

## Original Article

# Effect of postinjury intravenous or intrathecal methylprednisolone on spinal cord excitatory amino-acid release, nitric oxide generation, PGE<sub>2</sub> synthesis, and myeloperoxidase content in a pig model of acute spinal cord injury

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**Study design:** Prospective, randomized, *in vivo* acute spinal cord injury in pigs.

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

**Objectives:** To determine whether postinjury methylprednisolone could reduce the generation of known mediators of secondary neurological injury.

**Methods:** Intrathecal microdialysis probes were used to sample cerebrospinal fluid (CSF) for measurement of PGE<sub>2</sub>, glutamate, and citrulline (a byproduct of nitric oxide generation), before and after spinal cord injury in anesthetized pigs. The spinal cord was removed at the end of the study for measurement of myeloperoxidase and methylprednisolone concentrations. Animals were randomly allocated to receive intravenous methylprednisolone (30 mg/kg bolus then 3.4 mg/kg/h), intrathecal methylprednisolone (5 mg bolus then 5 mg/h), or saline, beginning 30 min after the spinal cord was injured by using a modification of the Allen weight drop technique.

**Results:** Spinal cord injury significantly increased the amount of glutamate, PGE<sub>2</sub>, myeloperoxidase, and citrulline, recovered from the CSF dialysates. However, neither intravenous nor intrathecal methylprednisolone administered after injury had any effect on the magnitude of the increase in any of the measured biochemicals. Intrathecal methylprednisolone administration produced a spinal cord methylprednisolone concentration that was eight times greater, and a plasma concentration that was 32 times less, than that achieved with intravenous administration.

**Conclusions:** Contrary to earlier animal studies in which methylprednisolone was administered either before or immediately after spinal cord injury, we found no effect of intravenous or intrathecal methylprednisolone on any of the parameters measured when administered 30 min postinjury.

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**Keywords:** PGE<sub>2</sub>; nitric oxide; glutamate; aspartate; myeloperoxidase; methylprednisolone

## Introduction

Multiple animal studies have shown that high-dose methylprednisolone treatment of acute spinal cord injury results in a reduction in biochemical mediators of secondary neurological injury<sup>1–6</sup> and significantly improved neurological function.<sup>7–11</sup> Unfortunately, results in humans have been less sanguine. For example, while the National Acute Spinal Cord Injury Studies (NASCIS I, II, and III) demonstrated improvement in

neurological function in patients given high-dose methylprednisolone within 8 h of spinal cord injury,<sup>12–17</sup> the improvement was modest at best. In addition, multiple other clinical studies have failed to demonstrate any neurological benefit of high-dose methylprednisolone therapy following acute spinal cord injury in humans.<sup>18–21</sup>

One possible explanation for the differences in methylprednisolone's efficacy when comparing human and animal data is that in animal studies, methylprednisolone is almost invariably administered prior to spinal cord injury, or immediately after injury.<sup>2–4,6</sup> In

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contrast, in the human clinical situation, the time necessary for patient transport and work-up can delay methylprednisolone administration until many hours after the spinal cord injury. Consequently, it is unclear to what extent the experimental animal data can be extrapolated to the human clinical setting.

To address this issue, we developed a pig model of acute spinal cord injury that permits measurement of multiple mediators of secondary neuronal injury (ie, nitric oxide (NO), PGE<sub>2</sub>, glutamate, aspartate, myeloperoxidase). These mediators were chosen for study because their concentration had been shown to be reduced by methylprednisolone therapy in animal models wherein methylprednisolone was administered prior to, or immediately after, spinal cord injury. We compared the effect of spinal cord injury on the elaboration of these biochemical mediators in control animals and in two groups of animals treated 30 min after spinal cord injury, with either intravenous or intrathecal methylprednisolone. We chose the 30-min time point because we reasoned that this was the earliest time after injury that humans could possibly receive high-dose methylprednisolone (eg, from paramedics during transport). In addition, we compared the conventional intravenous route of methylprednisolone administration with the intrathecal route to determine whether the much higher methylprednisolone concentrations achieved with intrathecal delivery offered any advantage over intravenous administration.

## Materials and methods

The University of Washington Animal Care and Use Committee approved all studies. Guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care were followed throughout. Farm-bred pigs of both genders weighing 18–22 kg were used. Animals were housed as groups in rooms with 12-h light–dark cycles, *ad libitum* access to water, and twice daily feedings of pig chow.

### *Surgical preparation*

On study days, the animals were anesthetized by mask inhalation of isoflurane (6% inspired concentration) in oxygen, paralyzed with intramuscular succinylcholine (200 mg), and orotracheally intubated. Anesthesia was maintained with 1.5% isoflurane (inspired concentration) in oxygen. The lungs were mechanically ventilated to maintain end-tidal CO<sub>2</sub> at 36–40 mmHg. Temperature was maintained at 37–38°C by a servo-controlled heating pad connected to a rectal temperature probe.

An intravenous catheter was placed in a femoral artery via cut down for blood pressure monitoring, and a second catheter was placed in the adjacent femoral vein for maintenance fluid and methylprednisolone administration. Maintenance fluid (lactated Ringer's) was administered at 4 ml/kg/h, and contained pancuronium bromide (0.06 mg/ml) to maintain muscle paralysis.

A 1.5 cm length of spinal cord was exposed by laminectomy at the T13 vertebral level and the site was covered with a saline-soaked gauze until subsequent weight drop injury. Laminotomies measuring approximately 0.5 cm<sup>2</sup> were made on the right and left sides of the L2 and T5 vertebrae to permit microdialysis probe insertion into the subarachnoid space (see Microdialysis probe manufacture below). One dialysis probe was inserted through each laminotomy. Microdialysis probes inserted at the L2 level were directed cephalad to lie on the right and left side of the spinal cord segment that was to be injured. Microdialysis probes inserted at T5 were also directed cephalad and served as a control site, distant from the injury. Samples from one microdialysis probe at each level were used for measuring PGE<sub>2</sub> concentrations, while samples from the second microdialysis probe were used for measuring glutamate, aspartate, and citrulline concentrations.

An epidural catheter (Arrow International, Reading, PA, USA) was also inserted 1 cm into the subarachnoid space, through a laminotomy at L4 to permit intrathecal methylprednisolone or saline infusion (see Drug administration below). The laminotomies were then sealed with cyanoacrylate glue.

### *Microdialysis probe manufacture*

Microdialysis probes were prepared from cellulose microdialysis fibers (Spectrum Medical Industries, 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 2 cm of the fiber, thus creating a 2-cm dialysis window. Epoxy was spread evenly over the fiber by running a 2 cm length of polyethylene 10 (PE-10) tubing over the fiber while the epoxy was still wet. PE-10 tubing had an inside diameter of 280 μm, and thus, the dialysis probe had a final outside diameter of 280 μm. To facilitate placement of the dialysis probes within the cerebrospinal fluid (CSF) and to prevent kinking of the dialysis fiber, a 90 μm diameter tungsten wire was inserted into the lumen of the dialysis probe, and bent at the center of the dialysis window, thereby creating a microdialysis loop. A 0.5 mm cone-shaped length of silicone caulk was placed approximately 0.5 mm from the dialysis window. To prevent CSF leak, this elastic cone was wedged into the meningeal hole through which the probes were inserted into the CSF. All probes were allowed to 'cure' for at least 12 h before implantation, and were used within 48 h of manufacture.

### *Microdialysis technique*

Mock CSF (NaCl 140 mEq, NaHCO<sub>3</sub> 25 mEq, KCl 2.9 mEq, MgCl<sub>2</sub> 0.4 mEq, urea 3.5 mEq, glucose 4.0 mEq, CaCl<sub>2</sub> 2.0 mEq, pH 7.38–7.42, 295 mOsm) was pumped through the dialysis probes at 10 μl/min. Mock CSF was oxygenated and pH adjusted by bubbling with 95% O<sub>2</sub>/5% CO<sub>2</sub>.

Beginning 30 min after starting dialysis, dialysate samples were collected from both probes at 15 min intervals (150  $\mu$ l samples) for subsequent measurement of excitatory amino acids and PGE<sub>2</sub> concentrations. To prevent amino-acid and prostaglandin metabolism, the dialysate samples were collected into plastic bullet tubes immersed in a water ice bath. Immediately after completing the collection of each sample, the bullet tube was removed from the ice bath, capped, and placed on water ice until frozen at  $-20^{\circ}\text{C}$  for later analysis.

#### *Spinal cord injury*

Baseline dialysis samples were collected for 105 min during when the spinal cord was injured using a weight drop technique. Specifically, a cylindrical steel 'impactor' measuring 1 cm in diameter and 3 cm in length was gently set on the surface of the previously exposed spinal cord (T13). A 1.25 cm diameter aluminum tube was positioned vertically over the impactor; and that served as a guide to ensure that the dropped weight hit the impactor squarely. The inner surface of the tube had previously been 'lubricated' with graphite to minimize friction between the dropped weight and the impactor. The steel weight dropped through the guide tube and onto the impactor weighed 25 g and was dropped from a height of 45 cm. The impactor and the weight were immediately removed from the injured spinal cord and the cord covered with a saline-soaked gauze.

#### *Drug administration*

Animals were prospectively randomized into one of the three groups. Group 1 served as the control and received intrathecal saline (100  $\mu$ l/min  $\times$  1 min, followed by 100  $\mu$ l/h for the remainder of the study). Group 2 received intravenous methylprednisolone (30 mg/kg bolus over 10 s, followed by a 5.4 mg/kg/h intravenous infusion; methylprednisolone concentration = 50 mg/ml) and an intrathecal saline infusion at the same rate as the control group. Group 3 received intrathecal methylprednisolone (100  $\mu$ l/min  $\times$  1 min, followed by 100  $\mu$ l/h for the remainder of the study; methylprednisolone concentration = 50 mg/ml). Methylprednisolone/saline administration was begun 30 min after spinal cord impact.

#### *PGE<sub>2</sub>, amino-acid, myeloperoxidase, and methylprednisolone assay*

**Myeloperoxidase assay** Myeloperoxidase activity was assayed using a variation of the technique described by Carlson *et al.*<sup>22</sup> Briefly, immediately at the end of the study, the descending aorta was cannulated with a 12 G catheter, and the animal perfused under pressure (300 mmHg) with 5 l of ice-cold saline to remove all blood.

After saline perfusion, the injured spinal cord section and two 1 cm sections cephalad and caudad of the

injured segment were removed. As a control, a 1 cm spinal cord section 10 cm cephalad of the injured segment was also removed. These specimens were weighed and placed in ice-cold 50 mM phosphate-buffered saline (PBS) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The volume of PBS + HTAB was 10 ml/g tissue. The tissue was then homogenized for 1 min at  $4^{\circ}\text{C}$  with a Teflon/glass homogenizer, and the homogenate centrifuged at 2000  $g$  for 10 min. The resulting supernatant was removed and the pellet resuspended in 1 ml PBS + HTAB and the specimen homogenized and centrifuged again as described above. The pellet was resuspended, sonicated for 10 s, exposed to three freeze/thaw cycles in an acetone-dry ice bath, sonicated for an additional 10 s, and centrifuged at 10 000  $g$  for 30 min. The resultant supernatant was immediately frozen at  $-20^{\circ}\text{C}$  until assayed for myeloperoxidase. This extraction method produced a myeloperoxidase recovery averaging  $77 \pm 11\%$ .

The myeloperoxidase assay solution (50 mM PBS, 0.167 mg/ml *o*-dianisidine, 0.0005% hydrogen peroxide) was made up fresh each day. The assay solution (2.9 ml) was warmed to  $38^{\circ}\text{C}$  and placed in a spectrophotometer cuvette. The sample to be assayed (0.1 ml) was added to the assay solution, the solution mixed by inverting the cuvette five times, and the absorbance at 460 nm recorded every 15 s for 3 min. Myeloperoxidase catalyzed a reaction to generate an oxygen free radical from hydrogen peroxide and the free radical reacted with *o*-dianisidine to produce a colored product that absorbs light at 460 nm. The rate at which the colored product is produced is proportional to the amount of myeloperoxidase present. The absorbance was plotted against time, and the method of least squares was used to determine the slope through the data points. Myeloperoxidase content was determined by comparing the measured slope with the slope generated from standard solutions containing known amounts of myeloperoxidase (Calbiochem, San Diego, CA, USA).

**Glutamate, aspartate, and citrulline assay** Glutamate, aspartate, and citrulline concentrations were measured using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbonate (AQC) derivatization and a high-performance liquid chromatography (HPLC)/mass spectroscopy assay modified from DeAntonis *et al.*<sup>23</sup> and Cohen *et al.*<sup>24</sup>

Briefly, the derivatizing reagent, AQC, was synthesized according to the method of Cohen *et al.*<sup>24</sup> Dialysate samples were thawed on ice and 20  $\mu$ l was added to 12  $\times$  75 mm glass tubes. A measure of 20  $\mu$ l of internal standard (20 ng/ $\mu$ l phosphoserine in water) was added to each tube. Borate buffer (40  $\mu$ l, 0.2 M, pH 8.8) was added to each tube, followed by 20  $\mu$ l AQC reagent. The solution was mixed well and incubated at room temperature for 1 min and then mixed again. The resultant solution was transferred to an autosampler vial and capped with a Teflon-lined crimp cap. The

solution was incubated at 55°C for 10 min and allowed to cool to room temperature. A measure of 10  $\mu$ l of this solution was injected onto the HPLC.

The HPLC (Agilent Technologies series 1100; Palo Alto, CA, USA) was fitted with a C18 column (Restek Allure model 9164572; Bellefonte, PA, USA), and the mobile phase consisted of 85% 20 mM ammonium acetate, 0.02% *N,N*-dimethylhexylamine (pH 5.5), and 15% acetonitrile at a flow rate of 0.250 ml/min from 0 to 5.2 min and then 0.3 ml/min until 8 min. Post-time flow was 0.250 ml/min for 1.5 min. The column temperature was 45°C and the sample temperature 25°C.

The mass spectrometer (Agilent Technologies series 1100; Palo Alto, CA, USA) was set in high-resolution mode using selective ion monitoring of ions 304 *m/z* (aspartate), 318 *m/z* (glutamate), 346 *m/z* (citrulline), and 356 *m/z* (phosphoserine). Fragmentor was 70 V, gain 1, and dwell 294 ms, for all. Nitrogen drying gas flow was 10 l/min at 350°C. Quadrupole temperature was 100°C. Nebulizing gas was nitrogen at a pressure of 35 psig. Capillary voltage was 2500 V.

A standard curve was run for each amino acid using standards prepared in water from commercially purchased amino acids (Sigma-Aldrich, St Louis, MO, USA). The standards were run as described above for the dialysate samples.

**PGE<sub>2</sub> assay** PGE<sub>2</sub> concentration in CSF dialysate was analyzed using a commercial enzyme immunoassay kit (Assay Designs Inc., Ann Arbor, MI, USA) according to the manufacturer's directions.

**Methylprednisolone assay** Methylprednisolone concentration was measured in blood removed at the end of each experiment and spinal cord removed immediately after saline perfusion as described above. Following saline perfusion, an approximately 1 cm long section of spinal cord was removed 3 cm cephalad of the injured spinal cord segment and immediately frozen at -20°C until later analyzed for methylprednisolone content. On the assay day, the spinal cord specimens were thawed on ice, fluoxymesterone added (700 ng) as an internal standard, the specimen homogenized at 4°C, and the homogenate extracted into methylene chloride. The remainder of the assay for methylprednisolone in spinal cord was as follows for plasma.

Blood samples (3 ml) were centrifuged at 10 000 r.p.m. for 5 min, and the supernatant frozen at -20°C until assayed for methylprednisolone concentration.

Plasma samples (0.5 ml) were placed in a silanized 13  $\times$  100 screw-cap culture tubes, and 20  $\mu$ l of internal standard (fluoxymesterone 35 ng/ $\mu$ l) 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 deionized water 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  $\mu$ l of HPLC mobile phase and filtered using a 0.2  $\mu$ m nylon centrifuge filter; 50  $\mu$ l was injected on the HPLC.

The HPLC was a Hewlett Packard 1050 series (Hewlett Packard, Palo Alto, CA, USA) with auto-sampler and UV detector. The column was a Supelco LC-18-DB 150  $\times$  4.6 mm  $\times$  5  $\mu$ m (Sigma-Aldrich Co., St Louis, MO, USA). The mobile phase consisted of 65% 10 mM potassium phosphate monobasic and 35% acetonitrile pumped at 1 ml/min. The peaks were detected at 242 nm. Quantification was based upon peak height ratios between methylprednisolone and the internal standard, plotted as a linear regression.

### Statistical analysis

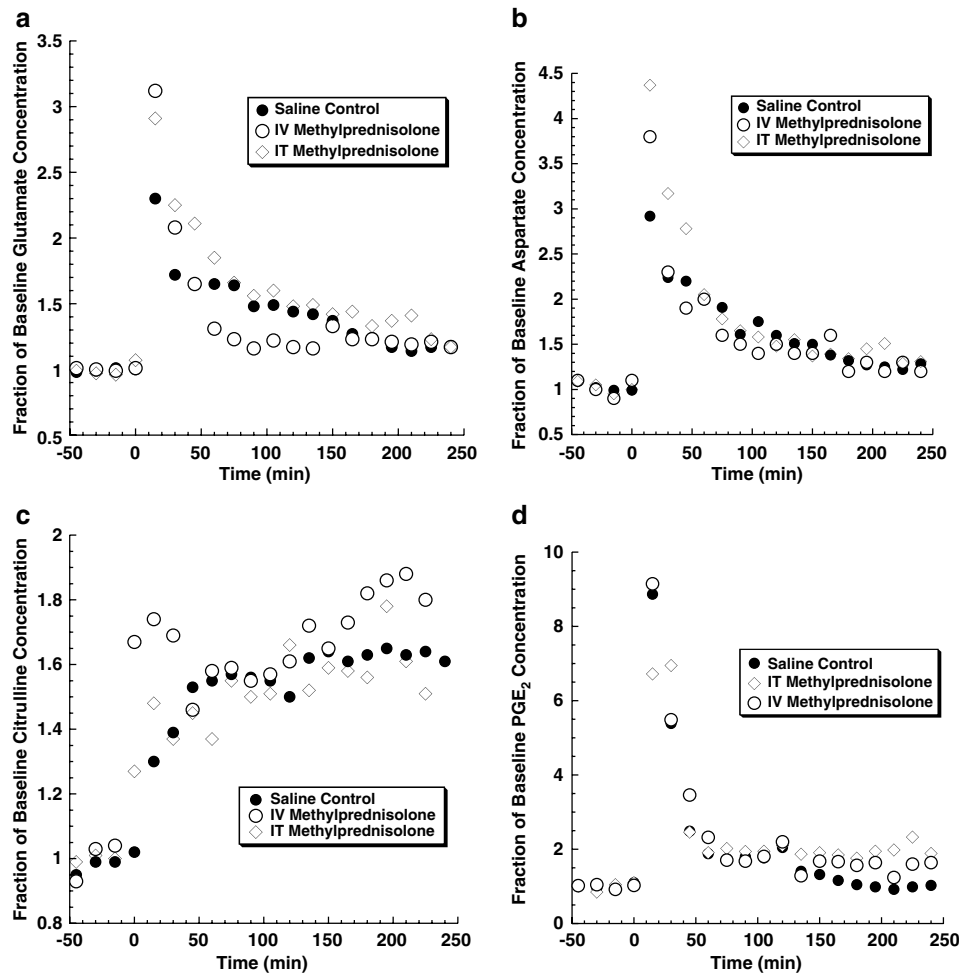
Excitatory amino-acid concentrations, citrulline concentrations, and PGE<sub>2</sub> concentrations were normalized by dividing each value by the respective baseline concentration. The baseline concentration was calculated as the average of the four values collected over the hour prior to spinal cord injury.

Differences among the three treatment groups in amino-acid, citrulline, and PGE<sub>2</sub> concentrations over time at the spinal cord injury site and at the proximal control site were assessed for statistical significance by repeated measures analysis of variance (ANOVA). Differences in peak concentrations and time to peak concentrations were assessed by ANOVA. Differences in myeloperoxidase and methylprednisolone concentrations were also assessed for statistical significance by ANOVA. Statview (SAS, Cary, NC, USA) statistical software was used throughout; *P* < 0.05 was considered statistically significant.

## Results

Peak methylprednisolone concentrations in plasma were significantly greater in the intravenous methylprednisolone group (3255  $\pm$  1106 ng/ml) than that in the intrathecal methylprednisolone group (100  $\pm$  56 ng/ml). In contrast, 'steady-state' spinal cord concentrations of methylprednisolone were significantly less in the intravenous group (111  $\pm$  26 ng/g) than that in the intrathecal group (924  $\pm$  1304 ng/g).

Spinal cord impact resulted in an immediate and statistically significant increase in glutamate, aspartate, citrulline, and PGE<sub>2</sub> concentrations, within the CSF adjacent to the injury site (Figure 1). However, compared to the control group, neither the intravenous or intrathecal methylprednisolone had any statistically significant effect on the time course, peak concentration, time to peak concentration, nor area under the concentration time curve (AUC) for any of these mediators of spinal cord injury (Figure 1, Table 1). This was true even if the statistical analysis was limited to the time period after methylprednisolone was administered. Interestingly, citrulline concentration remained elevated



**Figure 1** Glutamate (a), aspartate (b), citrulline (c), and PGE<sub>2</sub> (d) concentrations (expressed as fraction of baseline concentration) versus time for dialysate samples collected from CSF adjacent to the injured spinal cord segment (T13). Spinal cord injury occurred at time = 0 min. Baseline values were calculated as the average of the four dialysate samples whose collection ended at time = 0, -15, -30, and -45 min. There were no statistically significant differences among the three treatment groups for glutamate, aspartate, citrulline, or PGE<sub>2</sub>.

throughout the 4-h study period in all three groups, while concentrations of the excitatory amino acids and PGE<sub>2</sub> returned to near baseline.

The time course of glutamate, aspartate, and citrulline concentrations within the CSF adjacent to the thoracic control site was not significantly different among the three groups (Figure 2). Similarly, the peak concentrations, time to peak concentrations, and the AUC for glutamate, aspartate, and citrulline at the thoracic control site were not different among the three groups (Table 1). In contrast, the time course for PGE<sub>2</sub> in the intrathecal methylprednisolone group was significantly different than that of the control group and the intravenous methylprednisolone groups, which did not differ from one another (Figure 2). However, this difference in time course was not reflected by any differences among the three groups in peak PGE<sub>2</sub> concentration, time to peak PGE<sub>2</sub> concentration, or area under the PGE<sub>2</sub> concentration versus time curve at the thoracic control site (Table 1).

Figure 3 shows the average myeloperoxidase concentrations in spinal cord segments removed at the end of each experiment. There were no differences among the three treatment groups in the average myeloperoxidase concentrations in the various segments. For the saline and intrathecal methylprednisolone groups, myeloperoxidase concentration was greatest in the injured spinal cord segment and decreased significantly as a function of distance from the injured segment. Interestingly, this was not true for the intravenous methylprednisolone group. Although myeloperoxidase concentrations were also greatest in the injured spinal cord segment of the intravenous methylprednisolone group, myeloperoxidase concentrations in other segments were not significantly different as a function of distance.

## Discussion

Multiple animal studies have measured the effect of spinal cord trauma on one or more of the biochemical

**Table 1** Pharmacokinetic parameters

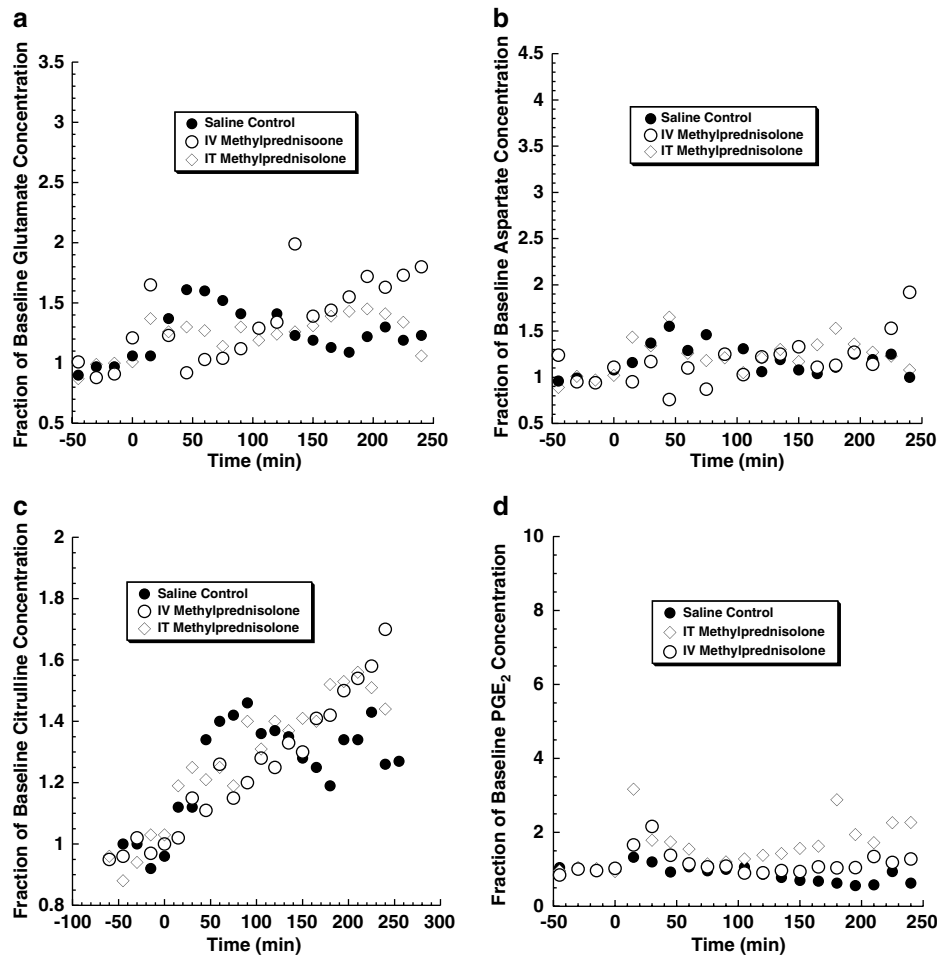
Sampling site	Group		
	Saline	Intravenous methylprednisolone	Intrathecal methylprednisolone
<i>CSF at injury site</i>			
<i>Peak concentration (fractions of baseline)</i>			
Aspartate	3.3 ± 1.7	4.8 ± 2.6	4.6 ± 3.8
Glutamate	2.6 ± 1.2	3.3 ± 1.5	3.1 ± 1.6
Citrulline	1.9 ± 0.4	2.2 ± 0.7	1.9 ± 0.7
PGE <sub>2</sub>	9.5 ± 8.7	10.8 ± 8.6	10.5 ± 10.6
<i>Time to peak concentration (min)</i>			
Aspartate	146 ± 28	138 ± 9.5	158 ± 56
Glutamate	145 ± 21	140 ± 10	153 ± 45
Citrulline	230 ± 72	272 ± 86	237 ± 85
PGE <sub>2</sub>	157 ± 43	188 ± 84	140 ± 7.5
<i>AUC (fraction of baseline × time)</i>			
Aspartate	389 ± 194	385 ± 118	408 ± 192
Glutamate	324 ± 167	308 ± 82	332 ± 176
Citrulline	353 ± 109	378 ± 152	344 ± 131
PGE <sub>2</sub>	448 ± 239	530 ± 263	559 ± 255
<i>CSF at proximal control site</i>			
<i>Peak concentration (fraction of baseline)</i>			
Aspartate	2.0 ± 0.8	2.5 ± 2.1	2.4 ± 1.6
Glutamate	1.9 ± 1.0	2.7 ± 2.0	2.0 ± 1.4
Citrulline	1.7 ± 0.5	1.8 ± 0.8	1.7 ± 0.6
PGE <sub>2</sub>	1.7 ± 1.3	4.7 ± 5.8	3.5 ± 2.5
<i>Time to peak concentration (min)</i>			
Aspartate	248 ± 82	245 ± 82	234 ± 84
Glutamate	251 ± 88	259 ± 99	193 ± 67
Citrulline	236 ± 66	311 ± 74	254 ± 83
PGE <sub>2</sub>	183 ± 66	178 ± 70	195 ± 80
<i>AUC (fraction of baseline × time)</i>			
Aspartate	277 ± 81	264 ± 124	291 ± 132
Glutamate	296 ± 127	317 ± 164	293 ± 154
Citrulline	300 ± 64	305 ± 95	309 ± 82
PGE <sub>2</sub>	195 ± 101	266 ± 154	394 ± 433

mediators of secondary spinal cord injury examined in this study. However, to our knowledge, this is the first study to simultaneously examine the early time course of such a large number of diverse biochemical responses to acute spinal cord injury. Consistent with earlier studies, this study demonstrates that acute spinal cord injury results in a significant increase in excitatory amino-acid release (glutamate and aspartate), proinflammatory prostanoid production (PGE<sub>2</sub>), NO generation (measured as citrulline by-product), and neutrophil content (measured as myeloperoxidase). However, we failed to find any significant effect of methylprednisolone administration, by either route, on any of these early mediators of secondary spinal cord injury. The reason for this is not entirely clear from the data, but may reflect the fact that for reasons of clinical relevance, we administered methylprednisolone 30 min after the spinal cord injury. In contrast, many of the animal studies that have demonstrated a benefit of methylprednisolone as a

treatment for acute spinal cord injury administered the drug either before, or immediately after, the injury.

For example, Anderson *et al*<sup>2</sup> demonstrated that methylprednisolone (30 mg/kg) administered 20 min before spinal cord compression significantly reduced eicosanoid (PGE<sub>2</sub>, PGF<sub>2α</sub>, 6-keto-PGF<sub>1α</sub>, thromboxane B<sub>2</sub>) concentrations in the injured tissue collected 30 min after compression. In a similar study using the same cat model, Saunders *et al*<sup>3</sup> also found that pretreatment with methylprednisolone (30 mg/kg) attenuated prostanoid production following spinal cord injury. Likewise, using a rat model in which methylprednisolone (30 mg/kg, then 5.4 mg/kg/h) was administered immediately after injury, Liu *et al*<sup>6</sup> also found that prostanoid (PGF<sub>2α</sub>) production was reduced by methylprednisolone treatment.

In contrast, and in keeping with our findings, Hall *et al*<sup>25</sup> reported that methylprednisolone (30 mg/kg) administered 30 min after injury had no effect on



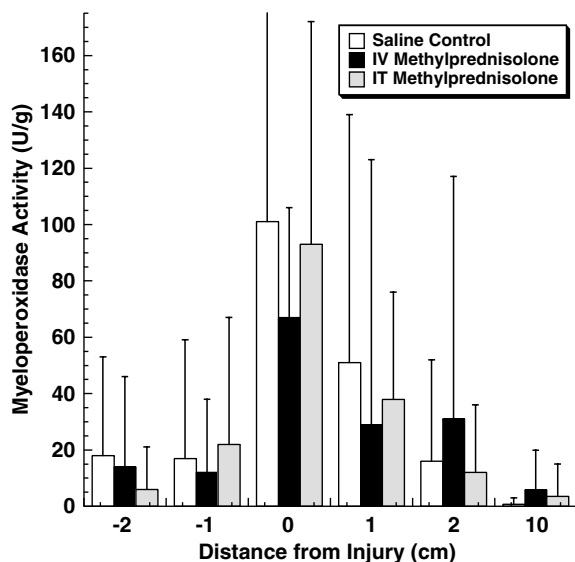
**Figure 2** Glutamate (a), aspartate (b), citrulline (c), and PGE<sub>2</sub> (d) concentration (expressed as fraction of baseline concentration) versus time for dialysate samples collected from CSF adjacent to the proximal control spinal cord segment (T5). Spinal cord injury occurred at time = 0 min. Baseline values were calculated as the average of the four dialysate samples whose collection ended at times = 0, -15, -30, and -45 min. There were no statistically significant differences among the three treatment groups for glutamate, aspartate, or citrulline concentrations. For PGE<sub>2</sub>, however, the intrathecal methylprednisolone group was significantly different than the intravenous methylprednisolone and the control groups, although there were no differences in PGE<sub>2</sub> concentrations among the three treatments at any individual time points

eicosanoid (PGE<sub>2</sub>, PGF<sub>2</sub>, 6-keto-PGF<sub>2α</sub>, thromboxane B<sub>2</sub>) concentrations in cat spinal tissue when measured at a single time point 1 h after injury. Thus, the data would suggest that methylprednisolone can reduce generation of PGE<sub>2</sub> after spinal cord injury, but only if given before or immediately after injury occurs. As methylprednisolone decreases prostanoid generation by inhibiting phospholipase A<sub>2</sub>-mediated cleavage of arachidonic acid from cell membranes and by blocking cyclooxygenase expression, the failure of methylprednisolone to impact PGE<sub>2</sub> production in our study and the study by Hall *et al* would suggest that the activity of these two enzymes is no longer rate limiting for prostanoid production 30 min after spinal cord injury.

NO is generated from L-arginine by NO synthase (NOS), and in the process, citrulline is generated. We chose to measure citrulline concentration as a marker for NO production because it is chemically stable and

easier to measure in dialysate samples. We examined NO production in this study because it has been implicated as a mediator of secondary neurological injury,<sup>26</sup> and inhibition of NOS activity has been shown to improve neurological outcome in a rat model of spinal cord injury.<sup>1</sup>

Our finding of an immediate increase in citrulline production is consistent with the findings of Liu *et al*,<sup>26</sup> who used an NO-sensitive electrode to demonstrate an immediate increase in NO concentration in spinal cord, following impact injury. However, our results differ from those of Liu *et al* in one regard. Specifically, Liu *et al* reported that NO concentrations returned to near baseline by approximately 2 h after injury, while we found that citrulline concentrations remained elevated throughout the 4 h of our study. These seemingly incongruous findings are not necessarily incompatible and can be explained by ongoing production of NO and



**Figure 3** Average myeloperoxidase content in spinal cord specimens obtained at the end of each experiment, as a function of distance from the injured segment (0 cm). There were no differences among the three treatment groups in myeloperoxidase content in any of the spinal cord segments. For the control and intrathecal methylprednisolone groups, myeloperoxidase content decreased significantly as a function of distance from the injured segment. This was not true of the intravenous methylprednisolone group in which there was no significant relationship between myeloperoxidase content and distance from the injured segment

rapid conversion to another form that was not measurable by the ion-selective electrode used by Liu *et al.* In fact, the data of Liu *et al.* support this hypothesis. That is, Liu *et al.* demonstrated a persistently elevated concentration of peroxynitrite ( $\text{ONOO}^-$ ) in spinal cord tissue throughout the 3 h when they collected data. Peroxynitrite is formed by reaction of NO with superoxide anion ( $\text{O}_2^-$ ), which is elevated in spinal cord injury.<sup>27–29</sup> Thus, the persistently elevated concentrations of the highly reactive  $\text{ONOO}^-$  anion observed by Liu *et al.* is best explained by ongoing production of NO in the presence of  $\text{O}_2^-$ . And, the failure of Liu *et al.* to measure the persistently elevated concentrations of free NO by ion-selective electrode would be explained by the rapid conversion of NO to peroxynitrite.

The source of the immediate increase in NO production in our study is unclear. NO is produced by NOS, an enzyme that exists in multiple locations and isoforms. Neuronal NOS (nNOS) and endothelial NOS (eNOS) are constitutively expressed enzymes. Inducible NOS (iNOS) is not constitutively expressed, but can be induced by a number of pathological processes including inflammation. Several studies have demonstrated that iNOS activity increases, following spinal cord injury, but that the increase is not significant until 12–72 h after injury; thus, iNOS would not seem to be responsible for the immediate increase in NO production observed by us and by Liu *et al.* The activity of the constitutively

expressed NOS enzymes is calcium-dependent and increased intracellular calcium concentrations result in increased NOS activity.<sup>30–32</sup> As neuronal injury results in an increase in intracellular calcium concentrations,<sup>33</sup> the activation of eNOS and nNOS via this mechanism is the likely explanation for the increase in NO production that we observed.

Excitatory amino-acid release in response to spinal cord injury has been implicated as a contributor to secondary neurological injury.<sup>34</sup> As previously demonstrated by others,<sup>4,5,34–40</sup> spinal cord injury in this study resulted in an immediate increase in concentrations of the excitatory amino acids glutamate and aspartate and a rapid return toward baseline over several hours. However, in our study, neither of the routes of methylprednisolone administration had any effect on glutamate or on aspartate release following trauma. This is true even if the analysis is limited to the period after methylprednisolone had been administered. This lack of a beneficial effect of methylprednisolone administration differs from the findings of others. However, studies demonstrating a benefit of methylprednisolone administered the drug before or immediately after the spinal cord injury. For example, Liu and McAdoo<sup>4</sup> demonstrated that methylprednisolone (30 mg/kg then 5.4 mg/kg/h) administered immediately after spinal cord injury in a rat model resulted in a significant decrease in glutamate release over time and a more rapid decline in aspartate concentrations. Our failure to find a similar benefit of methylprednisolone, even at the very high methylprednisolone concentrations achieved with intrathecal injection, suggests that the processes responsible for glutamate/aspartate release following spinal cord injury are no longer amenable to modification by methylprednisolone if administered 30 min after injury.

The inflammatory cell response to spinal cord injury has also been implicated as a mediator of secondary neurological injury.<sup>22,41,42</sup> For example, Carlson *et al.*<sup>22</sup> showed that the number of macrophages/microglia present in an area of acute spinal cord injury was correlated with the amount of tissue damage, and that myeloperoxidase content correlated with neutrophil influx. Using a rat spinal cord compression model, Taoka *et al.*<sup>42</sup> demonstrated that both inhibition of neutrophil binding to endothelial cells with anti-P-selectin antibody and induction of leukopenia with nitrogen mustard reduced neutrophil accumulation in injured spinal cord tissue, diminished myeloperoxidase activity in injured tissue, and improved animals' motor function. Similarly, Naruo *et al.*<sup>43</sup> used a rat spinal cord injury model to demonstrate that the anti-inflammatory prostaglandin,  $\text{PGE}_1$ , reduced intramedullary hemorrhages and myeloperoxidase content in injured spinal cord, and improved motor recovery. Importantly, both Naruo and Taoka administered their therapies prior to injury.

Based on these studies implicating activated inflammatory cells in the production of secondary neurological injury, we examined the effect of methylprednisolone on neutrophil accumulation, as reflected by myeloperox-



idase content, within the injured and uninjured segments of spinal cord. Consistent with the work of Carlson *et al.*,<sup>22</sup> we observed a significant increase in myeloperoxidase content in injured spinal cord, the magnitude of which decreased with increasing distance from the injury site. However, neither intravenous nor intrathecal methylprednisolone decreased the myeloperoxidase content of the injured spinal cord compared to control.

Corticosteroids inhibit neutrophil accumulation in damaged tissue by blocking injury-induced expression of adhesion molecules on vascular endothelial cells,<sup>44</sup> and by blocking expression of adhesion molecules on activated neutrophils.<sup>45</sup> Our failure to demonstrate that methylprednisolone reduced myeloperoxidase content in injured spinal cord may reflect the fact that the injury-induced expression of endothelial cell adhesion molecules is beyond glucocorticoid regulation by 30 min after injury. Alternatively, capillary disruption by the injury may render the endothelial cell unimportant in regulating neutrophil traffic into the injured tissue.

An additional goal of this study was to determine whether the intrathecal route of methylprednisolone administration conferred any advantage over the traditional intravenous route. The data demonstrate that the primary benefit of administering methylprednisolone intrathecally is a pharmacokinetic one. Specifically, compared to the intravenous route, intrathecal methylprednisolone administration resulted in an approximately 30-fold increase in methylprednisolone content in spinal cord tissue, while simultaneously producing a methylprednisolone plasma concentration that was nearly 10-fold lower. If methylprednisolone's therapeutic index is defined as the steady-state spinal cord concentration divided by the simultaneous plasma concentration, then intrathecal administration resulted in a greater than 300-fold increase in methylprednisolone's therapeutic index. This finding suggests that intrathecal administration may provide a means to take advantage of any beneficial effects of methylprednisolone (eg, reduced lipid peroxidation) while simultaneously avoiding the immunosuppressive side effects that accompany systemic methylprednisolone administration.

This could be important clinically because the one constant finding in all of the human clinical trials, regardless of whether they demonstrated a neurological benefit of high-dose methylprednisolone or not, was that methylprednisolone use was associated with significant morbidity because of its systemic immunosuppressive effects. In particular, the incidence of wound infection, sepsis, pneumonia, and the duration of intensive care unit stay have all been increased in patients receiving high-dose methylprednisolone.<sup>13,15,20,46-49</sup> Intravenous methylprednisolone must be administered in such high doses because, as we have previously shown, methylprednisolone entry into the central nervous system is actively opposed by P-glycoprotein present in the capillary endothelial cells comprising the brain- and spinal cord-blood barriers.<sup>50</sup> As P-glycoprotein actively pumps methylprednisolone out of the central nervous

system, systemically administered methylprednisolone must be given in very high doses to overwhelm the efflux pump's capacity and to achieve reasonable concentrations in the central nervous system.

Given that some of the direct (eg, hyperglycemia) and indirect (eg, sepsis-induced hypotension, fever) side effects of systemic methylprednisolone are known to contribute to secondary neurological injury, it is interesting to question whether the systemic side effects of methylprednisolone may work at cross purposes to any beneficial effects exerted within the spinal cord. If so, potentiation of secondary neurological injury by the side effects of systemic methylprednisolone administration might explain those studies that have failed to find any beneficial effect of high-dose methylprednisolone on neurological recovery following acute spinal cord injury. Thus, reducing systemic exposure to methylprednisolone by administering the drug directly into the spinal CSF may confer an important clinical benefit.

In summary, we measured the effect of intrathecal and intravenous methylprednisolone on the injury-induced increase in citrulline, glutamate, PGE<sub>2</sub>, and myeloperoxidase in a pig model of acute spinal cord injury. Neither intravenous nor intrathecal methylprednisolone had any demonstrable effect on the generation of any of these mediators of secondary neurological injury. This finding conflicts with multiple animal studies that have previously demonstrated that methylprednisolone can reduce the concentrations of these compounds in the setting of acute spinal cord injury. This seeming inconsistency is most readily explained by the fact that the earlier animal studies administered methylprednisolone either before or immediately after spinal cord injury, while we waited 30 min before administering methylprednisolone. Our findings would suggest that the potential beneficial effects of methylprednisolone administration in the setting of acute spinal cord injury are not mediated by early reductions in the mediators of secondary neurological injury measured in our study. Importantly, our data do not suggest that methylprednisolone is ineffective as a therapy for acute spinal cord injury; they simply suggest that the mechanism involves different mediators, for example, inhibition of lipid peroxidation. This study does, however, demonstrate that intrathecal methylprednisolone administration may be an effective method to increase methylprednisolone delivery to the injured spinal cord while simultaneously reducing systemic methylprednisolone concentrations and associated immunosuppressive side effects.

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## References

- 1 Yu Y, Matsuyama Y, Nakashima S, Yanase M, Kiuchi K, Ishiguro N. Effects of MPSS and a potent iNOS inhibitor

- on traumatic spinal cord injury. *Neuroreport* 2004; **15**: 2103–2107.
- 2 Anderson DK *et al.* Lipid hydrolysis and peroxidation in injured spinal cord: partial protection with methylprednisolone or vitamin E and selenium. *Central Nerv Syst Trauma* 1985; **2**: 257–267.
- 3 Saunders RD, Dugan LL, Demediuk P, Means ED, Horrocks LA, Anderson DK. Effects of methylprednisolone and the combination of alpha-tocopherol and selenium on arachidonic acid metabolism and lipid peroxidation in traumatized spinal cord tissue. *J Neurochem* 1987; **49**: 24–31.
- 4 Liu D, McAdoo DJ. Methylprednisolone reduces excitatory amino acid release following experimental spinal cord injury. *Brain Res* 1993; **609**: 293–297.
- 5 Farooque M, Hillered L, Holtz A, Olsson Y. Effects of methylprednisolone on extracellular lactic acidosis and amino acids after severe compression injury of rat spinal cord. *J Neurochem* 1996; **66**: 1125–1130.
- 6 Liu D, Li L, Augustus L. Prostaglandin release by spinal cord injury mediates production of hydroxyl radical, malondialdehyde and cell death: a site of the neuroprotective action of methylprednisolone. *J Neurochem* 2001; **77**: 1036–1047.
- 7 Gok A, Uk C, Yilmaz M, Bakir K, Erkutlu I, Alptekin M. Efficacy of methylprednisolone in acute experimental cauda equina injury. *Acta Neurochir (Wien)* 2002; **144**: 817–821 (discussion 821).
- 8 Perez-Espejo MA, Haghighi SS, Adelstein EH, Madsen R. The effects of taxol, methylprednisolone, and 4-aminopyridine in compressive spinal cord injury: a qualitative experimental study. *Surg Neurol* 1996; **46**: 350–357.
- 9 Behrmann DL, Bresnahan JC, Beattie MS. Modeling of acute spinal cord injury in the rat: neuroprotection and enhanced recovery with methylprednisolone, U-74006F and YM-14673. *Exp Neurol* 1994; **126**: 61–75.
- 10 Farooque M, Olsson Y, Holtz A. Effect of the 21-aminosteroid U74006F and methylprednisolone on motor function recovery and oedema after spinal cord compression in rats. *Acta Neurol Scand* 1994; **89**: 36–41.
- 11 Holtz A, Nystrom B, Gerdin B. Effect of methylprednisolone on motor function and spinal cord blood flow after spinal cord compression in rats. *Acta Neurol Scand* 1990; **82**: 68–73.
- 12 Bracken MB *et al.* Methylprednisolone and neurological function 1 year after spinal cord injury. Results of the National Acute Spinal Cord Injury Study. *J Neurosurg* 1985; **63**: 704–713.
- 13 Bracken MB *et al.* A randomized, controlled trial of methylprednisolone or naloxone in the treatment of acute spinal-cord injury. Results of the Second National Acute Spinal Cord Injury Study. *N Engl J Med* 1990; **322**: 1405–1411.
- 14 Bracken MB *et al.* Methylprednisolone or naloxone treatment after acute spinal cord injury: 1-year follow-up data. Results of the second National Acute Spinal Cord Injury Study. *J Neurosurg* 1992; **76**: 23–31.
- 15 Bracken MB *et al.* Administration of methylprednisolone for 24 or 48 h or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. *JAMA* 1997; **277**: 1597–1604.
- 16 Bracken MB *et al.* Methylprednisolone or tirilazad mesylate administration after acute spinal cord injury: 1-year follow up. Results of the third National Acute Spinal Cord Injury randomized controlled trial. *J Neurosurg* 1998; **89**: 699–706.
- 17 Bracken MB, Holford TR. Neurological and functional status 1 year after acute spinal cord injury: estimates of functional recovery in National Acute Spinal Cord Injury Study II from results modeled in National Acute Spinal Cord Injury Study III. *J Neurosurg Spine* 2002; **96**: 259–266.
- 18 Pointillart V *et al.* Pharmacological therapy of spinal cord injury during the acute phase. *Spinal Cord* 2000; **38**: 71–76.
- 19 Poynton AR, O'Farrell DA, Shannon F, Murray P, McManus F, Walsh MG. An evaluation of the factors affecting neurological recovery following spinal cord injury. *Injury* 1997; **28**: 545–548.
- 20 Gerndt SJ *et al.* Consequences of high-dose steroid therapy for acute spinal cord injury. *J Trauma* 1997; **42**: 279–284.
- 21 George ER, Scholten DJ, Buechler CM, Jordan-Tibbs J, Mattice C, Albrecht RM. Failure of methylprednisolone to improve the outcome of spinal cord injuries. *Am Surg* 1995; **61**: 659–663 (discussion 663–664).
- 22 Carlson SL, Parrish ME, Springer JE, Doty K, Dossett L. Acute inflammatory response in spinal cord following impact injury. *Exp Neurol* 1998; **151**: 77–88.
- 23 De Antonis KM, Brown PR, Cohen SA. High-performance liquid chromatographic analysis of synthetic peptides using derivatization with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. *Anal Biochem* 1994; **223**: 191–197.
- 24 Cohen SA, Michaud DP. Synthesis of a fluorescent derivatizing reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of hydrolysate amino acids via high-performance liquid chromatography. *Anal Biochem* 1993; **211**: 279–287.
- 25 Hall ED, Yonkers PA, Taylor BM, Sun FF. Lack of effect of postinjury treatment with methylprednisolone or tirilazad mesylate on the increase in eicosanoid levels in the acutely injured cat spinal cord. *J Neurotrauma* 1995; **12**: 245–256.
- 26 Liu D, Ling X, Wen J, Liu J. The role of reactive nitrogen species in secondary spinal cord injury: formation of nitric oxide, peroxynitrite, and nitrated protein. *J Neurochem* 2000; **75**: 2144–2154.
- 27 Liu D, Sybert TE, Qian H, Liu J. Superoxide production after spinal injury detected by microperfusion of cytochrome c. *Free Radic Biol Med* 1998; **25**: 298–304.
- 28 Luo J, Li N, Robinson JP, Shi R. The increase of reactive oxygen species and their inhibition in an isolated guinea pig spinal cord compression model. *Spinal Cord* 2002; **40**: 656–665.
- 29 Xu W *et al.* Increased production of reactive oxygen species contributes to motor neuron death in a compression mouse model of spinal cord injury. *Spinal Cord* 2005; **43**: 204–213.
- 30 Schmidt HH, Pollock JS, Nakane M, Forstermann U, Murad F.  $\text{Ca}^{2+}$ /calmodulin-regulated nitric oxide synthases. *Cell Calcium* 1992; **13**: 427–434.
- 31 Fleming I, Bauersachs J, Busse R. Calcium-dependent and calcium-independent activation of the endothelial NO synthase. *J Vasc Res* 1997; **34**: 165–174.
- 32 Weikert S *et al.* Rapid  $\text{Ca}^{2+}$ -dependent NO-production from central nervous system cells in culture measured by NO-nitrite/ozone chemoluminescence. *Brain Res* 1997; **748**: 1–11.

- 33 Stys PK. White matter injury mechanisms. *Curr Mol Med* 2004; **4**: 113–130.
- 34 Liu D, Xu GY, Pan E, McAdoo DJ. Neurotoxicity of glutamate at the concentration released upon spinal cord injury. *Neuroscience* 1999; **93**: 1383–1389.
- 35 Farooque M, Hillered L, Holtz A, Olsson Y. Effects of moderate hypothermia on extracellular lactic acid and amino acids after severe compression injury of rat spinal cord. *J Neurotrauma* 1997; **14**: 63–69.
- 36 Farooque M, Hillered L, Holtz A, Olsson Y. Effect of 21-aminosteroid on extracellular energy-related metabolites and amino acids after compression injury of rat spinal cord. *Exp Brain Res* 1997; **113**: 1–4.
- 37 Farooque M, Hillered L, Holtz A, Olsson Y. Changes of extracellular levels of amino acids after graded compression trauma to the spinal cord: an experimental study in the rat using microdialysis. *J Neurotrauma* 1996; **13**: 537–548.
- 38 Xu GY, Hughes MG, Ye Z, Hulsebosch CE, McAdoo DJ. Concentrations of glutamate released following spinal cord injury kill oligodendrocytes in the spinal cord. *Exp Neurol* 2004; **187**: 329–336.
- 39 Vera-Portocarrero LP *et al*. Rapid changes in expression of glutamate transporters after spinal cord injury. *Brain Res* 2002; **927**: 104–110.
- 40 Mills CD *et al*. AIDA reduces glutamate release and attenuates mechanical allodynia after spinal cord injury. *Neuroreport* 2000; **11**: 3067–3070.
- 41 Taoka Y, Okajima K. Role of leukocytes in spinal cord injury in rats. *J Neurotrauma* 2000; **17**: 219–229.
- 42 Taoka Y *et al*. Role of neutrophils in spinal cord injury in the rat. *Neuroscience* 1997; **79**: 1177–1182.
- 43 Naruo S *et al*. Prostaglandin E1 reduces compression trauma-induced spinal cord injury in rats mainly by inhibiting neutrophil activation. *J Neurotrauma* 2003; **20**: 221–228.
- 44 Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann G. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-eukocyte adhesion molecule 1 and intercellular adhesion molecule 1. *Proc Natl Acad Sci USA* 1992; **89**: 9991–9995.
- 45 Filep JG, Delalandre A, Payette Y, Foldes-Filep E. Glucocorticoid receptor regulates expression of L-selectin and CD11/CD18 on human neutrophils. *Circulation* 1997; **96**: 295–301.
- 46 Molano Mdel R, Broton JG, Bean JA, Calancie B. Complications associated with the prophylactic use of methylprednisolone during surgical stabilization after spinal cord injury. *J Neurosurg Spine* 2002; **96**: 267–272.
- 47 McCutcheon EP, Selassie AW, Gu JK, Pickelsimer EE. Acute traumatic spinal cord injury, 1993–2000A population-based assessment of methylprednisolone administration and hospitalization. *J Trauma* 2004; **56**: 1076–1083.
- 48 Matsumoto T, Tamaki T, Kawakami M, Yoshida M, Ando M, Yamada H. Early complications of high-dose methylprednisolone sodium succinate treatment in the follow-up of acute cervical spinal cord injury. *Spine* 2001; **26**: 426–430.
- 49 Galandiuk S, Raque G, Appel S, Polk Jr HC. The two-edged sword of large-dose steroids for spinal cord trauma. *Ann Surg* 1993; **218**: 419–425; (discussion 425–427).
- 50 Koszdin KL, Shen DD, Bernards CM. Spinal cord bioavailability of methylprednisolone after intravenous and intrathecal administration: the role of P-glycoprotein. *Anesthesiology* 2000; **92**: 156–163.