. Interestingly, we found no correlation between S100B serum level protein and total infarct size in the Cyclosporine I/R group. Our results suggest that S100B serum level is a state marker for the severity of the cerebral I/R rather than a trait marker for CsA responsiveness.

Our finding of elevated S100B serum levels in the Cyclosporine I/R group demonstrate that CsA may have substantial effects on I/R brain tissue damage and S100B serum levels. S100B protein is predominantly produced by astroglia cells and represents astrocytic activation [7]. Intra- or extracellular S100B mRNA and protein levels have been used as a parameter of astrocyte activation and death in several scenarios of brain injury [17]. The innate immune system of the CNS is composed of resident microglia and astrocytes, which are activated after I/R brain injury, and this activation predicts the severity of tissue damage [18]. At present, little is known about the effects of immunosuppressant medication, such as CsA, on glial cells within the CNS. The findings of another experimental study [19] indicate that CsA-treated rats had an increase in the number of astrocytes with activated morphologies along with reduction in the number of astrocytes with severely diffuse morphologies in the surrounding cortical and striatal border regions when compared with sham-treated animals following transient focal ischemia. While initial activation limits damage in the early stages of stroke, over-activated astrocytes form a persistent glial scar that disrupts nerve transmission and impedes later recovery [19]. CsA may interfere with the intricate pathways of astrocytic activation to the mRNA expression of S100B, and is considered to be a modulator of gene expression and function in astroglia.

A further finding of the present study is that CsA led to gradual decline in body weight of rats. Since S100B is not only expressed in the brain, but also in a number of peripheral tissues such as white fat, kidney epithelial cells, skeletal muscle, or heart [20], serum levels need not necessarily reflect individual variation of S100B expression in the brain. In the literature, adipocytes appear to be an important source of serum S100B, since the concentration of S100B in adipose tissue is as high as in nervous tissue [21]. Therefore, we assume that the changes in body weight between the 2 groups could be an additional explanation of the variations in S100B serum levels among the Cyclosporine I/R and Control I/R group that were found in our study.

Our study has several limitations. It has been reported that the time needed to reach maximum S100B serum levels may be as long as 2–3 days [22]. Several studies have shown that S100B serum levels measured in samples taken after 24 h of stroke onset had a strong correlation with the degree of neurological deficit and the final infarct size. In the majority of patients, serum levels returned to baseline within 9 days after the event. In our study, we investigated the concentrations of S100B protein after 60-min ischemia and 24-h reperfusion of
the brain. The long-term effect of therapy with CsA on sensorimotor function in this experimental setting is unknown. It is likely that serum levels of S100B protein are progressively changed after this study period. A longer follow-up might be required to ascertain further changes in the biomarker levels over time. Neuronal regeneration after an injury is a slow process and may be indirectly facilitated by therapy with CsA. Further, this study did not investigate the safety of CsA dosage, so the adverse effects of CsA therapy remain unclear. Cho et al. [23] showed that post-ischemia treatment with CsA could be useless or harmful. Finding the correct balance between the benefits and risks of CsA use, therefore, is still a major concern regarding the clinical use of CsA in the setting of acute ischemia-reperfusion brain injury. Another limitation of our study is that occlusion of the right MCA could be confirmed by monitoring the reduction in regional cerebral blood flow using a laser Doppler flowmeter, but this device was not available for our experiment.

Conclusions

The detection of high S100B serum levels after acute ischemic stroke and the correlation of S100B serum levels with total infarct size show that S100B protein could be used as a periphereral marker in charting the brain tissue damage following cerebral I/R injury. CsA seems to exhibit neuroprotective activity and affect both I/R brain tissue damage and S100B protein serum levels. Therefore, S100B serum level seems to be a state marker for the severity of the cerebral I/R rather than a trait marker for CsA responsiveness.

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Conflict of interest

None.

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