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NUCLEAR FACTOR ERYTHROID 2-RELATED FACTOR 2 AND CHOLINE ACETYLTRANSFERASE CO-EXPRESSION IN RAT SPINAL CORD NEURONS AFTER ISCHEMIA-REPERFUSION INJURY

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Spinal cord ischemia-reperfusion injury (IRI) results in overproduction of reactive oxygen species leading to tissue oxidative stress which impacts the neuronal network in the spinal cord as well as glial cells. We investigated the expression of Nuclear factor erythroid 2-related factor 2 (Nrf2) in neurons and glial cells after occlusion of the abdominal aorta followed by IRI as well as the time-dependent expression of Nrf2 in the same cells. The experimental method of transient aortic occlusion was carried out on rats by cross-clamping of the abdominal aorta for 45 minutes. The animals used for this study were sacrificed 1 h, 6 h, and 48 h after reperfusion to determine time-related changes of Nrf2 expression, as well as changes of astrocyte activity in the spinal cord. Immunofluorescence results showed an increase in the staining intensity of Nrf2 expression in the neurons following ischemia with highest intensity 48 h post-reperfusion and an increase in a number of reactive astrocytes. Western blot analysis showed that Nrf2 protein expression increased in a cytoplasmic and nuclear fraction as early as 1 h after reperfusion and remained active 48 h after, resulting in increased expression of the main Nrf2 target gene HO-1. In conclusion, substances that enhance expression of Nrf2 may have the potential to prevent cellular damage to the spinal cord caused by IRI.

The spinal cord is exceptionally vulnerable to ischemic injury caused by a variety of abnormalities, such as thoracoabdominal aortic aneurysm, atherosclerosis, embolism, cardiac arrest and iatrogenic causes such as surgery (1). Aortic cross-clamping during thoracoabdominal aortic aneurysm surgery additionally causes spinal cord ischemia followed by reperfusion which finally leads to ischemia-reperfusion injury (IRI) of the spinal cord tissue. IRI impacts neurons and glial cells in the spinal cord causing reversible or even irreversible cell damage (2). Transient ischemia in the spinal cord, lasting more than 30 min, is manifested as alteration or loss of motor function of the lower extremity. Such severe and unpredictable neurological complications include diverse neurological disorders, such as paraparesis or even paraplegia (3).

Although the exact pathophysiological mechanism that underlies spinal cord ischemia-reperfusion injury

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is not fully understood, it seems that oxidative stress and inflammatory response play a significant role in the progression of tissue damage. Spinal cord ischemia followed by reperfusion results in a sudden burst of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that aggravate tissue injury. Overproduction of these compounds overcomes endogenous anti-oxidant defense in a state of oxidative stress, causing oxidative damage to lipids, proteins, and DNA, and thus disruption of standard cell functions (4, 5). Oxidative stress is accompanied with an inflammatory response characterized by infiltration of inflammatory cells (macrophages, lymphocytes, neutrophils) to the injured tissue, which produces more ROS and RNS (6, 7).

To maintain redox balance, synthesis of antioxidant enzymes is increased. This is under control of Keap1/Nrf2/ARE signaling pathway, and heme oxygenase 1 (HO-1) is one of the most studied downstream proteins activated by it. The three cellular components involved in this pathway at the transcriptional level are Kelch-like ECH-associated protein 1 (Keap1), nuclear factor erythroid 2-related factor 2 (Nrf2), and antioxidant response elements (ARE). Research indicates that the Keap1/Nrf2/ARE signaling pathway is responsible for "turning on" the adaptive response against oxidative stress (8). Recent studies have shown that the Nrf2 pathway plays an essential protective role in a state of IRI of the brain, but few data are published on Nrf2 and spinal cord IRI (9-11). Furthermore, the expression of Nrf2 in different cells of the spinal cord, as well as time-related expression of Nrf2 in acute happening (1 h post-ischemia reperfusion) and up to 48 h of reperfusion, are not well known.

This study aims to determine the localization and expression of Nrf2 in neurons and glial cells after occlusion of the abdominal aorta followed by IRI, as well as the time-dependent expression of Nrf2 in the same cells.

MATERIALS AND METHODS

Animals

Thirty-two male Wistar rats (10–12 weeks old) were used in accordance with the protocols approved by the Ethics Committee of the Faculty of Medicine, University of Rijeka. The rats were housed two per cage in controlled conditions of temperature and humidity, exposed to a 12-h light/dark cycle with free access to food and water. During procedures, all efforts were made to minimize the suffering of the animals.

Spinal cord ischemia-reperfusion injury procedure and study design

The abdominal aorta was cross-clamped for 45 min to produce transient spinal cord ischemia in lumbar segments. Animals were anesthetized with *ip* injections of ketamine hydrochloride (0.1 mg/g body weight) and xylazine hydrochloride (0.02 mg/g body weight). The *ip* injection of cephalosporin (10 mg/kg) was administered before skin incision. Postoperative analgesia was provided with tramadol for 12 h; 150 U/kg of heparin was administered *sc* to all animals immediately before the procedure (the detailed procedure is described in our previous work (12).

Rats were randomly divided into two groups: (1) ischemia-reperfusion injury (IRI) group and (2) control (sham) group. The IRI group included 24 rats which were divided into three subgroups based on time post-reperfusion as follows: 1 h, 6 h, and 48 h. Each subgroup consisted of 8 animals. The sham group included eight rats subjected to anesthesia and laparotomy without ischemia and served as control.

At 1 h, 6 h or 48 h post-reperfusion animals were anesthetized, perfused transcardially with 0.9% saline and 4% paraformaldehyde (PFA) in 0.1 M phosphate-buffered saline (pH 7.4), and sacrificed. The spinal cord was extracted whole, and lumbar regions were fixed in 4% PFA for 24 h followed by embedding in paraffin wax for immunofluorescence.

Immunofluorescence microscopy

Immunofluorescence staining was carried out on 3-μm thick formalin-fixed, paraffin-embedded spinal cord tissue sections. Following deparaffinization, the antigen retrieval was performed using citric acid (pH 6.6) for 15 min in water bath at 95°C. Following, the sections were rinsed with fluent water for 15 min. Non-specific binding sites were blocked using 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). In the subsequent step, the sections were incubated with the primary antibodies shown in Table I. Incubation with suitable secondary antibodies followed, Alexa Fluor 488 goat anti-rabbit IgG (1:200, Invitrogen) and

Alexa Fluor 488 goat anti-mouse IgG (1:200, Invitrogen) were applied for 1 h at RT. The sections were washed using PBS 3 ×5 min after each step, and were then cover- slipped in fluorescence mounting media,

Microscopy and image analysis

Sections were observed under Olympus microscope BX51/BX52 (Tokyo, Japan) equipped with fluorescence and images captured using an Olympus camera. Images of the spinal cord ventral gray matter were analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA). From each animal, every fifth consecutive serial section was studied to avoid the risk of double quantification of the same cell in adjacent sections. In every animal, five sections of lumbar spine region were studied. The mean density of the somata of labeled cells were measured using integrated density divided by the analyzed area of the cell. Images were adjusted on the same brightness set for all dual labeled images. The quantity of astrocytes was taken from each section and pooled.

From each sample, astrocytes number were counted on four fields of 280 ×280 μm area, and the cell area percentage was calculated for astrocyte activity analysis.

Western blot analysis of Nrf2 and HO1

Nuclear and cytoplasmic fractionation was performed by the spinal cord tissue trypsinization (50 μg total) and washing twice in ice-cold PBS. After that, the sample was lysed on ice for 15 min in 100 mL of cytoplasmic lysis buffer (10 mM HEPES pH 7.4, 10 nM KCl, 0.01 mM EDTA, 0.1 mM EGTA, 2 mM DTT, 5 mM Na2VO4, 20 mM sodium β-glycerophosphate, 0.1% and Protease inhibitor cocktail. Nuclei were sedimented by centrifugation and the supernatant containing the cytoplasmic fraction removed, to which Urea and SDS were added to a final concentration of 2 M and 2% respectively, and the samples were denatured by boiling for 5 min. The nuclei were then washed in 1 ml of cytoplasmic lysis buffer to remove any contaminating cytoplasm, and re-sedimented. The nuclei were then lysed in 100 μl of denaturing lysis buffer (2% SDS, 2 M urea, 8% sucrose, 20 mM sodium β-glycerophosphate, 1mM NaF, and 5 mM Na2VO4).

Ten μg of cytoplasmic and 10 μg nuclear extracts were loaded to 12% polyacrylamide gel, submitted to SDS-PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane (Roche Diagnostics GmbH, Mannheim, Germany). After transfer, membranes were stained with Ponceau S dye, washed, and blocked with 5% non-fat dry milk in TBST buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween-20) for 1 h at RT. Membranes were further incubated overnight at 4°C with primary antibodies: rabbit polyclonal anti-heme oxygenase 1 (1:1000, Abcam,

Table I. *Primary antibodies used.*

Antibody	Species raised against	Manufacturer, catalog number	Antibody dilution
Nrf2 (nuclear factor erythroid 2 (NFE2)-related factor 2)	Rabbit polyclonal	Santa Cruz Sc-722	1:200
NeuN (neuronal nuclear antigen)	Mouse monoclonal	Milipore, MAB377	1:200
ChAT (Choline acetyltransferase)	Rabbit polyclonal	Abcam, ab6168	1:200
GFAP (glial fibrillary acidic protein)	Rabbit polyclonal	Abcam, ab7260	1:500
Iba1 (ionized calcium-binding adapter molecule 1)	Goat polyclonal	Abcam, ab5076	1:500
PLP1 (proteolipid protein 1)	Mouse polyclonal	Abcam ab9311	1:500
HO-1 (heme oxygenase 1)	Rabbit polyclonal	Abcam ab13243	1:1000
B-actin	Mouse monoclonal	Santa Cruz sc-47778	1:2000

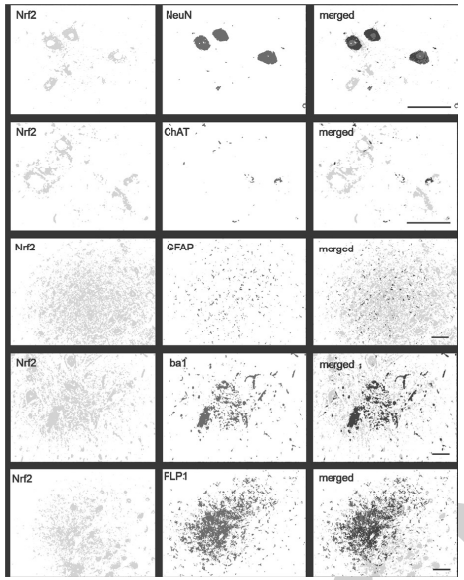

Fig. 1. *Double labelled (**A**) Nrf2 and NeuN, (**B**) Nrf2 and ChAT, (**C**) Nrf2 and GFAP, (**D**) Nrf2 and Iba1, (**E**) Nrf2 and PLP1 in cross sections of spinal cord lumbar region in control group and 1 h of IRI. Immunofluorescence images show co – expression of (A) Nrf2 and NeuN and (B) Nrf2 and ChAT in grey matter of spinal cord both in control and 1 h of IRI. The expression of Nrf2 is not observed in (C) GFAP, (D) Iba1 or (E) PLP1 positive cells. Although dark-field microscopy was used, images have been post-processed to enhance visibility. Scale bar = 50 and 100 μm.*

Cambridge, MA, USA), mouse monoclonal anti-β-actin (1:2000, Santa Cruz Biotechnology, Dallas, TX, USA) and rabbit polyclonal anti-Nrf2 (1:500, Santa Cruz Biotechnology, Dallas, TX, USA). The following day, membranes were washed and incubated for 1 h at RT with appropriate secondary antibodies: goat anti-mouse IgG-HRP (1:2000, Santa Cruz Biotechnology, Dallas, TX, USA), goat anti-rabbit IgG-HRP (1:2000, Santa Cruz Biotechnology, Dallas, TX, USA). The immunoblots were visualized by enhanced chemiluminescence using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) and quantified using ImageJ software. Band densities were normalized to β-actin for HO-1 and Ponceau S for Nrf2 and presented as relative densitometric units.

Statistical analysis

All statistical analyses were performed using Statistica software version 12 (StatSoft Inc., Tulsa, OK, USA). In densitometric analyses the values were expressed as relative optical densities of the related bands. Statistical significance for the Western blot data was calculated according to the one-way analysis of variance (ANOVA), followed by Duncan's multiple range post-hoc test. For the IF staining, the means of integrated density divided by analysed area of the cell were used for the Kruskal–Wallis analysis. Post-hoc group comparisons were accomplished utilizing the Mann–Whitney U test. Results are expressed as means ± SEM. In all comparisons, $P < 0.05$ was considered statistically significant.

All procedures were according to the ethical standards of the institutional and national responsible committee and with the Helsinki declaration of 1975, as revised in 2000.

RESULTS

Co-expression of Nrf2 and NeuN in the spinal cord after ischemia-reperfusion injury

NeuN and Nrf2 labeled cells were co-expressed

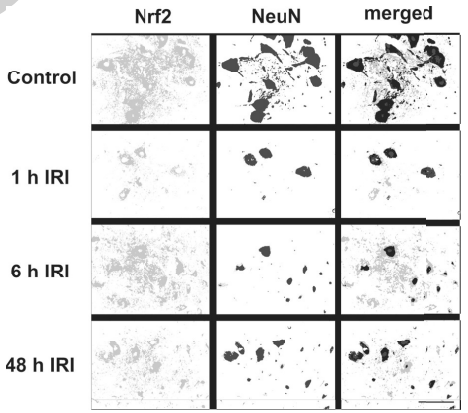

Fig. 2. *representative images of spinal cord sections stained with anti-nuclear factor erythroid 2-related factor 2 (nrf2) and the neuronal marker anti-neuronal nuclei (neun) by double-immunofluorescence labelling in control and at 1, 6 and 48 h after iri. although dark-field microscopy was used, images have been post-processed to enhance visibility. scale bar = 50 μm.*

in the grey matter of the spinal cord in control and IRI group. The co-expression was observed predominantly in the anterior horn and central part of the grey matter, while in the posterior horn of the grey matter only a few cells co-expressed NeuN and Nrf2. Immunoreactivity of Nrf2 was localized mostly in the cytoplasm or both cytoplasm and nucleus of neurons (Fig. 1). NeuN immunoreactivity was localized in the nucleus and peri-nuclear cytoplasm off all neurons in the grey matter, through the anterior, central and posterior horn. Neurons of the anterior horn were less immunoreactive for NeuN at 48 h of IRI, with mean density 45.2±11.7 A.U. (N = 8), however these values varied greatly ranging from 27A.U. to 86 A.U. (Fig. 2). Those variations may correspond to ischemic injury neurons suffered and consequent loss of NeuN reactivity.

Mean density of Nrf2 expression in cells co-

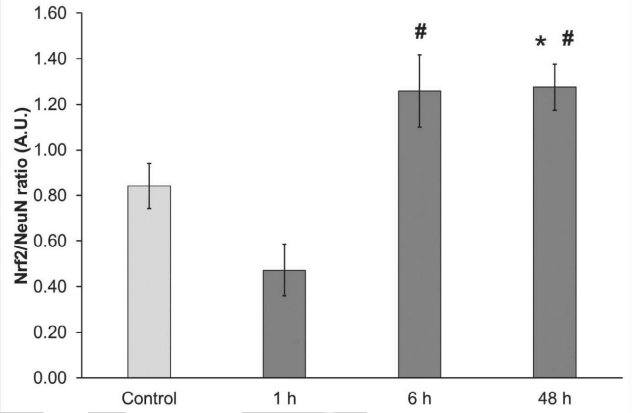

Fig. 3. *Nrf2/NeuN ratio (A.U.= arbitrary units) in control group and at 1h, 6 h and 48 h of IRI. control group; ischemia – reperfusion group (1 h, 6 h and 48 h). * p <0.005 vs control group; # p<0.005 vs 1 h of IRI. All data are expressed as average±SEM (N = 8)*

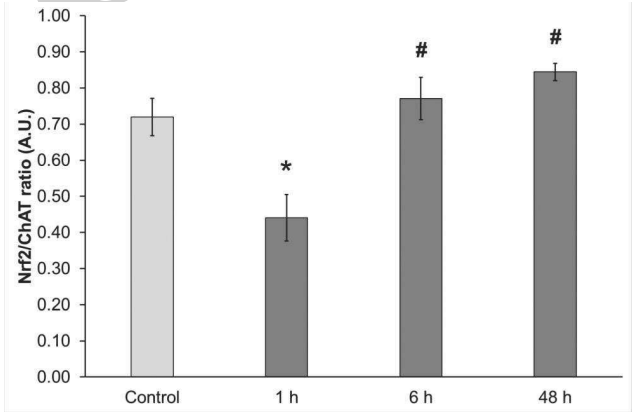

Fig. 4. *Nrf2/ChAT ratio (A.U.= arbitrary units) in control group and at 1 h, 6 h and 48 h of IRI. control group; ischemia – reperfusion group (1 h, 6 h and 48 h). * p <0.005 vs control group; # p<0.005 vs 1 h of IRI. All data are expressed as average±SEM (N = 8)*

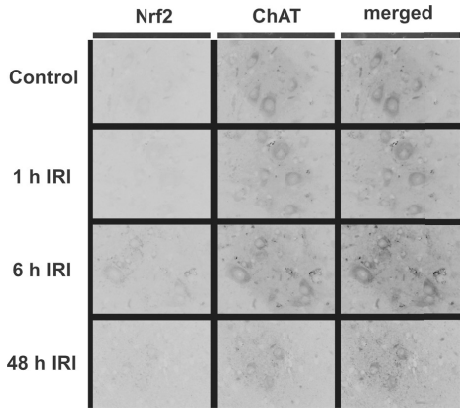

Fig. 5. *Representative images of spinal cord sections stained with anti-nuclear factor erythroid 2-related factor 2 (Nrf2) and the motor neuron marker anti-choline acetyltransferase (ChAT) by double-immunofluorescence labelling in control and at 1, 6 and 48 h after IRI. Although dark-field microscopy was used, images have been post-processed to enhance visibility. Scale bar = 50 μm.*

expressing NeuN was shown as Nrf2/NeuN mean intensity ratio in Fig. 3. In subacute time point, at 1 h of IRI, Nrf2/NeuN ratio was decreased compared to control group. The Nrf2/NeuN ratio at 6 h of IRI increased significantly compared to 1 h of IRI. The Nrf2/NeuN ratio at 6 h of IRI was significantly increased compared to 1 h of IRI. 48 h post-IRI Nrf2/NeuN ratio was significantly increased compared to control and 1 h of IRI.

Co-expression of Nrf2 and ChAT in the spinal cord after ischemia-reperfusion injury

Nrf2 and ChAT labeled cells were co-expressed in the grey matter of spinal cord in the control and IRI groups. All the cells expressing Nrf2 were ChAT-positive. The co-expression was observed in all motor neurons of the grey matter, mostly in the anterior and central horn, while in the posterior horn of the grey matter only a few cells co-expressed NeuN and Nrf2. Immunoreactivity of Nrf2 was localized mostly in the cytoplasm or in both cytoplasm and nucleus of neurons (Fig. 1). ChAT immunoreactivity was localised in cytoplasm and membrane of motor neuron bodies in mostly anterior and central horn, with few neurons positive in posterior horn. ChAT immunoreactivity had mean density 57.8±9.2 A.U. (N = 8) in controls, 41.5±14.2 A.U. (N = 8) at 1 h of IRI, 69.1±11.7 A.U. (N = 8) at 6 h of IRI, and 61.4±6.7 A.U. (N = 8) at 48 h of IRI.

Mean density of Nrf2 expression in cells co-expressing ChAT was shown as Nrf2/ChAT mean intensity ratio in Fig. 4. In subacute time point, at 1 h of IRI, the Nrf2/ChAT ratio was decreased compared to control group. The Nrf2/ChAT ratio at 6 h and 48 h of IRI increased significantly compared to 1 h of IRI.

Mean intensity of Nrf2 expression is increased in the spinal cord after ischemia-reperfusion injury

Immunoreactivity of Nrf2 was localized in the grey matter of spinal cord in the control and IRI groups. The localization of Nrf2 expression was observed in the cytoplasm, or both cytoplasm and nucleus of neurons, predominantly in the anterior and central horn of the spinal cord grey matter, but also in a few neurons of the posterior horn (Figs. 1, 2, 5).

The density of Nrf2 expression depended on the IRI time-point. After 1 h of IRI mean density of Nrf2 expression in neurons was decreased. After 6 h and 48 h of IRI Nrf2 expression in neurons was significantly increased compared to 1 h of IRI. Also at 48 h of IRI mean density of Nrf2 was significantly increased compared to the control group (Fig. 6.).

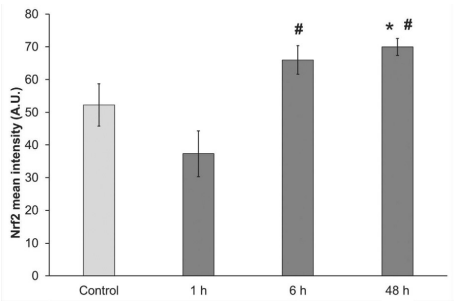

Fig. 6. *Nrf2 mean intensity (A.U.= arbitrary units) in control group and at 1 h, 6 h and 48 h of IRI. Control group; ischemia – reperfusion group (1 h, 6 h and 48 h). * p <0.005 vs control group; # p<0.005 vs 1 h of IRI. All data are expressed as average±SEM (N = 8)*

Co-expression of Nrf2 and GFAP, Iba1, PLP1 in the spinal cord after ischemia-reperfusion injury

To determine the possible expression of Nrf2 in GFAP, Iba1 or PLP1 positive cells in IRI of the spinal cord, we performed double labeled immunofluorescence. No double labeled Nrf2/GFAP, Nrf2/Iba1 or Nrf2/PLP1 expressions were observed in either grey or white matter of the spinal cord at 1, 6 and 48 h of IRI or in the control group (Fig. 1C-E).

Increase of astrocyte number and activity in white matter after IRI

During our experiments, we observed, in contrast to control animals, that animals in the IRI group exhibited an increase in GFAP positive cells in the spinal cord and also an increased percentage of GFAP positive cell area. The higher percentage of GFAP positive cell area confirms increased astrocyte activity. Analysis of GFAP positive cells number matter revealed moderate increase 6 h (627.2±27.4) after reperfusion and significant increase 48 h (784.3±21.2) after reperfusion compared to control animals (551±13.6). Analysis of GFAP positive area showed that astrocyte activity was increased in a lumbar segment of spinal cord 1 h (12%), 6 h (15%) and 48 h (17%) after reperfusion compared to control animals (8%) (Fig. 7).

nrf2 expression is increased in the spinal cord after IRI

To determine whether IRI induced activation of the Nrf2 transcription factor, we analyzed nuclear and cytoplasmic fractions of spinal cord lysates from IRI and sham animals for Nrf2 protein expression (Fig. 8). One hour after reperfusion, there was a decrease of the Nrf2 protein in the cytoplasmic and the nuclear fractions of IRI spinal cords. After 6 h of reperfusion Nrf2 protein levels increased in both cytoplasmic and nuclear fraction, while at 48 h after reperfusion Nrf2 protein expression stayed the same in cytoplasmic fraction and started to decline in nuclear fraction. Therefore, following IRI in the spinal cord, Nrf2 protein expression was significantly increased after 6 h and 48 h of reperfusion in the cytoplasm of cells followed by translocation into the nucleus.

IRI induces increased protein expression of Nrf2 signaling target HO-1

Fig. 9 shows the expression of Nrf2 regulated signaling protein HO-1 in sham-operated animals

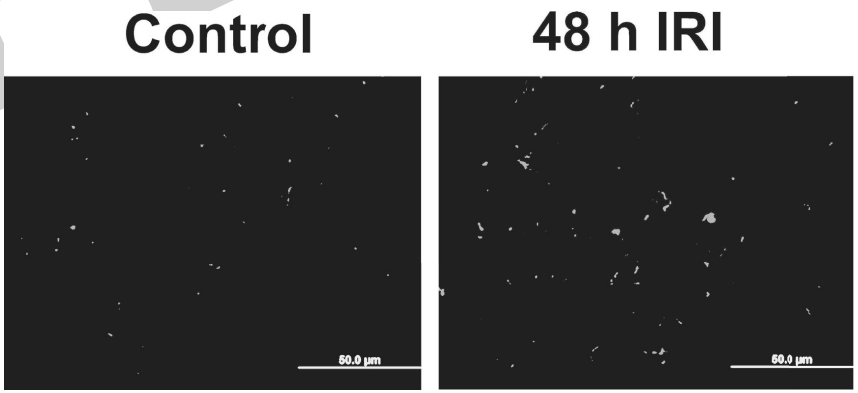

Fig. 7. *Representative images of spinal cord sections stained with anti- astrocyte marker glial fibrillary acidic protein (GFAP). Scale bar = 50 µm. At 48 h of IRI astrocyte number and activity is increased compared to control group.*

and animals that underwent IRI for 1 h, 6 h, and 48 h of reperfusion. After 6 h of reperfusion, there was a significant increase in the levels of HO-1 in spinal cord lysates and the level continued to increase 48 h after IRI compared to sham animals. Therefore, IRI induced increased protein expression of Nrf2 signaling target HO-1.

DISCUSSION

The present study demonstrated the expression of Nrf2 in neurons, an increase in the staining intensity of Nrf2 expression in the neurons following ischemia at all time-points of reperfusion and an increase in some reactive astrocytes. Ischemia followed by 1 hour of reperfusion decreased the level of Nrf2 expression in neurons but 6 hours after reperfusion the expression of Nrf2 was recovered and we observed an increase of Nrf2 expression in motoneurons. Astrogliosis was manifested as an increase in some GFAP positive cells as well as an increase in cellular volume.

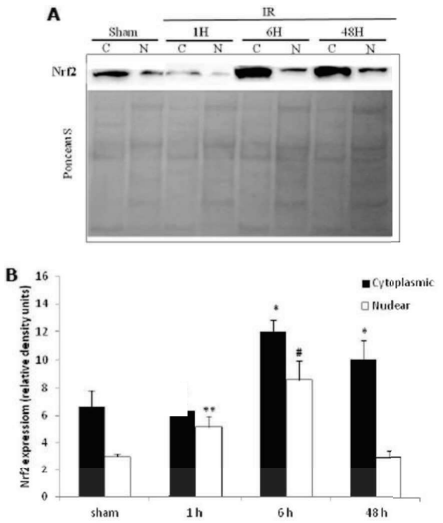

Fig. 8. *Ischemia reperfusion injury (IRI) induces expression of Nrf2 in cytoplasmic ■ (C) and nuclear □ (N) fractions of spinal cord. Representative immunoblots are shown. Data are presented as relative density units normalized to Ponceau S and expressed as mean±SEM. *p<0.05, **p<0.01 compared to sham; n= 8 per group.*

Moreover, our data showed that Nrf2 protein expression increases in cytoplasm and nucleus of spinal cord lysates 6 h post-reperfusion and remains active 48 h after, resulting in increased expression of the main Nrf2 target gene HO-1.

We used NeuN antibody to identify all neurons, ChAT to identify motoneurons, Iba1 to identify microglia, GFAP to identify reactive astrocytes and PLP1 to identify oligodendrocytes.

The spinal cord is extremely vulnerable to ischemic injury which could be caused by a variety of abnormalities (1). However, ischemia of the nervous tissue followed by reperfusion causes secondary injury manifested as neuronal apoptosis, inflammation, and astrogliosis (2). Although the exact pathophysiologic mechanism that underlies spinal cord I/R injury is not fully understood, it seems that oxidative stress (imbalance between reactive species and endogenous anti-oxidative defenses) plays an important role and contributes to spinal cord oxidative damage (3). Thoracoabdominal aortic aneurysm surgery may cause spinal cord ischemia-reperfusion injury because of transient aortic cross-clamping. Such scenario is consistent with the anatomy of the spinal cord blood supply. Except by anterior and posterior spinal arteries, the spinal cord from Th8 to the conus medullaris is supplied by segmental (radicular) arteries. The dominant segmental artery for supplying the lumbar and sacral part of the spinal cord is the artery of Adamkiewicz, which commonly arises from the posterior branch of the last intercostal artery or a lumbar artery which arises from the thoracic or abdominal aorta, respectively. In clinical practice, transient aortic cross-clamping may cause reversible or even irreversible spinal cord neuronal damage, manifested as alteration or loss of neurological motor function.

Nrf2 is ubiquitously expressed antioxidant transcription factor (13) which, under normal conditions (reduced intracellular conditions), is stabilized through binding to Keap-1 in the cytoplasm. During enhanced ROS formation, the cysteine residues in Keap-1 are modified or oxidized, causing the dissociation of Nrf2 and its translocation to the nucleus where it binds to ARE and, depending

upon the binding site, the different antioxidant genes are induced, including HO-1, NQO-1, SOD, GPx (9-11). Our results are in agreement with this, showing that IRI induces increased cytoplasmic and nuclear Nrf2 expression in the spinal cord after 6 h of reperfusion. Also Nrf2 expression stays elevated in both cellular compartments after 48 h of reperfusion. Data presented in this paper are the first showing Nrf2 activation in early phases of IRI in the spinal cord. The function of the Nrf2/ARE signaling pathway is important for protection in states of increased oxidative stress due to ischemia because enhanced ROS production induces activation of Nrf2 (14, 15). Therefore, increased expression of Nrf2 in the motoneurons after IRI may be implicated as a viable strategy for the promotion of neurological function recovery and a strategic target for IRI therapies (16).

The cell biology of the response to IRI is not well understood and the roles played by different cell types at different time-points in the progression of secondary degeneration during IRI are not well defined. Therefore, in the current study, we focused

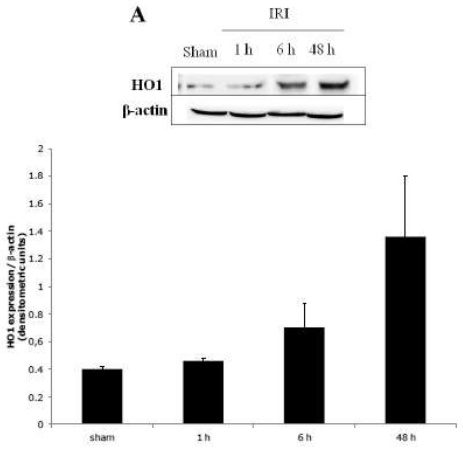

Fig. 9. *IRI induces increased protein level of the Nrf2 main signalling target HO-1. Immunoblots indicate that levels of HO-1 start to increase 6 h after reperfusion and continue to increase at 48 h. Data are presented as relative density units normalized to β-actin and expressed as mean±SEM; n= 8 per group.*

on identifying the cell types in the spinal cord for the expression of Nrf2 molecules and whether I/R injury affects the expression of the Nrf2 protein as a common antioxidative signaling pathway. We detected the expression of Nrf2 in the neurons in the gray matter of the spinal cord in the control group and after I/R injury. Nrf2 expression was significantly higher after 6 and 48 hours after IRI compared with the levels detected in the control group, thus implying the activation of the Nrf2 signaling pathway. Most of the neurons expressing Nrf2 molecules were motoneurons in the anterior horn and central part of the gray matter. IRI caused an increase in the density of Nrf2 expression in motoneurons after 6 and even more after 48 hours after reperfusion. Nrf2 expression was not detected in other cell types. Park et al. reported human neuronal cell protection by elevated Nrf2 activity and its downstream molecules HO-1 and NQO1, against oxygen-glucose deprivation/ reoxygenation (17). Dai et al. found that isoquercetin attenuates oxidative stress and neuronal apoptosis after ischemia-reperfusion injury via Nrf2 inhibiting NFκB inflammatory pathway (18). Li et al. showed that suppression of neuronal injury caused by oxygen-glucose deprivation/reoxygenation is achieved by activating Brg1/Nrf2/ARE signaling pathway via suppression of microRNA – 144 – 3p (19). Xun et al. reported that only overexpression of nuclear factor of activated T cells 5 (NFAT5) could protect astrocytes against oxidative damage by promoting the nuclear transport of Nrf2 from cytoplasm to cell nucleus, or NFAT regulates Nrf2 pathway in astrocytes (15). Other reports showed that the level of Nrf2 expression could also be increased in neurons, microglia, and astrocytes after treatment by some drugs (20).

Reactive astrocytosis, increasing GFAP positive cells, was a prominent feature of the cellular response to IRI. We found that astrocytes exhibit the expression of the glial fibrillary acid protein, hypertrophy, process extension and cell division. IRI induced an increase in the number of GFAP positive cells during the first 48 hours. Scar tissue formed by reactive astrocytes has long been implicated as a significant impediment to neuronal tissue repair after SC injury, however, a report by Faulkner et al.

showed that reactive astrocytes restrict inflammation, protect neurons and oligodendrocytes and preserve motor functions after SCI (21).

Our results suggested that ischemia of the spinal cord followed by reperfusion increased the level of Nrf2 expression in neurons, did not cause Nrf2 expression in other cell types and induced reactive astrocytosis.

Moreover, we demonstrated that IRI enhances activation of Nrf2 as well as its target gene HO-1 in the early phase after reperfusion, probably as a protective mechanism from oxidative stress.

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