Original Article

Cyclosporine-A-mediated inhibition of p-glycoprotein increases methylprednisolone entry into the central nervous system

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Study design: Prospective, randomized, pharmacokinetic study.

Objective: To determine if cyclosporine-A-mediated inhibition of p-glycoprotein would increase methylprednisolone entry into the central nervous system thereby permitting a reduction in the systemic methylprednisolone dose.

Setting: Department of Anesthesiology, University of Washington, Seattle, USA.

Methods: Microdialysis probes were used to obtain cerebrospinal fluid and gluteal muscle extracellular fluid samples for measurement of methylprednisolone concentration in pigs. At time zero, a methylprednisolone bolus was given and an infusion started. At 210 min, after reaching a stable methylprednisolone concentration, a cyclosporine-A bolus was given (either 10 or 30 mg/kg) and microdialysis samples collected until 420 min. Plasma samples were collected at 10, 30 min and then every 30 min until the study's end.

Results: Cyclosporine-A bolus produced a dose-dependant increase in methylprednisolone concentration in plasma, muscle and cerebrospinal fluid. Importantly, the magnitude of the increase in cerebrospinal fluid was significantly greater than the increase in plasma and muscle.

Conclusions: The relatively greater increase in cerebrospinal fluid concentrations of methylprednisolone is consistent with increased penetration of the blood–brain barrier secondary to cyclosporine-mediated p-glycoprotein inhibition. Theoretically, increased methylprednisolone entry into the central nervous system should allow a reduction in the systemic methylprednisolone dose and a consequent decrease in glucocorticoid-mediated side effects.

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Introduction

High-dose methylprednisolone begun within 8 h of spinal cord injury has been shown to improve neurological function.[1–4] However, its use is also associated with a significant increase in complications related to systemic immunosuppression. Specifically, the incidence of wound infection, sepsis, pneumonia, and the duration of intensive care unit stay are all increased in patients receiving high-dose methylprednisolone.[5–8] Importantly, some methylprednisolone-mediated morbidities/side effects put patients at increased risk of complications known to contribute to secondary neurological injury (eg, sepsis-induced hypotension,[9–11]

glucocorticoid-mediated hyperglycemia[12]). Consequently, methylprednisolone-related side effects may actually work at crosspurposes to any salutary neurological benefits of methylprednisolone therapy.

We have previously shown that methylprednisolone entry into the central nervous system is actively opposed by p-glycoprotein,[13] an active transporter present on the surface of capillary endothelial cells within the central nervous system and an important component of the blood–brain barrier.[14,15] Thus, when targeting the central nervous system, very large systemic doses of methylprednisolone must be administered to overwhelm p-glycoprotein's transport capacity and achieve therapeutic concentrations in the spinal cord.

With this in mind, one approach to improving the therapeutic index for methylprednisolone therapy would be to improve its entry into the central nervous system by blocking p-glycoprotein. Improved entry into

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the central nervous system would allow a reduction in the systemic methylprednisolone dose necessary to achieve therapeutic concentrations in the spinal cord and would decrease the risk of immunosuppressive side effects.

The goal of this study was to demonstrate just such a pharmacologic approach to improving the therapeutic index for systemic methylprednisolone therapy. To achieve this goal, we examined the effect of cyclosporine-A, a prototypical competitive inhibitor of p-glycoprotein, on methylprednisolone concentrations in plasma, cerebrospinal fluid and muscle.

Materials and methods

All studies were approved by the University of Washington Institutional Animal Care and Use Committee. Guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care were followed throughout.

Farm-bred, mixed-breed, pigs of both genders weighing 14.0 ± 2.6 kg were used. Animals were housed in rooms with 12 h light–dark cycles and given twice daily feedings of pig chow (Purina, St Louis, MO, USA) and *ad libitum* access to water.

Surgical preparation
On the study day, anesthesia was induced by mask inhalation of isoflurane (4–5%) in oxygen and the animals were orotracheally intubated after muscle relaxation by intramuscular succinylcholine (100 mg). Anesthesia was maintained with isoflurane (1.5–2%) in oxygen and the animals were mechanically ventilated to maintain end-tidal CO_2 at 36–38 mmHg (Datex Airway Gas Analyzer Type GAO, Datex, Helsinki, Finland). Femoral arterial and venous cannulae were placed via cutdown for arterial pressure monitoring, blood sampling, maintenance fluid administration (normal saline at 4 ml/kg/h) and study drug administration. Muscle relaxation was maintained by adding pancuronium bromide to the maintenance fluid infusion (1 mg/ml).

To permit placement of custom made microdialysis probes into the cerebrospinal fluid, the L5, L1, and T9 lamina were exposed and a small (2–3 mm) laminotomy was made to reveal the dura mater. A 1–2 mm hole was incised in the dura and arachnoid maters and a microdialysis probe was inserted into the cerebrospinal fluid. The dialysis probes were fixed in place by cyanoacrylate glue and dental acrylic was used to seal the laminotomies. Mock cerebrospinal fluid (NaCl 140 mEq, $NaHCO_3$ 25 mEq, KCl 2.9 mEq, $MgCl_2$ 0.4 mEq, urea 3.5 mEq, glucose 4.0 mEq, and $CaCl_2$ 2.0 mEq; pH 7.38–7.42; 295 mOsm) was pumped through the dialysis probes at 10 μl/min.

In addition to the dialysis probes placed in the cerebrospinal fluid, a probe was also placed percutaneously in a gluteal muscle. Since muscle capillaries do not express p-glycoprotein, this sample site served as a control to distinguish p-glycoprotein-related effects of cyclosporine-A from non-p-glycoprotein-related effects.

Microdialysis probe manufacture
Custom loop microdialysis probes were made as previously described.[13,16–18] Briefly, the probes were made from cellulose dialysis fibers (Spectrum Medical Industries Inc., Houston, TX, USA) with a 215 μm inside diameter, a 235 μm outside diameter, and a molecular weight cutoff of 6000 Da. Epoxy cement was used to coat all but the center 20 mm of the dialysis fiber, thus creating a 20 mm 'dialysis window'. The epoxy was spread evenly by running a 2 cm length of PE-10 tubing over the fiber while the epoxy was still wet. After the epoxy had cured, a 90 μm diameter tungsten wire (Hamilton Company, Reno, NV, USA) was placed in the lumen of the dialysis probe and the probe bent in half. The wire allowed the probe to be bent without occluding the lumen. A silicone elastomer was spread in the shape of a cone at the base of the probe; this served to plug the hole in the meninges through which the probe was inserted. The probes were allowed to 'cure' for 24 h before use and all probes were used within 72 h of manufacture.

Study paradigm
Following surgical preparation, the animals received a 2.7 mg/kg methylprednisolone bolus followed immediately by a 2.7 mg/kg/h continuous infusion of methylprednisolone. This infusion continued throughout the 420 min of the study. At the 210 min point, in a randomized fashion, the animals received either a 10 or a 30 mg/kg bolus of cyclosporine-A.

Dialysate was collected continuously as 10 min samples (ie, 100 μl) from all four microdialysis probes. Arterial blood samples (3 ml) were collected at time 0, 10, and 30 min and then every 30 min for the remainder of the study.

Drug assays
Dialysate (40 μl) was mixed with 20 μl of internal standard (fluoxymesterone 1 ng/μl) and placed in an autosampler vial. A 50 μl portion of this sample was injected on the HPLC.

Plasma samples (0.5 ml) were placed in a silanized 13×100 screw-cap culture tube and 20 μl of internal standard (fluoxymesterone 35 ng/μl) and 4 ml of methylene chloride were added. The samples were vortexed, the aqueous layer discarded, and 1.5 ml of 0.1 N NaOH and 1.5 ml of deionized water was added. The samples were vortexed, the tubes centrifuged and the aqueous layer again discarded. The methylene chloride phase was decanted into a clean silanized culture tube and evaporated under a stream of air at 40°C. The residue was dissolved in 100 μl of HPLC mobile phase and filtered using a 0.2 μm nylon centrifuge filter. A 50 μl portion was injected on the HPLC.

The HPLC consisted of a Hewlett Packard 1050 series with autosampler, pump, and UV detector. The column

was a Supelco LC-18-DB 150 mm × 4.6 mm × 5 μm. The mobile phase consisted of 65% 10 mM potassium phosphate mono-basic and 35% acetonitrile pumped at 1 ml/min. The peaks are detected at 242 nm. Quantification is based upon peak height ratios between methylprednisolone and the internal standard, plotted as a linear regression.

The interday coefficient of variation was 5–7% and the limits of quantitation were 5 ng/ml for plasma and 10 ng/ml for dialysate.

Statistical analysis

Steady-state methylprednisolone concentrations in blood, cerebrospinal fluid dialysate and gluteal muscle dialysate were determined by averaging the concentration present in samples collected over the hour before the cyclosporine bolus (ie, between 150 and 210 min). Steady-state methylprednisolone concentration after the cyclosporine-A bolus was determined by averaging the concentration present in dialysate and blood samples collected over the final hour of the study (ie, between 360 and 420 min). These steady-state concentrations were used for all comparisons of methylprednisolone concentration within and between sampling sites.

The statistical significance of the change in steady-state methylprednisolone concentration within each sample site after cyclosporine-A administration was determined by paired t-test. Differences between sampling sites in the magnitude of the change in methylprednisolone concentration was determined by analysis of variance. A P-value <0.05 was considered statistically significant. Data are reported as mean $\pm$ SD.

Results

Figure 1a and b shows the raw concentration *versus* time data for both the 10 and 30 mg/kg cyclosporine groups. Differences in methylprednisolone concentrations among plasma, muscle, and cerebrospinal fluid sampling sites were all statistically significant with the highest concentrations in plasma and the lowest in cerebrospinal fluid. Figure 2a and b shows the same data normalized for the methylprednisolone concentration at the time that the cyclosporine-A bolus was administered.

The coefficient of variation of the eight microdialysis samples used to calculate steady-state cerebrospinal fluid methylprednisolone concentrations for each animal between 150–210 and 360–420 min averaged $8.5 \pm 5.0\%$. The eight microdialysis samples used to calculate steady-state muscle methylprednisolone concentrations for each animal during the same time periods averaged $10.9 \pm 5.6\%$. The three plasma samples used to calculate steady-state methylprednisolone concentrations for each animal during the same time periods averaged $7.9 \pm 7.0\%$. These small coefficients of variation are comparable to the interday variability in the reproducibility of the methylprednisolone assay and indicate that drug concentrations were steady over the time periods analyzed.
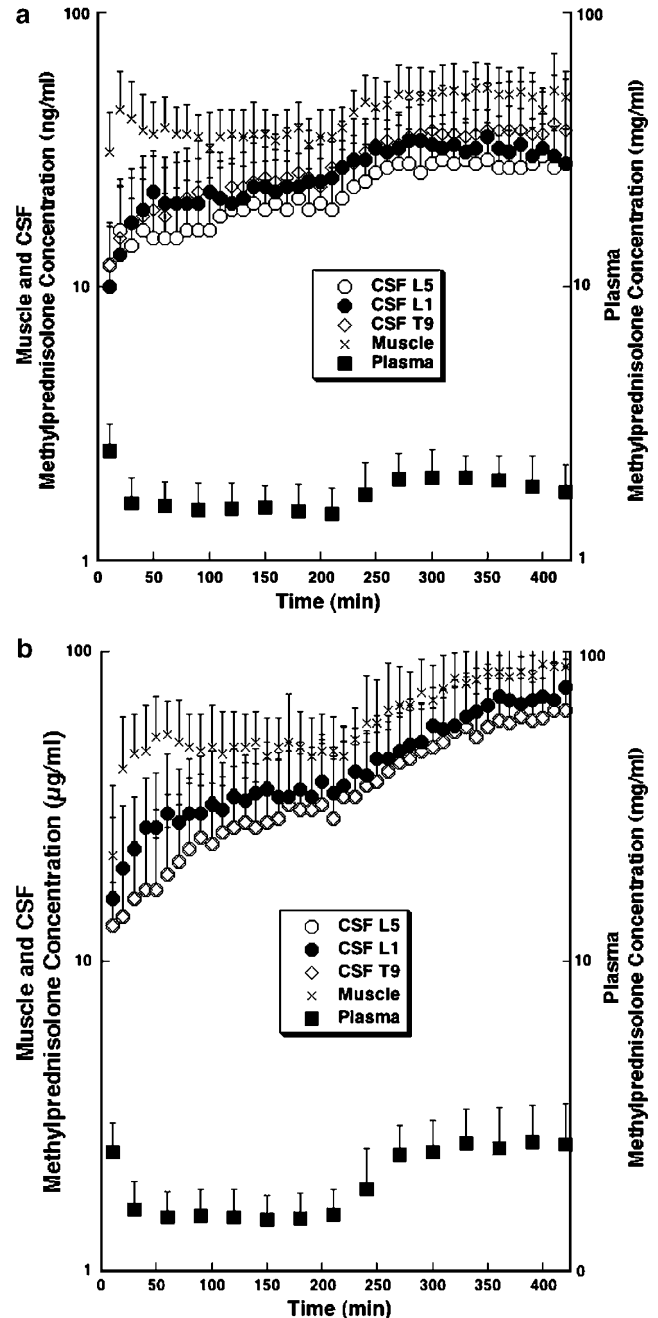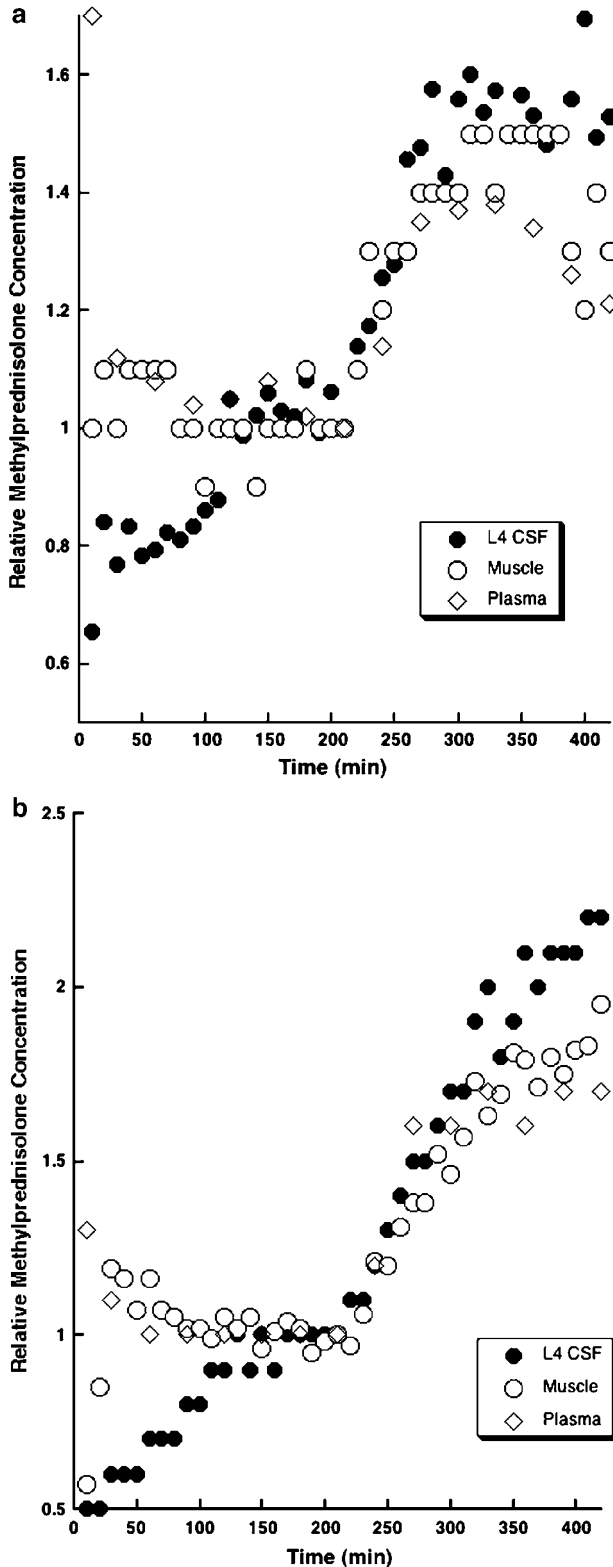

Figure 1 (a) Methylprednisolone concentration in cerebrospinal fluid dialysate, muscle dialysate, and plasma before and after 10 mg/kg cyclosporine bolus administered at time-210 min. Plasma concentrations were significantly greater than muscle concentrations, which were significantly greater than CSF concentrations. (b) Methylprednisolone concentration in cerebrospinal fluid dialysate, muscle dialysate, and plasma before and after 30 mg/kg cyclosporine bolus administered at time = 210 min. Plasma concentrations were significantly greater than muscle concentrations which were significantly greater than CSF concentrations

Both cyclosporine doses produced statistically significant increases in methylprednisolone concentration in muscle, plasma and all cerebrospinal fluid sampling

sites. At all sampling sites, the magnitude of the increase in methylprednisolone concentration was significantly greater for the 30 mg/kg cyclosporine-A dose than for the 10 mg/kg dose (Table 1).

Figure 2 (**a**) Relative methylprednisolone concentration in cerebrospinal fluid dialysate (L4 level), muscle dialysate, and plasma before and after 10 mg/kg cyclosporine bolus administered at time = 210 min. Methylprednisolone concentrations are expressed as a fraction of the concentration present in the sample collected at each site at the time of the cyclosporine bolus. (**b**) Relative methylprednisolone concentration in cerebrospinal fluid dialysate (L4 level), muscle dialysate, and plasma before and after 30 mg/kg cyclosporine bolus administered at time = 210 min. Methylprednisolone concentrations are expressed as a fraction of the concentration present in the sample collected at each site at the time of the cyclosporine bolus
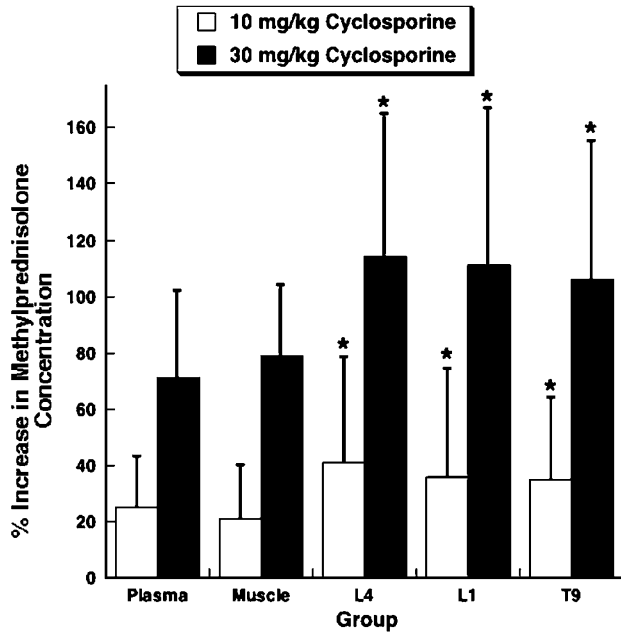
Importantly, the percentage increase in methylprednisolone concentration in cerebrospinal fluid compartments following cyclosporine-A was significantly greater than the simultaneous percentage increase in the plasma concentration of methylprednisolone (Figure 3; Table 1). This was true for both cyclosporine-A doses. In contrast, the percentage increase in methylprednisolone concentration in muscle was not different than the simultaneous percentage increase in the plasma concentration of methylprednisolone at either cyclosporine-A dose (Figure 3; Table 1).

Discussion

We have previously shown that methylprednisolone entry into the CNS is opposed by p-glycoprotein expressed by capillary endothelial cells of the blood–

Table 1 Effect of cyclosporine on steady-state methylprednisolone concentrations

| | | 10 mg/kg | | |
| | | *Methylprednisolone concentration (ng/ml)* | | |
Sample site		*Pre-cyclosporine*	*Post-cyclosporine*	*% Increase*
Plasma		1495 ± 357	1859 ± 461*	$25 + 16$
Muscle		35.6 ± 7	42.6 ± 19*	21 ± 19
CSF	L4	21 ± 8	28 ± 9*	41 ± 41[a]
	L1	24 ± 8	31 ± 10*	36 ± 38[a]
	T9	29 ± 13	37 ± 19*	35 ± 29[a]
		30 mg/kg		
Plasma		1494 ± 302	2599 ± 870*	72 ± 31
Muscle		48 ± 14	86 ± 20*	79 ± 25
CSF	L4	31 ± 16	63 ± 22.4*	114 ± 51[a]
	L1	35 ± 14	72 ± 29*	111 ± 56[a]
	T9	36 ± 14	66 ± 28*	106 ± 49[a]

*$P < 0.05$ compared to pre-cyclosporine for that sample site
[a]The percentage increase in methylprednisone concentrations is significantly greater then the simultaneous percentage increase in methylprednisolone concentration in plasma

Figure 3 Percentage increase in steady-state methylprednisolone concentrations following 10 and 30 mg/kg cyclosporine bolus. Steady-state methylprednisolone concentration was defined as the average concentration present for the 60 min prior to cyclosporine bolus and for the final 60 min of the study

brain/spinal cord barrier.[13] Our finding in this study that, prior to cyclosporine administration, methylprednisolone concentrations in muscle (which does not express p-glycoprotein) exceed simultaneous concentrations in CSF is consistent with this earlier observation. In addition, our finding that cyclosporine-A produced a greater percentage increase in cerebrospinal fluid concentrations of methylprednisolone than occurred in plasma or muscle suggests that p-glycoprotein inhibition is an effective method to increase methylprednisolone delivery to the CNS.

However, the increase in cerebrospinal fluid methylprednisolone concentration that resulted from cyclosporine-A was not solely the result of p-glycoprotein inhibition. Cyclosporine-A also increased plasma concentrations of methylprednisolone. The increase in methylprednisolone plasma concentration likely resulted from inhibition of hepatic metabolism of methylprednisolone by cytochrome P450-3A4.[19] Most p-glycoprotein inhibitors, including cyclosporine-A, are also competitive cytochrome P450-3A4 inhibitors. In fact, some investigators consider the two proteins to act in a coordinated and synergistic fashion within some tissues (eg, gastrointestinal epithelium, hepatocytes) to limit absorption or promote elimination of a large number of molecules.[20–22]

The cyclosporine-mediated increase in methylprednisolone plasma concentration produced a comparable increase in methylprednisolone concentration in muscle (Figure 3). Since muscle does not express p-glycoprotein, and therefore does not actively exclude methyl-

prednisolone, one would expect methylprednisolone concentrations in muscle to parallel those in plasma, which is what was found. In contrast, within the cerebrospinal fluid the increase in methylprednisolone concentration following cyclosporine-A bolus was significantly greater than can be explained by the simultaneous plasma methylprednisolone increase (Figure 2).

The relatively greater increase in cerebrospinal fluid methylprednisolone concentration compared to the increase in plasma concentration can best be explained by a reduction in the barrier to methylprednisolone entry into the CNS. The most likely mechanism for a reduction in the efficacy of the barrier to methylprednisolone entry is cyclosporine-A-mediated inhibition of p-glycoprotein. Thus, these data indicate that p-glycoprotein inhibition represents a potential method to enhance methylprednisolone entry into the CNS.

Since spinal cord injury increases permeability of the blood–spinal cord barrier,[23,24] it is reasonable to consider whether p-glycoprotein inhibition is necessary to increase methylprednisolone concentrations in the injured spinal cord. However, Braughler and Hall demonstrated that methylprednisolone entry into injured spinal tissue was greater than in uninjured tissue only if methylprednisolone was administered within 1 h of injury. If administered more than 1 h after injury, methylprednisolone concentration in injured spinal tissue was no different than in uninjured tissue. These findings would suggest that methylprednisolone permeability across the blood–spinal cord barrier is only very transiently increased and that methylprednisolone entry into the spinal cord is likely to be increased even in the injured spinal cord by p-glycoprotein inhibition.

The potential clinical advantage of improving methylprednisolone entry into the CNS from the plasma is that the methylprednisolone dose could be decreased with the potential for a reduction in glucocorticoid-mediated systemic side effects. In particular, reduced immune suppression would be expected to decrease the risk of wound infection, sepsis, and pneumonia that has been noted in multiple studies in which high-dose methylprednisolone was used in the treatment of acute spinal cord injury. Importantly, a reduction in the incidence or severity of these infectious complications may well have a positive impact on neurologic recovery. For example, hypotension, which frequently complicates sepsis, has been identified as an important contributor to secondary neurologic injury.[11]

Similarly, methylprednisolone-induced hyperglycemia may contribute to secondary neurologic injury. Animal models have demonstrated that hyperglycemia is an important contributor to secondary neurologic injury,[12,25] and hyperglycemia has been implicated as a contributor to poor neurologic outcome in humans.[26,27] For example, Lam et al[27] observed that head-injured patients with a postoperative blood glucose concentration greater than 200 mg/dl had a significantly worse neurologic outcome than did patients with a lower blood glucose concentration. Rovlias and Kotsou[26]

have reported very similar results. Consequently, use of a p-glycoprotein inhibitor that increases methylprednisolone entry into the CNS and thereby permits a reduction in the systemic methylprednisolone dose may improve neurological outcome.

The ideal p-glycoprotein inhibitor would be a drug that has little or no toxicity and which does not simultaneously inhibit the related cytochromes involved in drug metabolism. While pharmaceutical companies are working on just such a drug, it is not yet clinically available. We could have chosen other clinically available drugs (eg, digoxin, verapamil, vinca alkaloids, daunamycin, etc) as competitive p-glycoprotein inhibitors, but these drugs are acutely toxic or fatal in the doses needed to produce demonstrable p-glycoprotein inhibition *in vivo*. Thus, cyclosporine-A was an appropriate choice for a 'proof of principle'. However, these data should not be construed as advocating simultaneous administration of cyclosporine-A and methylprednisolone in humans with spinal cord injury. Although cyclosporine-A has been shown to improve neurological function when it is administered alone in animal models of acute neurological injury,[28–36] its coadministration with high-dose methylprednisolone is untested in humans and the potential for significant morbidity secondary to profound immunosuppression is a concern.

In summary, this study demonstrates that coadministering the p-glycoprotein inhibitor, cyclosporine-A, increases entry of methylprednisolone into the CNS, although it also increases plasma methylprednisolone concentration by inhibiting methylprednisolone metabolism. The findings suggest that current efforts by the pharmaceutical industry to develop more potent and less toxic p-glycoprotein inhibitors that do not simultaneously inhibit cytochrome p-450 may improve the efficacy of methylprednisolone in treating acute spinal cord trauma.

Acknowledgements

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