Characterization of spinal HSP72 induction and development of ischemic tolerance after spinal ischemia in rats

Dasa Cizkova, Jason B. Carmel, Kenji Yamamoto, Osamu Kakinohana, Dongming Sun, Ronald P. Hart, and Martin Marsala

Abstract

Induction of heat shock protein (HSP72) has been implicated in the development of ischemic tolerance in several tissue organs including brain and spinal cord. In the present study, using an aortic balloon occlusion model in rats, we characterized the effect of transient noninjurious (3 or 6 min) or injurious intervals (10 min) of spinal ischemia followed by 4-72 h of reflow on spinal expression of HSP72 and GFAP protein. In a separate group of animals, the effect of ischemic preconditioning (3 or 6 min) on the recovery of function after injurious interval of spinal ischemia (10 min) was studied.

After 3 min of ischemia, there was a modest increase in HSP72 protein immunoreactivity in the dorsal horn neurons at 12 h after reperfusion. After 6 min of ischemia, a more robust and wide spread HSP72 protein expression in both dorsal and ventral horn neurons was detected. The peak of the expression was seen at 24 h after ischemia. At the same time point, a significant increase in spinal tissue GFAP expression was measured with Western blots and corresponded morphologically with the presence of activated astrocytes in spinal segments that had been treated similarly. After 10 min of ischemia and 24 h of reflow, a significant increase in spinal neuronal HSP72 expression in perinecrotic regions was seen. Behaviorally, 3 min preconditioning ischemia led to the development of a biphasic ischemic tolerance (the first at 30 min and the second at 24 h after preconditioning) and was expressed as a significantly better recovery of motor function after exposure to a second 10-min interval of spinal ischemia. After 6 min ischemic preconditioning, a more robust ischemic tolerance at 24 h after preconditioning then seen after 3-min preconditioning was detected.

These data indicate that 3 min of spinal ischemia represents a threshold for spinal neuronal HSP72 induction, however, a longer sublethal interval (6 min) of preconditioning ischemia is required for a potent neuronal HSP72 induction. More robust neurological protection, seen after 6 min of preconditioning ischemia, also indicates that HSP72 expression in spinal interneurons seen at 24 h after preconditioning may represent an important variable in modulating ischemic tolerance observed during this time frame.

Keywords: Ischemia; Heat shock protein; Tolerance

Introduction

Transient spinal cord ischemia and subsequent loss of neurological function (spastic or flaccid paraplegia) represent a serious complication associated with transient aortic cross-clamp (as used in repair of aortic aneurysm). In clinical studies, it has been demonstrated that the incidence of paraparesis or developed spastic or flaccid paraplegia ranges between 12% and 40% in patients with extensive thoracoabdominal aortic aneurysm repair (Picone et al., 1986; Svensson et al., 1986). Although spontaneous recovery of function was noted in a fraction of patients that displayed motor dysfunction early after reflow, this injury is irreversible in a majority of cases. In accordance with these data, experimental studies using monkey, cat, dog, rabbit or rat spinal ischemia models show that aortic occlusion will lead to a comparable dysfunction, including transient motor weakness or permanent spastic or flaccid paraplegia (Homma et al., 1979; Marsala et al., 1991; Matsushita and...
Consideration of the clinical importance of spinal ischemia has led to efforts to characterize the potency of numerous pharmacological, surgical and physical interventions in an effort to reduce spinal neuronal degeneration during the periods of transient spinal cord ischemia. In general, these treatments can be divided into categories targeted to (i) decrease spinal metabolic rate (hypothermia, intrathecal local anesthetics, excitatory amino acid receptor blockade), (ii) increase spinal cord blood flow (by-pass, intrathecal or intra-arterial vasodilatory treatment and (iii) to decrease reflow-mediated neuronal degeneration (antioxidant therapy) (Anderson et al., 1985; Robertazzi and Cunningham, 1998; Svensson, 1997; Zvara, 2002). More recently, a phenomenon of induced ischemic tolerance, that is, development of higher ischemic tolerance against otherwise injurious intervals of ischemia, has been described in several tissue organs including brain, heart, kidney and spinal cord. This process has been attributed in part to the cellular expression of inducible form of heat shock protein 72 (HSP72).

In brain ischemia studies, it has been shown that in certain neuronal pools, the ability of neurons to express HSP72 is associated with a higher resistance of these neurons against ischemia-induced degeneration (Kawagoe et al., 1993; Simon et al., 1991). More importantly, exposure of brain tissue to even short-lasting, noninjurious, conditioning ischemia is effective in inducing HSP72 and these populations of cells then display an increased resistance to a subsequent injurious interval of ischemia, that is, induction of ischemic tolerance (Kato et al., 1991; Nishi et al., 1993). It has also been shown that the duration of the anoxic depolarization (i.e. shift in DC potential) produced during the preconditioning ischemia is a key determinant in HSP72 induction (Ikeda et al., 1994). Comparable development of ischemic tolerance in a rat, rabbit and dog spinal ischemia model has been described (Abraham et al., 2000; Matsuyama et al., 1997; Sakurai et al., 1998). However, several other studies have demonstrated that this ischemic tolerance can develop in the absence of a significant HSP72 expression, suggesting that other factors can also be independently involved in this process (Kobayashi et al., 1995; Zvara et al., 1999).

Recently, we characterized a simple and reliable rat spinal ischemia model employing a balloon occlusion of the descending aorta. In this model, 10 min of spinal ischemia leads to development of spastic paraplegia and well-defined spinal neurodegenerative changes characterized by a selective loss of small- and medium-sized interneurons in lumbosacral segments. Ischemic intervals shorter then 8 min have no significant effect and all animals display normal motor function and no detectable histopathological changes in the spinal cord (Taira and Marsala, 1996). Using the rabbit and dog spinal ischemia model, we have observed comparable selective interneuronal degeneration and corresponding loss of motor function (Marsala et al., 1989, 1993; Lukacova et al., 1997). The selective vulnerability of spinal interneurons to ischemia and the ability of preconditioning ischemia to induce ischemic tolerance would suggest that such an ischemia-tolerant state might be associated with increased activity or expression of HSP72 in spinal interneurons.

Accordingly, using rat spinal ischemia models, we sought to characterize (i) the changes in spinal HSP72 expression after increasing intervals (3, 6 or 10 min) of spinal ischemia, (ii) the laminar distribution and structural specificity (neuronal or glial origin) of HSP72 expression and (iii) the effect of preconditioning ischemia (3 or 6 min) on the induction of ischemic tolerance measured by the correlation of neurological and histopathological analysis.

**Material and methods**

All work described herein was accomplished according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

**Induction of spinal ischemia**

Male Sprague–Dawley rats (300–365 g; Harlan Industries, Indianapolis, IN) were used. To induce spinal cord ischemia, a previously described technique was used (Taira and Marsala, 1996). Animals were anesthetized with 3% halothane in an O2/room air mixture (1:1) in an induction box and were maintained with 1–1.5% halothane delivered by an inhalation mask. For core temperature measurements, a rectal probe was inserted 6 cm into the rectum. Following anesthetic induction, a 2F Fogarty catheter (Am.V. Muller; CV 1035) was passed through the left femoral artery to the descending thoracic aorta so that the tip reached the level of the left subclavian artery. This corresponds to a distance of 10.8–11.4 cm from the site of insertion. To measure distal blood pressure (DBP, i.e., below the level of occlusion), the tail artery was cannulated with a PE-50 catheter. The left carotid artery was cannulated with a 20-gauge polytetrafluoroethylene catheter for blood withdrawal. To induce spinal cord ischemia, the intraaortic balloon catheter was inflated with 0.05 ml of saline and systemic hypotension (40 mm Hg) was induced by withdrawing arterial blood (10.5–11 cc). The efficacy of the occlusion was evidenced by an immediate and sustained drop in the DBP measured in the tail artery. After ischemia, the balloon was deflated, and the blood was reinfused over 60 s. After blood reinfusion, 4 mg of Protamine sulfate was administered subcutaneously. Stabilization of the arterial blood pressure was then monitored for an additional 10 min after which arterial lines were removed and wounds were closed. In control animals, the balloon catheter was placed into the descending thoracic aorta but was not inflated. At the end of the survival period, animals were euthanized with pentobarbital, spinal cervical and lumbosacral spinal cord removed by hydroextrusion and...
immediately frozen in −70°C penthane for later Western blot analysis or were perfusion fixed with 4% paraformaldehyde for spinal HSP72 and GFAP immunohistochemistry (see Immunohistochemistry). Experimental groups and survival times are summarized in Table 1.

Systematic characterization of spinal HSP72 expression after increasing intervals of spinal ischemia

Halothane anesthetized rats were exposed to 3, 6 or 10 min of spinal ischemia as described. At 4, 12 or at 72 h after ischemia, animals were sacrificed and spinal cord analyzed for HSP72 and GFAP expression by immunohistochemistry and/or by Western blot.

Induction of preconditioning ischemia and recovery of function

Halothane anesthetized rats were exposed to 3 or 6 min of aortic occlusion (preconditioning ischemia) as described. At 30 min, 2 h or at 24 h after preconditioning, a second 10-min interval of aortic occlusion was induced (Table 1). Animals were then allowed to recover and survived for 7 days. During this period, the recovery of motor function was assessed. Motor function was quantified by assessment of ambulation and placing and stepping responses. The final index was the sum of the scores (walking with lower extremities plus placing and stepping reflex). The maximum deficit score (score of 6) corresponded to fully developed spastic paraplegia. Ambulation: (0, normal; 1, toes flat under the body when walking, but ataxia present; 2, knuckle walking; 3, movement in lower extremities but unable to knuckle walk; 4, no movement, drags lower extremities. The placing/stepping reflex (Coordination lifting and placing response): (0, normal; 1, weak; 2, no stepping).

Perfusion fixation and HSP72 and GFAP immunohistochemistry

At the end of the survival periods, rats were anesthetized with pentobarbital (40 mg/kg, ip) and phenytoin (25 mg/kg, ip) and transcardially perfused with saline for 1–2 min followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Four hours after perfusion fixation, the spinal cords were removed and postfixed in the same fixative overnight at 4°C. After postfixation, tissue was cryoprotected in a graded sucrose solution (10%, 20% and 30%). Frozen transverse spinal sections (10–25 μm) were then cut. Immunohistochemistry was performed using the avidin-biotin/horseradish peroxidase technique (Mouse ABC kit, Vector) or with immunofluorescence. For immunohistochemistry, free floating sections (25 μm) were placed in PBS, 0.1 M (pH = 7.4) containing 5% horse serum (HS), 0.2% Triton X-100 and 0.2% bovine serum albumin (BSA) for 3 h at room temperature to block the nonspecific protein activity. This was followed by overnight incubation at 4°C with primary antibodies. Two different HSP70 antibodies were used in the present study. First, HSP72/73 monoclonal mouse antibody (Stressgene) and the second HSP70 polyclonal rabbit antibody (Chemicon, CA) were used. Both antibodies recognize 70-kDa protein, corresponding to the inducible HSP70. While a modest labeling of ventral α-motoneurons was seen in control animals after using rabbit polyclonal antibody, both antibodies showed no or minimal immunoreactivity in the intermediate zone or in the dorsal horn in control animals. For identification of astrocytes, GFAP monoclonal or polyclonal antibody (Oncogene Science, Sigma; diluted 1:700; 1:1500) was used. After incubation with primary antibodies, sections were washed the next day in PBS three times, then incubated in biotinylated horse anti-mouse secondary antibody 1:200 for 2 h. Bound specific antibodies reacted with the avidine-biotin peroxidase solution for 1 h and were visualized by using 0.05% 3,3,4,4-diaminobenzidine hydrochloride (DAB) in 0.05 M Tris buffer containing 0.001% H₂O₂.

In double labeling experiments, an immunofluorescence technique was used. Ten-micrometer sections were cut and

Table 1

<table>
<thead>
<tr>
<th>Experimental groups and design</th>
<th>Analysis</th>
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<tbody>
<tr>
<td>Group A1 (n = 6): 3 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>−30 min reflow−10 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>Group A2 (n = 6): 3 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>−2 h reflow−10 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>Group A3 (n = 6): 3 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>−24 h reflow−10 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>Group B1 (n = 6): 6 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>−50 min reflow−10 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>Group B2 (n = 6): 6 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>−2 h reflow−10 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>Group B3 (n = 6): 6 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>−24 h reflow−10 min ischemia</td>
<td>MDI</td>
</tr>
<tr>
<td>Group C1 (n = 6): 3 min ischemia and 4 h survival</td>
<td>ICH</td>
</tr>
<tr>
<td>Group C2 (n = 6): 3 min ischemia and 12 h survival</td>
<td>ICH/WB</td>
</tr>
<tr>
<td>Group C3 (n = 3): 3 min ischemia and 24 h survival</td>
<td>ICH/WB</td>
</tr>
<tr>
<td>Group D1 (n = 6): 6 min ischemia and 4 h survival</td>
<td>ICH</td>
</tr>
<tr>
<td>Group D2 (n = 6): 6 min ischemia and 12 h survival</td>
<td>ICH</td>
</tr>
<tr>
<td>Group D3 (n = 6): 6 min ischemia and 24 h survival</td>
<td>ICH/WB</td>
</tr>
<tr>
<td>Group D4 (n = 5): 6 min ischemia and 72 h survival</td>
<td>ICH</td>
</tr>
<tr>
<td>Group D1 (n = 6): 10 min ischemia and 30 min survival</td>
<td>ICH</td>
</tr>
<tr>
<td>Group D2 (n = 6): 10 min ischemia and 12 h survival</td>
<td>ICH</td>
</tr>
<tr>
<td>Group D3 (n = 6): 10 min ischemia and 24 h survival</td>
<td>ICH</td>
</tr>
</tbody>
</table>

MDI, motor deficit index; ICH, immunohistochemistry; WB, Western blot.
collected on Fischer Plus slides. Slides were then placed into a 100% humidified chamber for 5 min to allow sufficient attachments of the sections to the glass surface. Secondary goat anti-rabbit or mouse antibodies conjugated to fluorescent marker (Alexa 488 or 594; 4 μl/ml; Molecular Probes) were used. All blocking and antibody preparations were made in 1× PBS/0.2% TX/5% goat serum. Following 3 h, block sections were incubated in primary antibody for 72 h at 4°C followed by PBS/TX wash (3 × 5 min) and incubation with secondary antibody for 1 h at RT. For double labeling experiments, primary antibodies from different species were applied simultaneously. In control experiments, primary antibodies were omitted. For general nuclear staining, DAPI (3 μl/ml) was added to the final secondary antibody solutions. After staining, sections were dried at RT and covered by Prolong (Molecular Probes). Fluorescence slides were analyzed using a Leica fluorescence microscope. Images (512 × 512 pixels) were obtained with SPOT digital camera and processed by Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA). To identify colocalization of HSP72 immunoreactivity with GFAP antibody, images were captured with a DeltaVision deconvolution microscope system (Applied Precision, Inc.) The system includes a Photometics CCD mounted on a Nikon microscope. In general, 75 optical sections spaced by 0.1 or 0.2 μm were taken. Lenses used were 20× and 40× (NA 1.3). The data sets were deconvolved and analyzed using SoftWorx software (Applied Precision, Inc.) on a Silicon Graphics Octane workstation. Deconvolution microscopy was done in the UCSD Cancer Center Digital Imaging Core.

For systematic quantification of neuronal loss in animals in the behavioral part of the study, the L2–L5 lumbar segments were used. Frozen transverse sections (60 μm thick) were sequentially cut and individually collected from each L2–L5 segment. Sections were stained with NeuN (mouse monoclonal, 1:1000; Chemicon) antibody using a standard immunofluorescence technique. For quantification, every fifth section from each spinal segment was used. The number of persisting interneurons (laminae IV–VII) and α-motoneurons was counted and averaged for each segment and animal. The accuracy of the laminar and segmental level in the individual sections used for neuronal counting was confirmed by comparing analyzed sections to standard laminar and segmental histological maps as described by Molander and Grant (1995). Computer-based quantification of α-motoneurons and interneurons was performed using Image Pro 4.5 software (Media Cybernetics).

**Western blot analysis**

Spinal cord samples were homogenized with a tissue sonicator for 5 min. The supernatant was collected after centrifugation (12,000 × g). Protein samples were prepared and applied to SDS-PAGE and transferred to a nylon filter. Western blot was performed using HSP70 monoclonal antibody (Stressgene) or monoclonal GFAP antibody (Sigma) and ECL+ (Amersham). The filter was scanned by FluorImager 595 (Amersham) and quantified with NIH Image.

**Statistics**

Statistical analysis of physiological data was carried out by one-way ANOVA for multiple comparisons followed by Dunnett’s post hoc test. For the analysis of neurological outcome and spinal histopathology, nonparametric tests were used. For individual studies (Group A1–3, B1–3, C1–3), tests for overall main effects were performed with the Kruskal–Wallis test. Significant main effects (P < 0.05) were probed further through sequential comparisons of each test condition to the adjacent test condition (e.g., 3 or 6 min preconditioning on recovery at 1 and 4 h and 1–7 days).

**Fig. 1.** Motor deficit index assessed at 1 h to 7 days in animals after 3 min (upper panel) or 6 min (lower panel) of preconditioning ischemia followed by injurious interval of ischemia (10 min) at extended period of reflow after preconditioning (i.e. 120 min or 24 h). The most pronounced neuroprotection in animals after 3-min preconditioning and 30 min of reflow as well as in animals after 6 min of preconditioning and 24 h of reflow can be seen (M.D.I. = 6, complete paraplegia; 0, complete recovery).
days) using the comparison of experimental Mann–Whitney tests (unpaired two-group test). Data were expressed as mean ± SD.

In Western blot analysis, the differences between groups were compared using Student’s *t* test or ANOVA. Data were represented as mean ± SEM. The statistical significance was set at *P* < 0.05.

Results

Effect of ischemic preconditioning on the recovery of function: experimental groups A and B

**Group A: 3 min preconditioning (Fig. 1)**

Animals exposed to a 10-min injurious interval of ischemia at 30 min of reflow after 3 min preconditioning ischemia showed a significant recovery of motor function if compared to sham-preconditioned animals (Fig. 1, upper panel). At 24 h after ischemia, seven of eight animals showed ability to stand and walk and continued to improve for the whole 7 days period of survival.

In animals preconditioned with 3 min of ischemia and exposed to a second injurious interval of ischemia at 2 h of reflow, no significant recovery of function was detected.

**Table 2**

<table>
<thead>
<tr>
<th>Segment</th>
<th>Control (n = 3)</th>
<th>Spastic*</th>
<th>Preconditioning*</th>
</tr>
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<tbody>
<tr>
<td>L2</td>
<td>352 (± 25)</td>
<td>334 (± 12)</td>
<td>348 (± 10)</td>
</tr>
<tr>
<td>L3</td>
<td>450 (± 15)</td>
<td>315 (± 20)</td>
<td>436 (± 27)</td>
</tr>
<tr>
<td>L4</td>
<td>522 (± 30)</td>
<td>310 (± 23)</td>
<td>480 (± 14)</td>
</tr>
<tr>
<td>L5</td>
<td>555 (± 27)</td>
<td>522 (± 22)</td>
<td>549 (± 13)</td>
</tr>
<tr>
<td>L6</td>
<td>510 (± 15)</td>
<td>490 (± 9)</td>
<td>540 (± 20)</td>
</tr>
</tbody>
</table>

*Counts of interneurons were done in animals which developed spasticity (groups A2, A3, B1, B2) and in animals which showed recovery of motor function (groups A1, C3) (see Table 1 for experimental groups).

In animals preconditioned with 3 min ischemia and exposed to a second injurious interval of ischemia at 24 h, only partial recovery of function was seen at 7 days. The majority of these animals (6/7) were not able to stand and displayed only a knuckle walking pattern.

**Group B: 6 min preconditioning (Fig. 1)**

In animals preconditioned with 6 min of ischemia and exposed to a second injurious interval of ischemia at 30 min or 2 h after preconditioning, no significant recovery of function was detected at any time point.

If the exposure to the injurious ischemia was delayed to 24 h, the majority of animals displayed a gradual and
significant recovery of motor function, with the most pronounced effect seen at 7 days after injury. Quantitative and qualitative histopathological analysis of the spinal cords at 7 days of survival revealed that animals that had no significant recovery of function showed a significant loss of small- and medium-sized interneurons in laminae IV–VII (Figs. 2B and E; Table 2). This was in contrast to animals with a significant recovery of function. In these animals, only minimal or no neuronal loss was seen (Fig. 2C; Table 2).

**HSP72 immunohistochemistry**

**Three minutes of spinal ischemia (Fig. 3)**

Using two different antibodies, no or only modest HSP72 immunoreactivity in ventral α-motoneurons was seen in control sham-operated animals (Figs. 3A and B). At 12 h after ischemia, a modest HSP72 staining in neurons localized in the superficial dorsal horn was detected (Fig. 3C, arrows). Small- and medium-sized interneurons as well as large α-motoneurons showed no HSP72 immunoreactiv-

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![Image of HSP72 immunohistochemistry](image-url)

Fig. 3. (A–K) Spinal HSP72 immunohistochemistry in animals exposed to 3 or 6 min of ischemia followed by 12, 24 or 72 h of reflow. (A and B) Control animals: only modest HSP72 immunoreactivity in the ventral α-motoneurons can be seen. Interneurons localized in the dorsal horn or in the intermediate zone were HSP72 negative. (C) Three minutes of ischemia and 12 h of reflow: only modest HSP72 immunoreactivity in neurons on the base of the dorsal horn can be seen (arrows). (D) Six minutes of ischemia and 12 h of reflow: a clear expression of HSP72 in dorsal horn neurons can be seen. (E, F, G) Six minutes of ischemia and 24 h reflow: a robust HSP72 induction in neurons localized in the dorsal horn but also in the central gray matter can be seen. (H) Six minutes of ischemia and 72 h of reflow: general decrease in HSP72 immunoreactivity in spinal gray matter can be seen if compared to 24 h of reflow. Continuing HSP72 expression in a subpopulation of dorsal horn neurons and in interneurons in the intermediate zone can still be seen (arrows). (I) Camera lucida drawing of HSP72-positive neurons after 6 min ischemia and 12 h of reflow (I), 24 h (II) and 72 h (III) of reflow. (J, K) Western blot analysis of HSP72 protein in L2–L5 segments after 3 or 6 min of ischemia and different periods of reflow: (J) lanes 1–3: control spinal cord; lanes 4, 5: 3 min ischemia and 24 h reflow; lanes 6, 7: 6 min ischemia and 24 h reflow; lanes 8, 9: 3 min ischemia and 12 h reflow. Note a significant increase in HSP72 expression after 6 min ischemia and 24 h of reflow (lanes 6, 7) which corresponds with a dense HSP72 immunopositivity in spinal interneurons at the same time point.
ity. Similarly, no detectable HSP72 immunostaining was seen in the neuropil. At 24 h after ischemia, HSP72-positive neurons were no longer present and the overall HSP72 staining pattern was comparable to that seen in control-sham operated animals.

**Six minutes of spinal ischemia (Figs. 3 and 4)**

At 12 h of reflow after 6 min of spinal ischemia, a clear neuronal increase in HSP72 immunoreactivity was seen. These neurons were typically localized in the dorsal horn among laminae I–V (Fig. 3D). Occasionally, HSP72-positive neurons in the intermediate zone (lamina VII) were detected. At 24 h after ischemia, the HSP72 immunoreactivity was seen in both the dorsal horn and the intermediate zone. This immunoreactivity was particularly expressed in medium-sized and small interneurons localized in the medial but also lateral part of the intermediate zone (Figs. 3E, F, G and I). Several neurons with an intense immunoreactivity in the neuronal cytoplasm, axons and dendrites were also identified (Fig. 3G). An additional group of neurons with intense HSP72 immunoreactivity in the pericentral region surrounding the central canal was also identified (Fig. 3E). At 72 h after ischemia, the intensity and the number of HSP72 immunoreactive neurons was decreased, however, intensely immunoreactive HSP72 neurons were still present in the superficial dorsal horn (laminae II and III) (Fig. 3H). These results correlate well with the rapid appearance of Hsp70 mRNA after 3 or 6 min ischemic
treatment as assessed by microarrays (Carmel et al., accompanying manuscript). Similarly, Western blot analysis revealed a significant increase in HSP72 expression in spinal parenchyma at 24 h of reflow after 6 min of ischemia.

Analysis of spinal glial fibrillary acidic protein (GFAP) showed a clear upregulation at 24 h after ischemia as determined by Western blot analysis (Fig. 4G) and immunohistochemistry. In comparison with sham-operated animals (Figs. 4A and C), the highest degree of astrocytic activation in the intermediate zone and in the superficial dorsal horn was seen (Figs. 4B and D). Using double labeling with GFAP and HSP72 antibody coupled with deconvolution microscopy, a specific HSP72 expression in neurons but not in astrocytes was revealed (Fig. 4F). Expression of iHSP70 was not seen in astrocytes (Fig. 4E).

**Group C: 10 min of spinal ischemia (Fig. 5)**

After 10 min ischemia and 12 h of reflow, an increased HSP72 immunoreactivity was seen in both dorsal and ventral horn. This immunoreactivity was expressed more at the periphery of both dorsal and ventral horns.

At 24 h, consistent with the presence of extensive necrotic changes in this experimental group, HSP72 immunopositivity showed a specific pattern. In the central gray matter (necrotic areas), a clear loss of HSP72 reactivity was seen (Figs. 5A and B, asterisk). In contrast, an intense HSP72 immunoreactivity was detected in the regions surrounding necrotic cavities (penumbral region). Only in some sections a persisting, heavily stained HSP72 immunoreactive neurons, surrounded by HSP72-negative neuropil were detected (Fig. 5C). Staining of the adjacent sections with GFAP antibody showed similar decreases in GFAP immunoreactivity in the regions of central necrosis (Fig. 5D, black circle).

**Discussion**

In the present study, using a rat aortic occlusion model, two different preconditioning ischemic intervals (3 and 6 min) were tested for their ability to induce ischemic tolerance against a subsequent injurious interval (10 min) of spinal ischemia. A second injurious interval of ischemia was induced after increasing intervals of reflow after preconditioning. The most robust protection was seen in animals that were preconditioned with 3 min of ischemia and followed

![Fig. 5. (A-D) Spinal HSP72 and GFAP immunohistochemistry in animals exposed to 10 min of ischemia followed by 24 h of reflow. (A, B) A clear lack of HSP72 immunoreactivity in the central gray matter, corresponding to the necrotic region can be seen (asterisk). In contrast, an intense HSP72 staining in the perinecrotic areas and in dorsal horn can be detected. (C) Persisting interneurons localized at the periphery or in the center of necrotic regions showed a continuing expression of HSP72 protein (arrows). (D) Loss of GFAP immunoreactivity in the central necrotic regions can be seen (black circle).](image-url)
by the second ischemia 30 min later. Gene expression analysis of both 3 and 6 min ischemic treatment identified several groups of mRNAs with putative protective function (see Carmel et al., accompanying manuscript). This protective effect was lost when the reflow interval after preconditioning was extended to 120 min and partially reappeared at 24 h after preconditioning. After 6 min of preconditioning ischemia, a significant ischemia-tolerant state at 24 h (but not at 30 or 120 min) was observed and correlated with a robust spinal neuronal expression of HSP72.

Mechanisms of ischemic tolerance: possible role of HSP

As demonstrated in the present study, 6 min of preconditioning spinal ischemia evoked an intense HSP72 protein expression in lumbosacral neurons at 24 h after preconditioning. This expression was detected in all laminar levels with the most dense expression seen in small neurons in the superficial dorsal horn and in the dorsolateral part of the gray matter. Using deconvolution microscopy, no HSP72 expression in astrocytes was detected. Animals exposed to the injurious interval of spinal ischemia during this time frame (i.e. at 24 h after 6-min preconditioning) showed a robust ischemic tolerance. In contrast to the 6 min preconditioning ischemia, the majority of HSP72 immunoreactive neurons were localized in the superficial dorsal horn at 12 h after 3-min preconditioning. Behaviorally, animals in this group displayed only partial ischemic tolerance if the second ischemia was performed at 24 h after preconditioning.

Similarly, using the rabbit spinal ischemia model, a comparable preconditioning effect of short duration non-injurious ischemia was demonstrated. In these studies, it was shown that 10–12.5 min of preconditioning spinal ischemia provided protection against 15–30 min of spinal ischemia at 12–48 h and that this ischemia-tolerant state coincides with the presence of HSP70-72 expression in spinal cord neurons (Munyao et al., 1998; Sakurai et al., 1998). Similar data were reported using a dog spinal ischemia model (Matsuyama et al., 1997).

In addition to spinal ischemia studies, comparable results after ischemic preconditioning in several cerebral ischemia models have been reported. The interval of peak HSP72 protein expression measured after preconditioning cerebral ischemia is coincidental with the time course of ischemic tolerance (Kato et al., 1991; Kawagoe et al., 1992; Nishi et al., 1993). It has also been shown that overexpression of HSP72 in neurons, induced by delivery of defective herpes simplex virus encoding hsp72 gene, is associated with a significantly better survival of striatal neurons after focal cerebral ischemia and of hippocampal, dentate gyrus neurons after systemic kainic acid administration (Yenari et al., 1998). Comparable reduction in the infarcted volume after permanent middle cerebral artery occlusion in transgenic mice overexpressing the rat HSP70 (HSP70tg mice) was reported (Rajdev et al., 2000). These data provide convergent support for a potential protective role of HSP72 in the development of ischemic tolerance.

However, in a more recent study using a rabbit spinal ischemia model and 6 min of ischemic preconditioning, a lack of ischemic tolerance was described despite a significant increase in spinal tissue HSP72 expression at 24 h after preconditioning (de Haan et al., 2000). These data suggest that, depending on the duration of preconditioning ischemia, there may be differences in the magnitude of HSP72 upregulation in specific neuronal pools and that these differences may ultimately define the robustness of the ischemia-tolerant state. Similarly, in the present study, there was a clear HSP72 mRNA upregulation after both 3- and 6-min preconditioning, however, an intense neuronal HSP72 protein expression was only seen after the 6-min preconditioning interval (see accompanying manuscript; Carmel et al.).

Interestingly, the expression of inducible HSP is not the only candidate for mediating ischemic tolerance. It has been shown that the development of ischemic tolerance is also present in the cerebral cortex after topical application of 2 M KCl and this effect is present in the cortical neurons that show no HSP72 mRNA upregulation (Kobayashi et al., 1995). In the same study, a clear correlation between regional c-fos expression and the reduction of neuronal necrosis was seen. It has been hypothesized that activation of c-fos, which encodes several transcriptional factors, can mediate upregulation of other neuroprotective genes that can potentially be involved in the observed protection in the absence of a detectable HSP expression. We have seen a similar c-fos expression after 6 min of spinal cord ischemia with the most intense and long-lasting expression seen in the interneuronal pool localized among laminae V–VII in L2–L5 spinal segments (Yang et al., 2000). Microarrays also identify several groups of potentially protective genes, including metallothioneins, specific transcription factors, and other immediate-early genes (Carmel et al., accompanying manuscript). More recently, we have found that a 6-min preconditioning ischemia is also associated with a biphasic spinal parenchymal BDNF and GDNF upregulation as determined by ELISA measurement and immunohistochemistry (Tokumine et al., 2003). A significant upregulation of BDNF and GDNF was seen at 2 and 48 h after preconditioning. These data suggest a possible role of these growth factors in neuronal protection. Accordingly, it has been reported that intracerebroventricular infusion of BDNF or GDNF provides a significant neuroprotection after global cerebral ischemia or after middle cerebral occlusion in the rat (Kiprianova et al., 1999; Wang et al., 1997).

Similarly, using a spinal ischemia model in rabbit, adeno viral-mediated GDNF gene delivery has been demonstrated to provide a significant reduction in α-motoneuronal degeneration and this effect was associated with an increased neuronal GDNF expression (Sakurai et al., 2000).
Loss of HSP expression after injurious interval of spinal ischemia

After an injurious interval of spinal ischemia without preconditioning, a clear loss of HSP72 and GFAP immunoreactivity was detected in the areas of gray matter necrosis. In contrast, an intense HSP72 and GFAP expression was seen in the penumbral region.

Similarly, as seen in the present study, a comparable expression pattern of HSP72 protein was reported in global and focal cerebral ischemia models in the rat (for review, see Kinouchi et al., 1993; Sharp et al., 1993). These systematic studies demonstrate that the induction of HSP70 protein in specific hippocampal regions correlates with cellular vulnerability to ischemia after increasing intervals of global ischemia. CA1 neurons, which are very vulnerable to ischemia, express HSP70 after the briefest periods of ischemia but this expression is lost after more severe ischemia. After severe ischemia, HSP70 expression is present only in hippocampal capillary endothelial cells and not in any infarcted elements including neurons in CA1, CA3, CA4, dentate granule neurons or in glia.

Focal ischemia that leads to the development of infarcts induces HSP70 in endothelial cells of cerebral blood vessels in the regions of infarction and in neurons (but also in astrocytes) in the penumbral region. This neuronal HSP72 expression pattern appears to be similar to that seen after 10 min of spinal ischemia and leads to the development of central necrosis and increased penumbral HSP72 expression.

Stimulus for ischemic HSP72 induction

Receptor-coupled induction

During the period of transient spinal ischemia, there is a significant increase in the extracellular concentration of several excitatory amino acids (EAA) including glutamate and aspartate (Marsala et al., 1994; Simpson et al., 1990). Increased levels of these excitatory neurotransmitters activate several classes of glutamate receptors (NMDA, non-NMDA). Activation of glutamate receptors has been shown to be a potent stimulus for the induction of immediate early and late genes in the brain and spinal cord. Exposure of granule cerebellar cells to NMDA evokes transient HSP72 expression. This expression is blocked by NMDA receptor antagonists and corresponds with the protection of the cell to subsequent neurotoxic concentrations of EAAs (Marini and Paul, 1992). Systemic administration of AMAA [(+/-)-2-amino-3-hydroxy-5-methyl-4-isoxazoleacetic acid: a potent systemically active NMDA receptor analog] at doses with no excitotoxic effect, induces HSP72-mRNA between 2.5 and 5 h after administration in CA1 pyramidal cells, with HSP72 proteins present at 24 h after injection (Planas et al., 1995). This enhanced expression was blocked by MK-801. In accord with these data, we found that intrathecal delivery of NMDA induces spinal interneuronal and α-motoneuronal HSP72 protein expression, with the maximum effect observed at 24 h after injection (Sasara et al., 2004).

Lack of neuronal HSP72 expression after simple neuronal depolarization

In our previous study, we showed that in the rat ischemic model, used in the present study, the onset of anoxic depolarization is measured at about 2 min after induction of spinal ischemia (Sasara et al., 2004). However, as demonstrated in the present study, even 3 min of preconditioning ischemia induces only minimal neuronal HSP72 expression, suggesting that a brief depolarization of spinal neurons per se is not sufficient stimulus for HSP72 expression. In accord with this observation, we have also shown that intrathecal injection of depolarizing concentrations of KCl will lead to HSP72 expression in spinal glial cells only with no expression in spinal neurons (Sasara et al., 2004). Similarly, a lack of HSP72 upregulation after topical application of 2 mM KCl on cerebral cortex has been reported (Kobayashi et al., 1995). In addition, systematic studies in brain showed that after flurothyl-induced status epilepticus, there is a duration- and degree-dependent induction of HSP72 in neurons localized in several brain regions, including frontoparietal and visual cortex, dentate hilus cells, CA1 and CA3 pyramidal neurons, and certain thalamic and amygdaloid nuclei. In the same study, it was also shown that region-specific expression of HSP72 correlates in part with the brain regions which displayed seizure-induced injury (Lowenstein et al., 1990). Jointly, these data suggest that the potency, frequency as well as the duration of applied stimulus is the key determinant in neuronal HSP72 induction and expression and that a brief physiological stimulus even if sufficient to induce transient neuronal depolarization will not be effective in inducing neuronal HSP72 expression.

Development of acute ischemic tolerance

Interestingly, in the present study, the most robust protection was seen after 3 min ischemic preconditioning followed by 30 min reflow. This result is similar to that published by Zvara et al. (1999), using the same ischemic model as used in the current study. However, at present, the mechanism of this protection is not clear. We speculate that it may result in part from downregulation of spinal NMDA receptors during the acute post-reflow phase after preconditioning ischemia. It has been reported that after traumatic spinal injury, there is a significant decrease in spinal [3H]MK-801 binding at 4 h after injury followed by its normalization at 24 h (Sun and Faden, 1994). This downregulation was attributed to a significant glutamate release measured at the site of traumatic injury (Liu et al., 1991; Marsala et al., 1995). Similarly, a significant increase in spinal parenchymal glutamate release was described in rabbit and rat spinal ischemia models, suggesting a similar mechanism in the NMDA receptor downregulation after preconditioning ischemia (Marsala et al., 1994; Simpson et al., 1990).
An additional mechanism may include local release of adenosine and spinal activation of adenosine A1 receptors during the immediate reflow period after preconditioning. In a recent study, we have found that intrathecal pretreatment with an adenosine A1 antagonist (DPCPX) was effective in partially blocking the development of acute ischemic tolerance, while treatment with an adenosine A1 agonist (2-chloroadenosine) provided partial protection (Yamamoto et al., 1998). Similar neuroprotective effects of adenosine and resulting activation of A1 receptor in vivo cerebral ischemia models or in vitro hippocampal slices were previously reported (Heurteaux et al., 1995; Nakamura et al., 2002; Perez-Pinzon et al., 1996). It has also been demonstrated that activation of K (ATP) channels is required for adenosine-mediated neuroprotection (Reshef et al., 2000).

Finally, a parallel study of spinal cord ischemia has identified several novel genes, including B-cell translocation gene 2 (BTG2), early growth response 1 (egr-1), nerve growth factor inducible B (NGFI-B), mitogen-activated protein kinase phosphatase (ptpn16), as well as Hsp70 and the metallothioneins (Carmel et al., accompanying manuscript). These candidates represent additional mechanisms that may contribute to the mediation of acute and/or delayed ischemic tolerance.

Conclusion and clinical relevance of spinal ischemic tolerance

In the present study, we demonstrated that short-lasting noninjurious intervals of spinal ischemia induced a significant ischemic tolerance against a second injurious interval of aortic occlusion. The time frame of the ischemia-tolerant state depended on the duration of preconditioning ischemia. While 3 min of ischemic preconditioning provided a significant protection at 30 min after reflow, 6 min of pre-conditioning ischemia was only associated with a significant protection at 24 h after preconditioning. This protection correlated with spinal neuronal expression of HSP72. Although several other factors likely contribute to the development of ischemic tolerance, the ischemia-induced depolarization, excessive activation of excitatory amino acid receptors and/or voltage-dependent neuronal depolarization appear to represent a common denominator in this process. Identification of the potency of the individual manipulations in their ability to induce ischemic tolerance will represent the key element in a successful use of these procedures to provide spinal protection in patients undergoing aortic cross-clamping.

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