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

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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 non-injurious (3 or 6 min) or injurious intervals (10 min) of spinal ischemia followed by 4– 72 hrs 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 hrs 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 hrs 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 hrs 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 hrs 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 hrs 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 hrs after preconditioning may represent an important variable in modulating ischemic tolerance observed during this time frame.

Introduction

Transient spinal cord ischemia and subsequent loss of neurological function (spastic or flaccid paraplegia) represents 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 12-40% in patients with extensive thoracoabdominal aortic aneurysm repair (36, 49). 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 (10, 22, 26, 49, 50, 59).

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 vasodilatatory treatment, and iii) to decrease reflow-mediated neuronal degeneration (anti-oxidant therapy) (3, 40, 48, 60). More recently, a phenomenon of induced ischemic tolerance i.e. 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).

Heat shock protein (HSP) is a member of a family of molecules called chaperonins. These molecules are highly conserved across a number of eukaryotic cell lines (7). Inducible forms of HSP (i.e. HSP72) were first identified in Drosophila as a covariant of the ability of the insect to withstand an otherwise lethal environmental temperature after a brief conditioning exposure to a just sublethal temperature (4, 52). Although the mechanism of this protection is not completely clear, it has been shown that HSP molecules stabilize protein conformations in the face of cell exposure to extreme conditions, including hyperthermia and ischemia (28).

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 (14, 45). More importantly, exposure of brain tissue to even short lasting, non-injurious, conditioning ischemia is effective in inducing HSP72 and these populations of cells then display an increased resistance to a subsequent injurious interval of ischemia i.e. induction of ischemic tolerance (12, 33). 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 (11). Comparable

development of ischemic tolerance in a rat, rabbit and dog spinal ischemia model has been described (2, 27, 43). 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 (16, 61).

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 minutes have no significant effect and all animals display normal motor function and no detectable histopathological changes in the spinal cord (50). Using the rabbit and dog spinal ischemia model we have observed comparable selective interneuronal degeneration and corresponding loss of motor function (21, 25). 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/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 when determined by correlated 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.

1) 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 (50). Animals were anesthetized with 3% halothane in an O_2/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 polytetrafluororthylene catheter for blood withdrawal. To induce spinal cord ischemia, the intra-aortic balloon catheter was inflated with 0.05mL 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 sec. 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.

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

3) Induction of preconditioning ischemia and recovery of function: Halothane anesthetized rats were

exposed to 3 min or 6 min of aortic occlusion (preconditioning ischemia) as described. At 30 min, 2 hrs or at 24 hrs 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).

4) Perfusion fixation and HSP72 and GFAP immunohistochemistry: At the end of the survival periods, rats were anesthetized with pentobarbital (40 mg/kg; i.p.) and phenytoin (25 mg/kg, i.p.) and transcardially perfused with saline for 1-2 min followed by 4% paraformaldehyde in 0.1M 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 (10-25µm) 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 (25µm) were placed in PBS, 0.1M (pH=7.4) containing 5% horse serum (HS), 0.2% Triton X100, and 0.2% bovine serum albumin (BSA) for 3 hrs at room temperature to block the non-specific protein activity. This was followed by over night 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) was 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 3 times, then incubated in biotinylated horse anti mouse secondary antibody 1:200 for 2 hrs. Bound specific antibodies reacted with the avidine-biotin peroxidase solution for 1h and were visualized by using 0.05% 3,3,4,4-diaminobenzidine hydrochloride (DAB) in 0.05 M Tris buffer containing 0.001 % H_2O_2.

In double labeling experiments an immunofluorescence technique was used. 10 µm sections were cut and collected on Fischer Plus slides. Slides were then placed into a 100% humidified chamber for 5

minutes 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; 4µl/ml; Molecular Probes) were used. All blocking and antibody preparations were made in 1xPBS/0.2%TX/5% goat serum. Following 3 hrs block sections were incubated in primary antibody for 72 hrs at 4°C followed by PBS/TX wash (3x5min) and incubation with secondary antibody for 1 hr 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 (3µl/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 x 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 Photometrics CCD mounted on a Nikon microscope. In general 75 optical sections spaced by 0.1 or 0.2 µm were taken. Lenses used were 20x and 40x (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 (60µm thick) were sequentially cut and individually collected from each L2-L5 segment. Sections were stained with NeuN (mouse monoclonal, 1:1000, Chemicon, CA) 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 (29). Computer based quantification of α-motoneurons and interneurons was performed using Image Pro 4.5 software (Media Cybernetics).

5) Western Blot analysis: Spinal cord samples were homogenized with a tissue sonicator for 5 minutes. The supernatant was collected after centrifugation (12,000 x 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.

6) Statistics: Statistical analysis of physiological data was carried out by One-way ANOVA for multiple comparisons followed by Dunnett post hoc test. For the analysis of neurological outcome and spinal histopathology non-parametric tests were used. For individual studies (Group A1-3, B 1-3, C 1-3) 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 effect on recovery at 1 and 4 hrs and 1-7 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 hrs after ischemia 7 of 8 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 hrs of reflow no significant recovery of function was detected.

In animals preconditioned with 3 min ischemia and exposed to a second injurious interval of ischemia at 24 hrs 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 hrs after preconditioning no significant recovery of function was detected at any time point. If the exposure to the injurious ischemia was delayed to 24 hrs the majority of animals displayed a gradual and significant recovery of motor function, with the most pronounced effect seen at 7 days after injury.

INSERT FIGURE 1 ABOUT HERE

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 (Fig.2 B, 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.2 C; Table 2).

INSERT FIGURE 2 A - E ABOUT HERE

HSP72 immunohistochemistry:

(Group C: 3 min of spinal ischemia) (Fig.3):

Using 2 different antibodies, no or only modest HSP72 immunoreactivity in ventral α-motoneurons was seen in control sham-operated animals (Fig.3 A, B). At 12 hrs after ischemia a modest HSP72 staining in neurons localized in the superficial dorsal horn was detected (Fig.3 C; arrows). Small and medium-sized interneurons as well as large α-motoneurons showed no HSP72 immunoreactivity. Similarly no detectable HSP72 immunostaining was seen in the neuropil. At 24 hrs 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.

INSERT FIGURE 3 A - K ABOUT HERE

(Group D: 6 min of spinal ischemia) (Fig.3, 4):

At 12 hrs 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 between lamina I-V (Fig.3 D). Occasionally, HSP72 positive neurons in the intermediate zone (lamina VII) were detected. At 24 hrs 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 (Fig.3 E, F, G, I). Several neurons with an intense immunoreactivity in the neuronal cytoplasm, axons and dendrites were also identified (Fig.3 G). An additional group of neurons with intense HSP72 immunoreactivity in the pericentral region surrounding the central canal was also identified (Fig. 3 E). At 72 hrs 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-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 hrs of reflow after 6 min of ischemia.

Analysis of spinal glial fibrilary acidotic protein (GFAP) showed a clear upregulation at 24 hrs after ischemia as determined by westren blot analysis (Fig.4 G) and immunohistochemistry. In comparison with sham operated animals (Fig.4 A, C) the highest degree of astrocytic activation in the intermediate zone and

in the superficial dorsal horn was seen (Fig.4 B, D). Using double labeling with GFAP and HSP72 antibody coupled with diconvolution microscopy a specific HSP72 expression in neurons but not in astrocytes was revealed (Fig.4 F). Expression of iHSP70 was not seen in astrocytes (Fig.4 E).

INSERT FIGURE 4 A - G ABOUT HERE

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

After 10 min ischemia and 12 hrs 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 hrs, 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 (Fig.5 A, 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. 5 C). Staining of the adjacent sections with GFAP antibody showed similar decreases in GFAP immunoreactivity in the regions of central necrosis (Fig.5 D; black circle).

INSERT FIGURE 5 A - D ABOUT HERE

DISCUSSION

In the present study, using a rat aortic occlusion model, two different preconditioning ischemic intervals (3 and 6 minutes) 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 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 hrs after preconditioning. After 6 min of preconditioning ischemia a significant ischemia-tolerant state at 24 hrs (but not at 30 min 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 hrs 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 hrs 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 hrs after 3 min preconditioning. Behaviorally animals in this group displayed only partial ischemic tolerance if the second ischemia was performed at 24 hrs 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 a 10-12.5 min of preconditioning spinal ischemia provided protection against 15-30 min of spinal ischemia at 12-48 hrs and that this ischemia-tolerant state coincides with the presence of HSP70-72 expression in spinal cord neurons (30, 42). Similar data were reported using a dog spinal ischemia model (27).

In addition to spinal ischemia studies, comparable results after ischemic preconditioning in several cerebral ischemia models were reported. The interval of peak HSP72 protein expression measured after preconditioning cerebral ischemia is coincidental with the time course of ischemic tolerance (12, 13, 33). Importantly, manipulations which lead to suppression or blockade of HSP72 expression after application of

preconditioning ischemia were effective in blocking the development of an ischemia-tolerant state. Continuous infusion of anti-HSP72 antibody into the lateral ventricle suppressed the protective effect evoked by ischemic preconditioning, as measured by the density of surviving CA1 cells after an injurious ischemic exposure (32). Similar results were observed with quercetin. Though this agent has a number of actions (including the ability to inhibit nitric oxide synthase), (6) it can also inhibit HSP72 expression (54). Jointly these data provide support for a possible role of HSP expression in ischemic tolerance observed after 6 min preconditioning and 24 hrs of reflow.

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 hrs after preconditioning (8). 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 min and 6 min preconditioning, however, an intense neuronal HSP72 protein expression was only seen after the 6 min peconditioning 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 2M KCL and this effect is present in the cortical neurons that show no HSP72 mRNA upregulation (16). 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 which 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 between laminae V-VII in L2-L5 spinal segments (58). 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 (51). A significant upregulation of BDNF and GDNF was seen at 2 hrs and at 48 hrs after preconditioning. These data suggest a possible role of these growth factors in neuronal protection. Accordingly, it has been reported that intracerebro-ventricular infusion of BDNF or GDNF provides a significant neuroprotection after global cerebral ischemia or after middle cerebral occlusion in the rat (15, 56).

Similarly, using a spinal ischemia model in rabbit, adenoviral-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 (41).

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.

In contrast to our present data, not completely uniform loss of HSP72 expression was described in cerebral ischemia models after injurious intervals of ischemia (17). In a rat global cerebral ischemia model, the expression of HSP72 (and HSP72 mRNA) was present not only in the regions which were resistant to ischemia but also in areas which showed delayed degeneration (CA1 pyramidal cells) (14, 45, 53). In contrast, in the gerbil global cerebral ischemia model, much lower immunoreactivity for HSP72 was seen in CA1 neurons following 10 min ischemia (55). In another study, using the same cerebral ischemia model in gerbils, a persistent HSP72 mRNA upregulation was described during an extended period of reflow. This indicates that the translation block can account for the loss of protein immunoreactivity in this model (1, 34). In accord with these data, a comparable suppression of protein synthesis was reported in spinal gray matter after an injurious interval of spinal ischemia in rabbits (5). This may account for the loss of HSP72 reactivity in this region seen in the present study.

Regarding GFAP induction, a significant GFAP upregulation in astrocytes, localized in the penumbral region, was demonstrated after middle cerebral artery occlusion in rats (18). It has also been demonstrated that these astrocytes were HSP72 negative, contrasting to a high neuronal HSP72 immunoreactivity seen in the peri-necrotic regions. Similar results were noted in the present study when no colocalization of HSP72 with GFAP staining was detected.

Stimulus for ischemic HSP72 induction

Several stimuli may trigger neuronal expression in the course of preconditioning ischemia and reperfusion:

i) 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 (24, 46). 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 (20). Systemic administration of AMAA ((+/-)-2-amino-3-hydroxy-5-methyl-4-isoxazoleacetic acid: a potent systematically active NMDA receptor analog) at doses with no excitotoxic effect, induces HSP72-mRNA between 2.5-5 hr after administration in CA1 pyramidal cells, with HSP72 proteins present at 24 hours after injection (37). This enhanced expression was blocked by MK-801. In accord with these data, we found that intrathecal delivery of NMDA induces spinal inter-neuronal and α-motoneuronal HSP72 protein expression, with the maximum effect observed at 24 hr after injection (44).

ii) Simple neuronal depolarization: Because NMDA receptor occupancy is associated with neuronal depolarization, general membrane depolarization may be an adequate stimulus for HSP-induction. Topical application of 2M potassium solution on the cortical surface evoked c-FOS mRNA and HSP72 mRNA induction at the site of spreading depolarization after 24 hrs (16). However, negative results with systemic pentyleneterazole (GABA-A receptor antagonist) at seizure-inducing doses suggest that all cellular excitation does not lead to HSP induction (38).

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., using the same ischemic model as used in the current study (61). 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 postreflow 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 hrs after injury followed by its normalization at 24 hrs (47). This downregulation was attributed to a significant glutamate release measured at the site of traumatic injury (19, 23). 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 (24, 46).

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 (57). Similar neuroprotective effects of adenosine and resulting activation of A1 receptor in *in vivo* cerebral ischemia models or in *in vitro* hippocampal slices were previously reported (9, 31, 35). It has also been demonstrated that activation of K (ATP) channels is required for adenosine-mediated neuroprotection (39).

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 non-injurious 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 preconditioning ischemia was only associated with a significant protection at 24 hrs 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.

Acknowledgments

This work was supported by NIH-NS32794 (M.M) and by the Slovak Academy of Sciences SAS 2/1064/20 (D.C). We would like to thank Tony L.Yaksh, PhD for his editorial assistance. JBC was a Howard Hughes Medical Student Fellow during the initiation of this project. RPH is supported by grants from the New Jersey Commission on Spinal Cord Research and the Spinal Cord Project of the W.M. Keck Center for Collaborative Neuroscience.

Figure Legends

Fig.1

Motor deficit index assessed at 1hr – 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. 30 min, 120 min or at 24 hrs). The most pronounced neuroprotection in animals after 3 min preconditioning and 30 min of reflow as well as in animal after 6 min of preconditioning and 24 hrs of reflow can be seen (M.D.I. = 6 - complete paraplegia; 0 – complete recovery).

Fig. 2 A - E

Light microphotograph of the transverse (30 μm) sections taken from L4 spinal segment of sham-operated animal (A), an animal without preconditioning ischemia and exposed to 10 min ischemia (B) and animal after 6 min of preconditioning ischemia followed by 10 min of ischemia at 24 hrs after preconditioning. Preconditioned animals survived for 7 days after exposure to second ischemia. A significant loss of spinal interneurons in the intermediate zone in an animal without preconditioning (B) can be seen (compare co control – A). In contrast number of normally appearing interneurons in the same laminar region in an animal after preconditioning can be detected (C). Systematic quantification of the neuronal loss between L2 – L6 segments revealed a positive correlation between number of persisting neurons and recovery of motor function (E).

Fig. 3 A - K

Spinal HSP72 immunohistochemistry in animals exposed to 3 or 6 min of ischemia followed by 12 hrs, 24 hrs or 72 hrs of reflow. **A, B** – control animals: only modest HSP72 immunoreactivity in the ventral A-motoneurons can be seen. Interneurons localized in the dorsal horn or in the intermediate zone were HSP72 negative. **C** – 3 min ischemia and 12 hrs of reflow : only modest HSP72 immunoreactivity in neurons on the base of the dorsal horn can be seen (arrows). **D** – 6 min ischemia and 12 hrs of reflow : a clear expression of HSP72 in dorsal horn neurons can be seen. **E , F, G** – 6 min ischemia and 24 hrs reflow : a robust HSP72 induction in neurons localized in the dorsal horn but also in the central gray matter can be seen. **H** – 6 min ischemia and 72 hrs of reflow: general decrease in HSP72 immunoreactivity in spinal gray matter can be seen if compared to 24 hrs of reflow. Continuing HSP72 expression in a sub-population 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 hrs (I), 24 hrs (II) and 72 hrs (III) of

reflow.

J, K – western blot analysis of HSP72 protein in L2-L5 segments after 3 min 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 hrs reflow; **lanes 6, 7** - 6 min ischemia and 24 hrs reflow; **lanes 8, 9** – 3 min ischemia and 12 hrs reflow. Note a significant increase in HSP72 expression after 6 min ischemia and 24 hrs of reflow (lanes 6, 7) which corresponds with a dense HSP72 immunopositivity in spinal interneurons at the same time point.

Fig. 4 A - G

Spinal GFAP and HSP72 immunohistochemistry in animals exposed to 6 min of ischemia followed by 24 hrs of reflow. **B, D** – 6 min ischemia and 24 hrs of reflow: in comparison to control (A, C) an increased GFAP immunoreactivity in reactive astrocytes was detected. **E, F**- confocal analysis of double stained (GFAP-red; HSP72 – green) spinal cord sections revealed lack of HSP72 expression in reactive astrocytes **G** – Western blot analysis of GFAP protein in L2-L5 segments in control animals and in animals after 6 min of ischemia and 24 hrs of reflow. A significant increase in GFAP expression in animals after 6 min ischemia and 24 hrs of reflow was measured.

Fig. 5 A - D

Spinal HSP72 and GFAP immunohistochemistry in animals exposed to 10 min of ischemia followed by 24 hrs 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 peri-necrotic 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).

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Table 1: Experimental groups and design

Experimental Groups	Analysis
Group A1 (n=6): 3 min isch → 30 min reflow -> 10 min ischemia	MDI
Group A2 (n=6): 3 min isch → 2 hrs reflow -> 10 min ischemia	MDI
Group A3 (n=6): 3 min isch → 24 hrs reflow -> 10 min ischemia	MDI
Group B1 (n=6): 6 min isch → 30 min reflow -> 10 min ischemia	MDI
Group B2 (n=6): 6 min isch → 2 hrs reflow -> 10 min ischemia	MDI
Group B3 (n=6): 6 min isch → 24 hrs reflow -> 10 min ischemia	MDI
Group C1 (n=6): 3 min ischemia and 4 hrs survival	IHCH
Group C2 (n=6): 3 min ischemia and 12 hrs survival	IHCH/WB
Group C3 (n=3): 3 min ischemia and 24 hrs survival	IHCH/WB
Group D1 (n=6): 6 min ischemia and 4-hrs survival	IHCH
Group D2 (n=6): 6 min ischemia and 12 hrs survival	IHCH
Group D3 (n=6): 6 min ischemia and 24 hrs survival	IHCH/WB
Group D4 (n=5) 6 min ischemia and 72 hrs survival	IHCH
Group E1 (n=6): 10 min ischemia and 30 min survival	IHCH
Group E2 (n=6): 10 min ischemia and 12 hrs survival	IHCH
Group E3 (n=6): 10 min ischemia and 24 hrs survival	IHCH

MDI – motor deficit index; IHCH - immunohistochemistry; WB - Western Blot;

Table 2

Interneurons (LIV-VII)			
Segment	Control (n=3)	Spastic (*)	Preconditioning (*)
L2	352 (±25)	334 (±12)	348 (±10)
L3	450 (±15)	315 (±20)	436(±27)
L4	522 (±30)	310 (±25)	480(±14)
L5	555 (±27)	522 (±22)	549(±13)
L6	510 (±15)	490 (±9)	540(±20)

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

Motor Deficit Index.

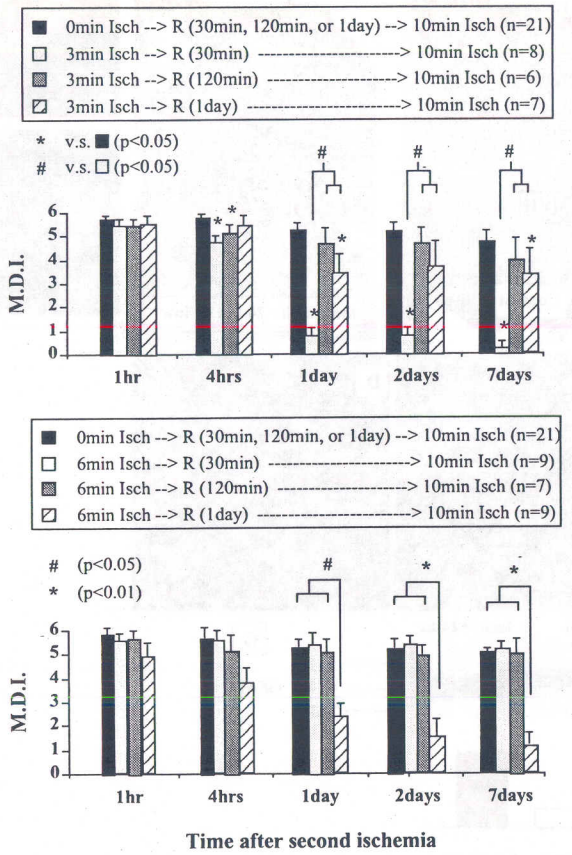

Fig.1

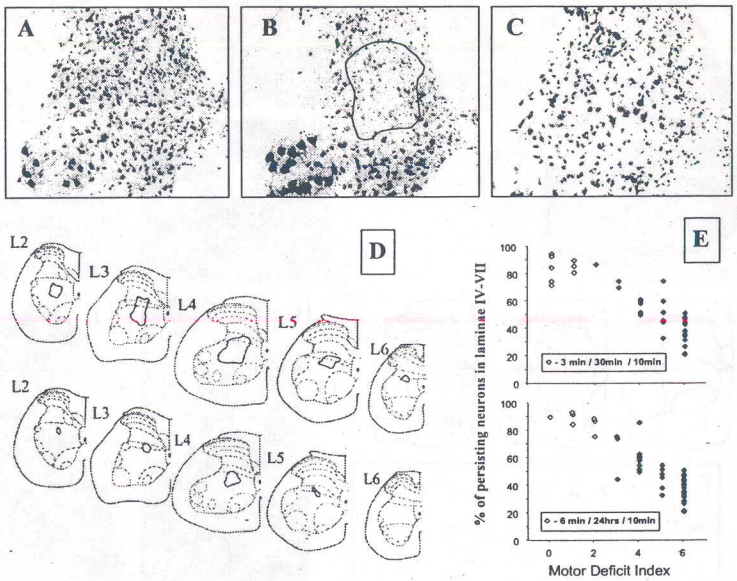

Fig. 2 A - E

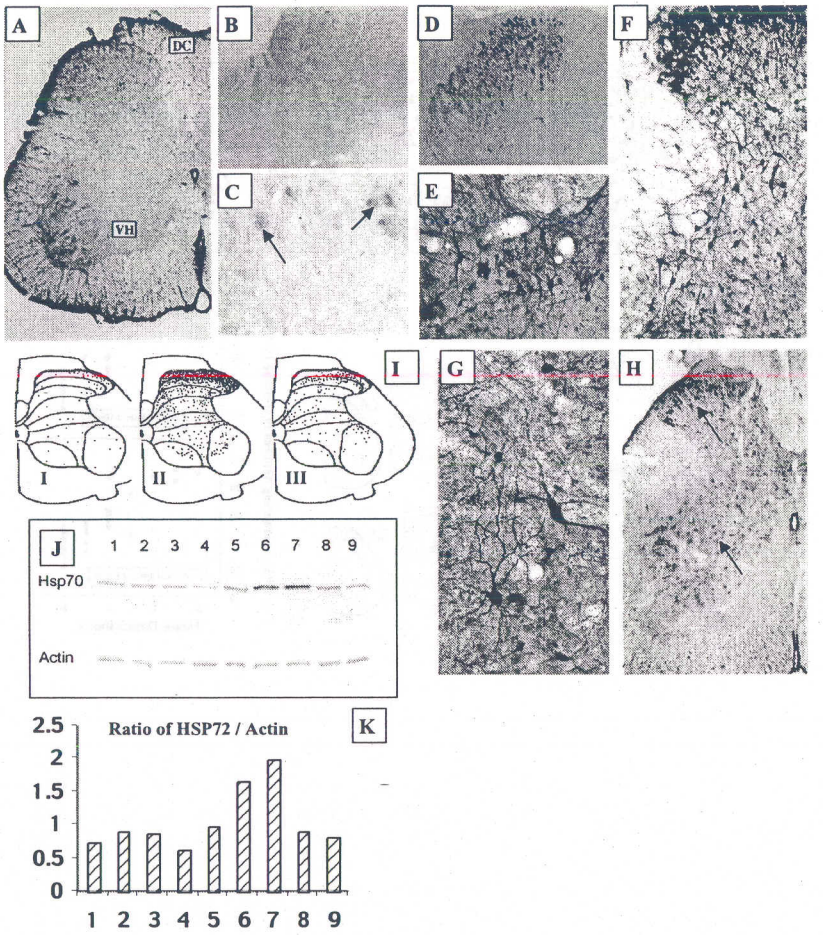

Fig. 3 A - K

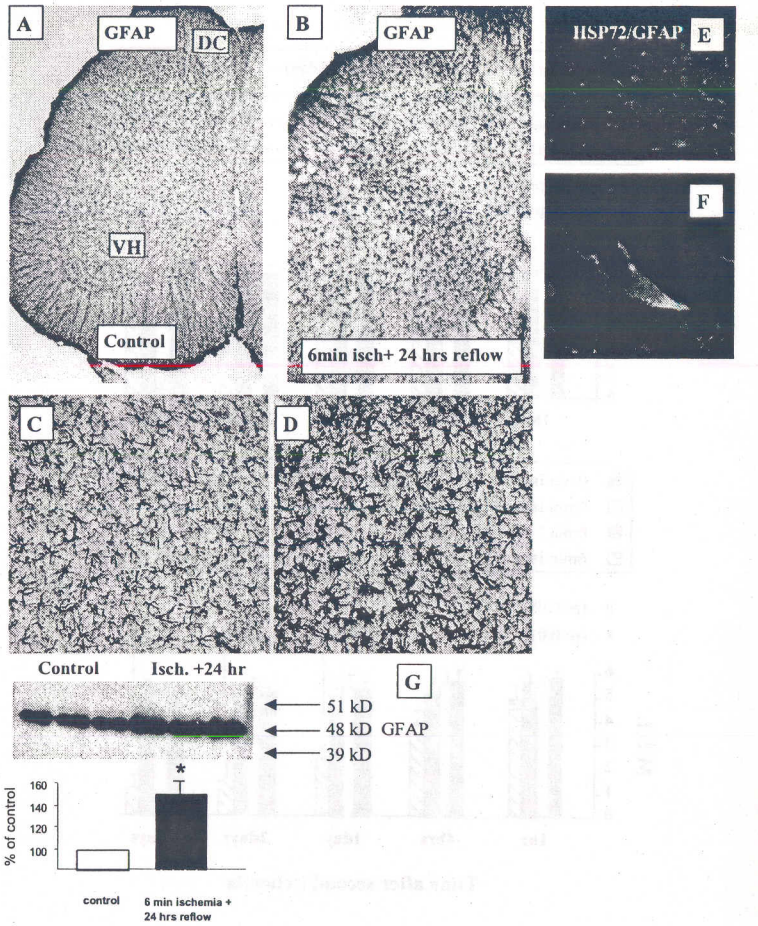

Fig.4 A - G

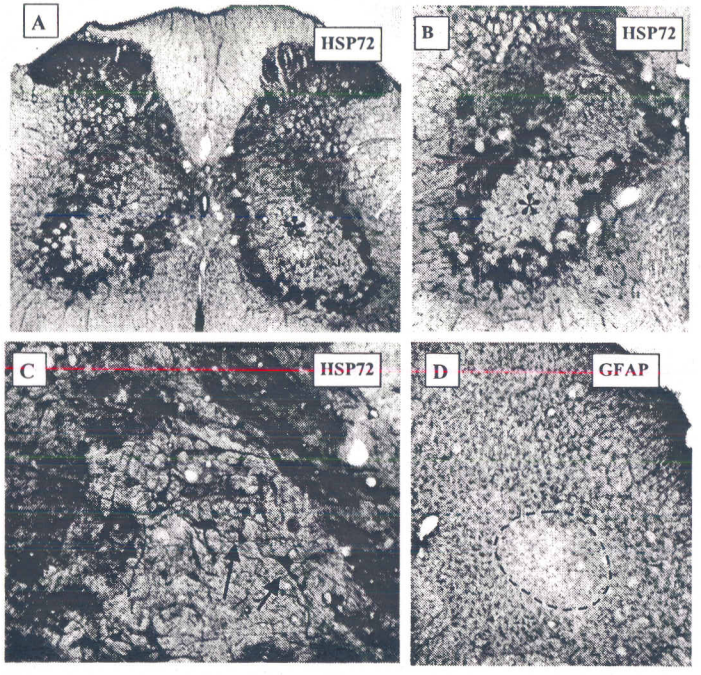

Fig. 5 A-D