Global brain ischemia in rats is associated with mitochondrial release and downregulation of Mfn2 in the cerebral cortex, but not the hippocampus

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Abstract. Mitochondria are crucial for neuronal cell survival and death through their functions in ATP production and the intrinsic pathway of apoptosis. Mitochondrial dysfunction is considered to play a central role in several serious human diseases, including neurodegenerative diseases, such as Parkinson's and Alzheimer's disease and ischemic neurodegeneration. The aim of the present study was to investigate the impact of transient global brain ischemia on the expression of selected proteins involved in mitochondrial dynamics and mitochondria-associated membranes. The main foci of interest were the proteins mitofusin 2 (Mfn2), dynamin-related protein 1 (DRP1), voltage-dependent anion-selective channel 1 (VDAC1) and glucose-regulated protein 75 (GRP75). Western blot analysis of total cell extracts and mitochondria isolated from either the cerebral cortex or hippocampus of experimental animals was performed. In addition, Mfn2 was localized intracellularly by laser scanning confocal microscopy. It was demonstrated that 15-min ischemia, or 15-min ischemia followed by 1, 3, 24 or 72 h of reperfusion, was associated with a marked decrease of the Mfn2 protein in mitochondria isolated from the cerebral cortex, but not in hippocampal mitochondria. Moreover, a translocation of the Mfn2 protein to the cytoplasm was documented immediately after global brain ischemia in the neurons of the cerebral cortex by laser scanning confocal microscopy. Mfn2 translocation was followed by decreased expression of Mfn2 during reperfusion. Markedly elevated levels of the VDAC1 protein were also documented in total cell extracts isolated from the hippocampus of rats after 15 min of global brain ischemia followed by 3 h of reperfusion, and from the cerebral cortex of rats after 15 min of global brain ischemia followed by 72 h of reperfusion. The mitochondrial Mfn2 release observed during the early stages of reperfusion may thus represent an important mechanism of mitochondrial dysfunction associated with neuronal dysfunction or death induced by global brain ischemia.

Introduction

Mitochondria play a key role in neuronal cell survival and death through their function in ATP production (1,2) and involvement in the intrinsic pathway of apoptosis (3). In the majority of cells, including neuronal cells, mitochondria form a dynamic tubular network that is continuously remodeled; they may either divide via binary fission and form separate entities, or fuse and form a more continuous network (4). Both the fusion, due to collision, and the fission, due to separation, of mitochondria are dependent on the intracellular movement of these organelles. In specific intracellular compartments, mitochondria are in close contact with the endoplasmic reticulum (ER), albeit without fusion, particularly in the ER mitochondria-associated membranes (MAM). ER-mitochondrial contacts strongly affect mitochondrial dynamics, as they favor mitochondrial constriction and consequent fission (5). Recent research results suggest that mitochondrial function depends on mitochondrial morphology, which can rapidly change in response to cellular conditions (6,7). Fused mitochondria, prevailing in healthy metabolically active cells, exhibit a higher ATP production that is attributed to optimized

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exchange of metabolites and mitochondrial DNA within the mitochondrial matrix. On the contrary, fragmented mitochondria, which are encountered in quiescent and metabolically inactive cells, exhibit reduced respiration and are associated with different pathological conditions (8). However, mitochondrial fission is crucial for cell division and for the generation of single mitochondria that can be transported along axons by the motor protein apparatus (9). The latter function is particularly important for neuronal cells. In addition, mitochondrial fission is crucial for the release of cytochrome *c* and other intermembrane space proteins during apoptosis, and for the elimination of damaged organelles from the mitochondrial network by autophagy (10). The fission/fusion equilibrium is controlled by specific mitochondrial proteins. Mitochondrial fission is mediated by the cytosolic GTPase dynamin-related protein 1 (DRP1), which is activated by various post-translational modifications (11) and consequently translocates to the outer mitochondrial membrane (12). Mitochondrial fusion involves two membranes that must be rearranged in a coordinated manner in order to maintain mitochondrial integrity. Inner mitochondrial membrane fusion is mediated by the mitochondrial dynamin-like GTPase (OPA1), whereas two mitofusins (Mfn), namely Mfn1 and 2, are involved in the process of mitochondrial outer membrane fusion. Despite Mfn1 and Mfn2 displaying high homology (81%) and identity (~60%), they have non-redundant functions (13). In addition to its role in fusion, Mfn2 plays a role in the control of the ER-mitochondria interaction (14), although the exact function of Mfn2 in this inter-organelle interplay remains a subject of intense discussion (15,16).

With respect to MAM, the first complex that tethers the ER and mitochondria was identified in mammalian cells and is the tripartite complex between the cytosolic chaperone glucose-regulated protein 75 (GRP75), the mitochondrial voltage-dependent anion-selective channel 1 (VDAC1), and the inositol 1,4,5-triphosphate receptor located at the ER membrane (17). MAM play a key role in the maintenance of lipid and Ca^{2+} homeostasis, in the initiation of autophagy and mitochondrial fission, and in sensing metabolic shifts (18).

Disturbances of mitochondrial dynamics and MAM are associated with mitochondrial dysfunction and are considered to be an important mechanism underlying several serious human diseases, including neurodegenerative diseases (19,20), such as Parkinson's (21) and Alzheimer's (22) disease and ischemic neurodegeneration (23). Alterations at the protein level of Mfn2 (24,25), DRP1 (26), OPA1 (27,28) and VDCA1 (29) have been documented in various models of brain ischemia. The aim of the present study was to investigate the impact of transient global brain ischemia on the expression of selected proteins involved in mitochondrial dynamics and MAM. Previous studies have investigated the impact of different types of brain ischemia on the total levels of the abovementioned proteins, but without focusing on their intracellular localization. Therefore, we focused on Mfn2, DRP1, VDAC1 and GRP75 with respect to their intracellular localization, and performed western blot (WB) analysis of both total cell extracts and mitochondria isolated from either the cerebral cortex or the hippocampus. In addition, Mfn2 intracellular localization was analyzed by laser scanning confocal microscopy.

Materials and methods

Ischemia-reperfusion. Animal studies were performed according to the guideline for Animal Care and Health of the State Veterinary and Food Department of the Slovak Republic (approval no. 2414/06-221/3). The experiments were conducted in accordance with Directive 2010/63/EU of the European Parliament and of the Council for the protection of animals used for scientific purposes.

A total of 50 adult male Wistar rats (Velaz, Ltd.) were used. All animals were maintained on a 12/12-h light/dark cycle. Food and water were available *ad libitum* until the beginning of the experiments. Animal health and behaviour were monitored regularly by a doctor of veterinary medicine. Transient global cerebral ischemia was produced using the four-vessel occlusion model. Briefly, on day 1, both vertebral arteries were irreversibly occluded by coagulation through the alar foramina following anesthesia with a mixture of 2% halothane, 30% O_2 and 68% N_2O. On day 2, both common carotid arteries were occluded for 15 min by small clips under anesthesia with a mixture of 2% halothane, 30% O_2 and 68% N_2O. Two minutes prior to carotid occlusion, the halothane was removed from the mixture. Body temperature was maintained by means of a homeothermic blanket. Global ischemia was followed by 1, 3, 24 or 72 h of reperfusion. During a short time of reperfusion (1 or 3 h), the animals were monitored by the experimenter. Animals surviving over a longer time of reperfusion (24 or 72 h) were monitored by a doctor of veterinary medicine. Control animals underwent the same procedure, apart from the carotid occlusion. The duration of the experiment was 1-4 days, depending on the time of reperfusion. With respect to WB analysis, control animals and animals undergoing ischemia and reperfusion were first anesthetized with a mixture of 2% halothane, 30% O_2 and 68% N_2O and then sacrificed by decapitation. The cerebral cortex and both hippocampi were dissected and processed immediately. With respect to fluorescence immunohistochemistry (FIHC), control and experimental animals were anesthetized, perfused transcardially with ice-cold 0.1 mol/l phosphate-buffered saline (PBS, pH 7.4) and fixed by perfusion with ice-cold 4% paraformaldehyde in PBS. The brains were removed, post-fixed with the same solution as mentioned above for 24 h at 4°C, and cryoprotected by infiltration using 30% sucrose for the next 24 h at 4°C.

Experimental groups of animals. The rats were randomized into the following groups: i) Control sham-operated rats (CNT; n=5 for WB and n=3 for FIHC); ii) rats that underwent a 15-min global brain ischemia (ISCH; n=5 for WB and n=3 for FIHC); iii) rats that underwent a 15-min global brain ischemia followed by 1 h of reperfusion (I1R; n=5); iv) rats that underwent a 15-min global brain ischemia followed by 3 h of reperfusion (I3R; n=5); v) rats that underwent a 15-min global brain ischemia followed by 24 h of reperfusion (I24R; n=5); and vi) rats that underwent a 15-min global brain ischemia followed by 72 h of reperfusion (I72R; n=5 for WB and n=3 for FIHC).

Preparation of protein extracts and isolation of mitochondria. Protein extracts were prepared by homogenization of either

the cerebral cortex or both hippocampi in homogenization buffer (10 mM Tris-HCl, pH=7.4, 1 mM EDTA and 0.24 M sucrose) using a Potter Teflon glass homogenizer. Total cell extracts were prepared by the addition of an appropriate volume of 6X RIPA buffer [6X PBS, 6% (v/v) Nonidet P-40, 3% (w/v) sodium deoxycholate, 0.6% (w/v) sodium dodecyl sulphate (SDS)] to the homogenate.

Mitochondria from both whole hippocampi were isolated by differential centrifugation as described previously (30). Non-synaptic mitochondria from the cerebral cortex were isolated by differential centrifugation using one-step Percoll gradient (16% Percoll in 0.25 M sucrose), as described previously (31). The protein concentration was determined by the protein Dc assay kit (Bio-Rad Laboratories, Inc.) with bovine serum albumin (BSA) as a standard.

WB analysis. Isolated proteins were separated by 10% SDS-PAGE. Following electrophoresis, the separated proteins were transferred onto nitrocellulose membranes using a semi-dry transfer protocol. The membranes were controlled for even load and possible transfer artefacts by staining with Ponceau Red solution. After blocking with BSA blocking buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 2% BSA), the membranes were first incubated for 90 min with primary mouse monoclonal antibodies against GRP75 (1:1,000, sc-133137), VDAC1 (1:1,000, sc390996), β-actin (1:2,000, sc-47778) (all from Santa Cruz Biotechnology, Inc.) and cytochrome *c* oxidase subunit 1 (COXI; 1 µg/ml, 459600, Invitrogen; Thermo Fisher Scientific, Inc.), rabbit polyclonal antibodies raised against Mfn2 (1:500; sc-50331), or goat polyclonal antibodies raised against DRP1 (1:500, sc-21804) (all from Santa Cruz Biotechnology, Inc.) dissolved in BSA blocking solution. Membranes incubated with primary antibodies were washed in TBS-T solution (50 mM Tris-Cl, pH 7.5, 150 mM NaCl and 0.05% Tween 20) and then incubated with secondary antibodies conjugated with horseradish peroxidase (1:5,000, Santa Cruz Biotechnology, Inc). After extensive washes with TBS-T solution (4 times, 15 min), the membranes were incubated in SuperSignal West Pico Chemiluminescent Substrate solution (Thermo Fisher Scientific, Inc.) for 3 min. Following exposure of the membranes to Chemidoc XRS (Bio-Rad Laboratories, Inc.), the intensities of the corresponding bands were quantified using Quantity One software (BioRad Laboratories, Inc.). The intensities of the bands of interest were normalized to the corresponding band intensities of either β-actin or COXI.

Detection of β3 tubulin and Mfn2 by FIHC. The brains from control and experimental rats were frozen and cut with a cryostat into 30-µm sections; the sections were mounted on Superfrost Plus glass slides (Thermo Fisher Scientific, Inc.). Mounted brain sections were permeabilized with a permeabilization solution (0.1% Triton X-100 with 10% BSA) for 1 h. Mouse monoclonal antibody against β3 tubulin (1:50; sc-80005; Santa Cruz Biotechnology, Inc.), as a specific marker of neuronal cell body cytoplasm and axon guidance, was used as a primary antibody. Mfn2 rabbit polyclonal antibodies (1:50; sc-50331; Santa Cruz Biotechnology, Inc.) were used to detect Mfn2. The tissue sections were incubated at 4˚C overnight in primary antibodies diluted in permeabilization solution.

Alexa Fluor 488 goat-anti-rabbit IgG (1:50; cat. no. 4412, Cell Signaling Technology, Inc.) was applied as a secondary antibody for Mfn2, and Alexa Fluor 594 goat-anti-mouse IgG (1:100, cat. no. 8890, Cell Signaling Technology, Inc.) was applied as a secondary antibody for β3 tubulin. Finally, the brain sections were cover-slipped with Fluoromount-G® medium containing 4',6-diamidino-2-phenylindole (DAPI, CA 0100-20, SouthernBiotech). In the absence of primary antibody, no immunoreactivity was observed. The slides were examined under an Olympus FluoView FV10i confocal laser scanning microscope (Olympus Corporation) equipped with an objective of x10 with a zoom up to a magnification of x40 and filters for fluorescein isothiocyanate for Alexa Fluor 488 (excitation: 499 nm; emission: 520 nm) and Texas Red (excitation: 590 nm; emission: 618 nm). Images were captured using Olympus Fluoview FV10-ASW software, version 02.01 (Olympus Corporation) and Quick Photo Micro software, version 2.3 (Promicra, s.r.o.) and further processed in Adobe Photoshop CS3 Extended, version 10.0 for Windows (Adobe Systems, Inc.).

The brightness and contrast of each image file were uniformly calibrated using Adobe Photoshop CS3 Extended, version 10.0 for Windows (Adobe Systems, Inc.). Values of background staining were obtained and subtracted from the immunoreactive intensities.

Statistical analysis. All statistical analyses were performed using GraphPad InStat V2.04a (GraphPad Software, Inc.). For the comparison of the ischemia-induced changes among all groups, one-way analysis of variance was first performed to determine any differences among all experimental groups. Additionally, an unpaired Tukey's test was used to determine differences between individual groups. The significance level was set at P<0.05.

Results

WB analysis of the levels of the selected proteins in total cell extracts from the cerebral cortex and hippocampus of control and experimental animals. In order to study the impact of global brain ischemia and ischemia followed by reperfusion on the levels of proteins involved in mitochondrial dynamics and MAM, WB analysis of total cell extracts from the cerebral cortex and hippocampus of control and experimental animals was performed. In total cell extracts prepared form the cerebral cortex, significantly decreased total levels of Mfn2 were observed after ischemia followed by 3 h (69.5% of control, P<0.05), 24 h (58.3% of control, P<0.01), and 72 h (65.9% of control, P<0.05) of reperfusion. In addition to Mfn2, the total level of VDAC1 was significantly increased after 72 h of reperfusion (417.7% of control, P<0.05). The levels of DRP1 were increased, whereas those of GRP75 were decreased after ischemia and after ischemia followed by reperfusion, but the observed changes were not statistically significant (Fig. 1). In hippocampal total cell extracts, ischemia followed by reperfusion led to an increase in the levels of the Mfn2 protein, although these changes were not statistically significant (Fig. 2). A statistically significantly increased level of VDAC1 was documented after 3 h of reperfusion (189.8% of control, P<0.01). The levels of other investigated proteins in hippocampal total cell extracts were unaltered after ischemia and after ischemia followed by reperfusion (Fig. 2).
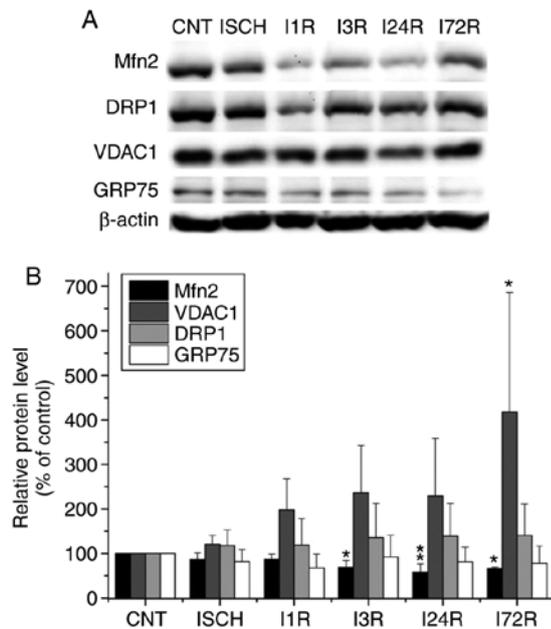
Figure 1. Effect of transient global brain ischemia on the total levels of the Mfn2, DRP1, VDAC1 and GRP75 proteins in rat cerebral cortex. (A) Experimental rats were subjected to 15 min of transient global brain ischemia (ISCH) or 15 min of transient global brain ischemia followed by 1 (I1R), 3 (I3R), 24 (I24R) or 72 h (I72R) of reperfusion. The pattern of protein expression was evaluated by western blot analysis of total cell extracts prepared from the cerebral cortex of control (CNT) and experimental rats, as described in Materials and methods. β-actin served as the loading control. (B) Quantification of the post-ischemic changes in Mfn2, DRP1, VDAC1 and GRP75 protein levels in total cell extracts isolated from rat cerebral cortex. The data were normalized to the β-actin level and are expressed relative to controls. Data are presented as means ± standard deviation (n=5 per group). *P<0.05 and **P<0.01, significantly different from control. Mfn2, mitofusin 2; DRP1, dynamin-related protein 1; VDAC1, voltage-dependent anion-selective channel 1; GRP75, glucose-regulated protein 75.

Figure 2. Effect of transient global brain ischemia on the total levels of the Mfn2, DRP1, VDAC1 and GRP75 proteins in the rat hippocampus. (A) Experimental rats were subjected to 15 min of transient global brain ischemia (ISCH) or 15 min of transient global brain ischemia followed by 1 (I1R), 3 (I3R), 24 (I24R), or 72 h (I72R) of reperfusion. The pattern of protein expression was evaluated by western blot analysis of total cell extracts prepared from both hippocampi of control (CNT) and experimental rats, as described in Materials and methods. β-actin served as the loading control. (B) Quantification of the post-ischemic changes in Mfn2, DRP1, VDAC1 and GRP75 protein levels in total cell extracts isolated from rat hippocampus. The data were normalized to the β-actin level and are expressed relative to controls. Data are presented as means ± standard deviation (n=5 per group). **P<0.01, significantly different from control. Mfn2, mitofusin 2; DRP1, dynamin-related protein 1; VDAC1, voltage-dependent anion-selective channel 1; GRP75, glucose-regulated protein 75.

WB analysis of the levels of the selected proteins in mitochondria isolated from the cortex and hippocampus of control and experimental animals. In addition to the analysis of total cell extracts, the levels of selected proteins were determined in mitochondria isolated from the cortex and hippocampus of control and experimental animals (Figs. 3 and 4). We observed that ischemia and ischemia followed by reperfusion led to a decrease in the levels of Mfn2 in mitochondria isolated from the cerebral cortex; this decrease was significant after ischemia (60.8% of control, P<0.001) and after 1 h (54.8% of control, P<0.001), 3 h (65.2% of control, P<0.01), 24 h (60.6% of control, P<0.001), and 72 h (66.7% of control, P<0.01) of reperfusion (Fig. 3). We also observed that ischemia followed by reperfusion led to a decrease in the levels of the VDAC1 protein in cortical mitochondria, although the changes were not statistically significant (Fig. 3). The levels of the other investigated proteins in the cortical mitochondria were not significantly altered after ischemia and after ischemia followed by reperfusion (Fig. 3). In hippocampal mitochondria, the levels of Mfn2, VDAC1 and GRP75 were increased after ischemia followed by reperfusion, but the changes were not statistically significant. The levels of DRP1 in hippocampal mitochondria were unaltered after ischemia and after ischemia followed by reperfusion (Fig. 4).

Immunoreactivity of Mfn2 and β3 tubulin in the hippocampus and in the M1 region of the rat brain cortex. To further confirm the WB results, immunofluorescence was used to detect any immunoreactivity of Mfn2 and β3 tubulin, as a neuronal cytoskeletal marker, in the hippocampal area and in the M1 region of the rat brain cortex. Representative images from control rats (CNT), rats that were subjected to global brain ischemia for 15 min (ISCH), and from rats that underwent reperfusion with a duration of 72 h after 15 min of global brain ischemia (I72R) are shown in Figs. 5 and 6. Signals corresponding to Mfn2 were predominantly located within the perikaryal cytoplasm, in the processes of histologically normal tissue in the M1 region of the cortex (Fig. 5B), and in the CA1 layer of the hippocampus (Fig. 6B). In the M1 region of ischemic rats (Fig. 5E), the intensity of the Mfn2 signal was slightly decreased. The localization of Mfn2 was shifted, and Mfn2 and β3 tubulin signals were co-localized in some neurons (Fig. 5F, arrows). In the CA1 layer of the hippocampus, the signal for Mfn2 was reduced compared with that of the control, and the localization of Mfn2 was transferred to the neuronal processes and the neurophil (Fig. 6E). In the M1 region of the cortex, a 15-min ischemia followed by 72 h of reperfusion resulted in a diminution of the Mfn2 signal, a finding that was consistent with the data from the WB analysis. The localization of Mfn2 was limited to the neuronal processes and the neurophil (Fig. 5H).
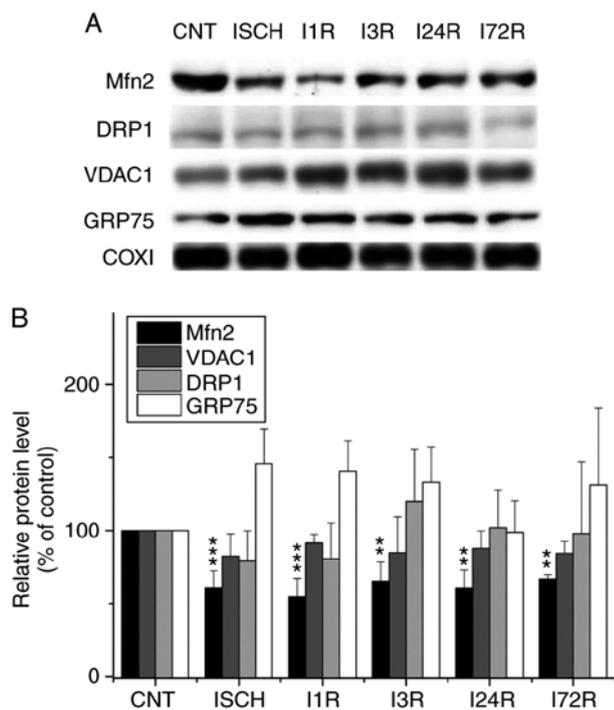
Figure 3. Effect of transient global brain ischemia on the levels of Mfn2, DRP1, VDAC1 and GRP75 proteins in mitochondria isolated from rat cerebral cortex. (A) Experimental rats were subjected to 15 min of transient global brain ischemia (ISCH) or 15 min of transient global brain ischemia followed by 1 (I1R), 3 (I3R), 24 (I24R), or 72 (I72R) of reperfusion. The mitochondrial levels of Mfn2, Drp1, VDAC1 and GRP75 were determined by western blot analysis of mitochondria isolated from cerebral cortex of control (CNT) and experimental rats as described in Materials and methods. COXI served as the loading control. (B) Quantification of the post-ischemic changes of Mfn2, DRP1, VDAC1 and GRP75 protein levels in mitochondria isolated from rat cerebral cortex. The data were normalized to the COXI level and are expressed relative to controls. Data are presented as means ± standard deviation (n=5 per group). **P<0.01 and ***P<0.001: significantly different from control. Mfn2, mitofusin 2; DRP1, dynamin-related protein 1; VDAC1, voltage-dependent anion-selective channel 1; GRP75, glucose-regulated protein 75; COXI, cytochrome c oxidase subunit 1.

Figure 4. Effect of transient global brain ischemia on the levels of the Mfn2, Drp1, VDAC1 and GRP75 proteins in mitochondria isolated from rat hippocampus. (A) Experimental rats were subjected to 15 min of transient global brain ischemia (ISCH) or 15 min of transient global brain ischemia followed by 1 (I1R), 3 (I3R), 24 (I24R), or 72 (I72R) of reperfusion. The mitochondrial levels of Mfn2, DRP1, VDAC1 and GRP75 were determined by western blot analysis of mitochondria isolated from hippocampus of control and experimental rats as described in Materials and methods. COXI served as the loading control. (B) Quantification of the post-ischemic changes of Mfn2, DRP1, VDAC1 and GRP75 protein levels in hippocampal mitochondria. The data were normalized to the COXI level and are expressed relative to controls. Data are presented as means ± standard deviation (n=5 per group). Mfn2, mitofusin 2; DRP1, dynamin-related protein 1; VDAC1, voltage-dependent anion-selective channel 1; GRP75, glucose-regulated protein 75; COXI, cytochrome c oxidase subunit 1.

In the CA1 layer of the hippocampus, Mfn2 immunoreactivity was decreased after 15 min of ischemia followed by 72 h of reperfusion (Fig. 6H), and was relocated to the periphery of the perikaryon, with minimal intersection with the neuronal processes or neurophil. The cytoskeleton of neurons in CA1 was highly disintegrated with no specific morphology in this group of rats (Fig. 6G).

Discussion

The focus of the present study was the effect of transient global brain ischemia on the expression and intracellular distribution of selected proteins involved in mitochondrial dynamics and MAM. It was demonstrated that ischemia for 15 min, as well as a 15-min ischemia followed by 1, 3, 24 and 72 h of reperfusion, were associated with a significant decrease of the Mfn2 protein in mitochondria isolated from the cerebral cortex, but not in hippocampal mitochondria. Moreover, the translocation of the Mfn2 protein to the cytoplasm immediately after global brain ischemia was documented in the neurons of the cerebral cortex using laser scanning confocal microscopy. The translocation of Mfn2 was followed by decreased expression of Mfn2

during reperfusion. In addition, significantly elevated levels of the VDAC1 protein were detected in total cell extracts isolated from the hippocampus of rats that had undergone 15 min of global brain ischemia followed by 3 h of reperfusion, and from the cerebral cortex of rats that had undergone 15 min of global brain ischemia followed by 72 h of reperfusion. The mitochondrial and total levels of DRP1 and GRP75 exhibited no significant changes in either the hippocampus or the cortex in all experimental groups.

Mfn2 is an important protein involved in the process of mitochondrial outer membrane fusion, which plays an important role in several intracellular pathways and in the pathogenesis of neurodegenerative diseases, metabolic disorders, cardiomyopathies and cancer (32). In addition, Mfn2 is involved in the process of mitophagy (33), which represents an important mechanism of mitochondria quality control that is often dysregulated in neurodegenerative diseases and ischemia-reperfusion injury (23). Various models of brain ischemia have been used to document the alterations in the total levels of the Mfn2 protein (24,25). As shown recently, Mfn2 downregulation causes mitochondrial dysfunction, altered Ca^{2+} homeostasis and enhanced Bax translocation to
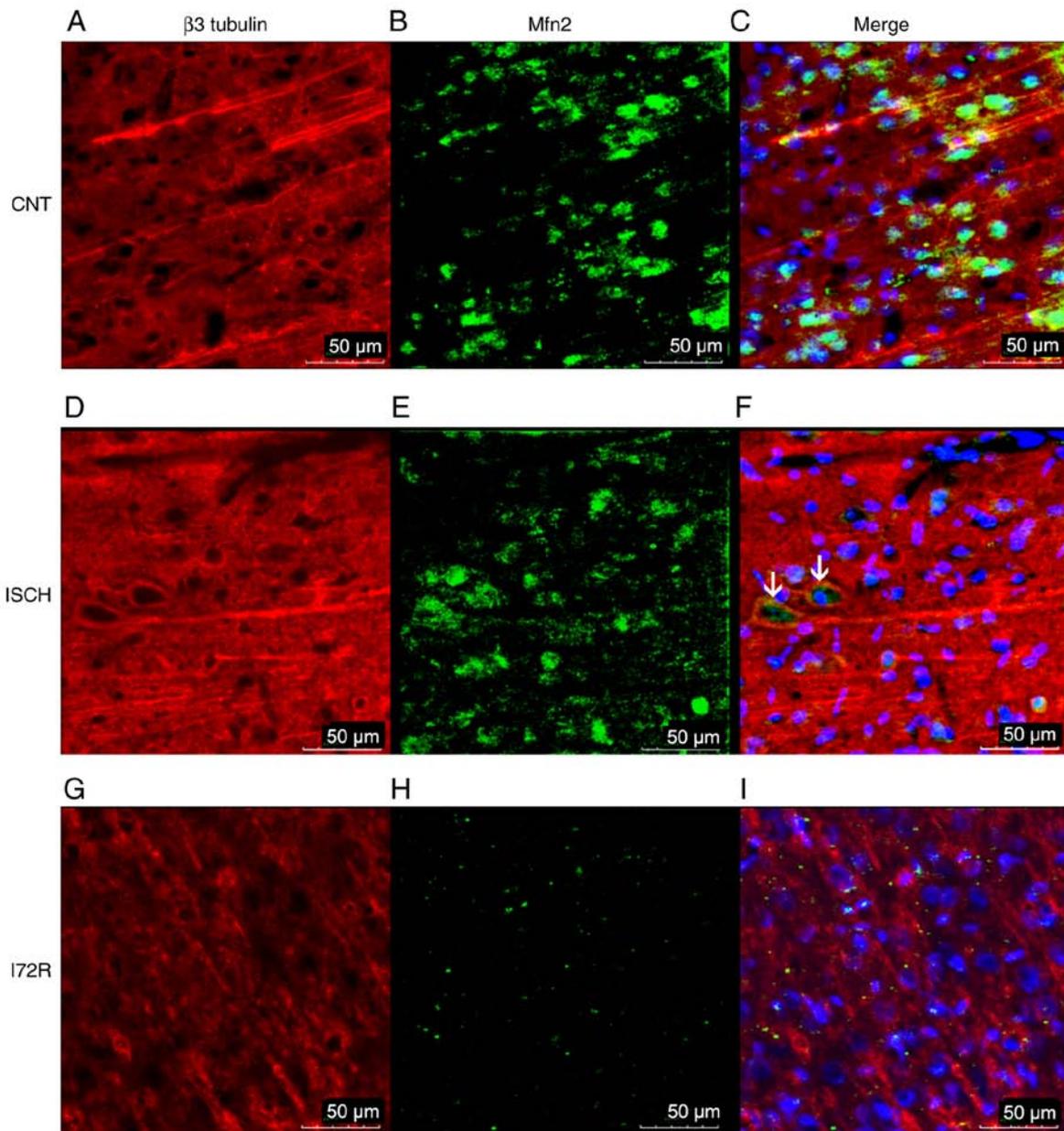
Figure 5. Fluorescence micrographs of cells positive for β3 tubulin (red) and Mfn2 (green) in the M1 region of rat brain cortex. Nuclei are co-stained with DAPI (blue). (A-C) Micrographs of brain slices of control rats (CNT), (D-F) rats that were subjected to global brain ischemia for 15 min (ISCH), and (G-I) rats that underwent reperfusion for 72 h after 15 min of global brain ischemia (I72R). The arrows indicate cells positive for β3 tubulin and Mfn2 colocalization. bar, 50 μm. Mfn2, mitofusin 2.

the mitochondria, resulting in delayed neuronal death (24). Since a significant decrease in Mfn2 was observed 6 h after middle cerebral artery occlusion for 90 min, Mfn2 reduction was suggested to be a late event during reperfusion, and its targeting may help reduce ischemic damage and expand the currently narrow therapeutic window in stroke (24). In the present study, the data of WB and laser scanning confocal microscopy analysis are in favor of the view that Mfn2 is released from mitochondria to the cytoplasm even after transient global brain ischemia for 15 min. The release of Mfn2 from mitochondria resulting in decreased mitochondrial level of Mfn2 was persistent during all investigated periods of reperfusion, and was followed by a decrease in the total Mfn2 level that was significant after 3 h of reperfusion. Interestingly, these changes in the level and mitochondrial localization of the

Mfn2 protein were observed in the cerebral cortex but not in the hippocampus, although pyramidal neurons of the CA1 layer belong to the most vulnerable cells with respect to global brain ischemia (34,35). The mechanism controlling mitochondrial localization and the release of Mfn2 from the mitochondria following global brain ischemia remains elusive. With respect to the total level of Mfn2, the suppression of the transcription of the Mfn2 gene has been described in a model of focal brain ischemia and *N*-methyl-D-aspartate-induced excitotoxicity of primary cortical neurons (24). On the contrary, Ca^{2+}-dependent activation of the cysteine protease calpain in response to glutamate resulting in the degradation of Mfn2 and Mfn2-mediated mitochondrial fragmentation that precedes glutamate-induced neuronal death has been shown in primary spinal cord motor neurons (36). In a model of global ischemia, Mfn2 was found
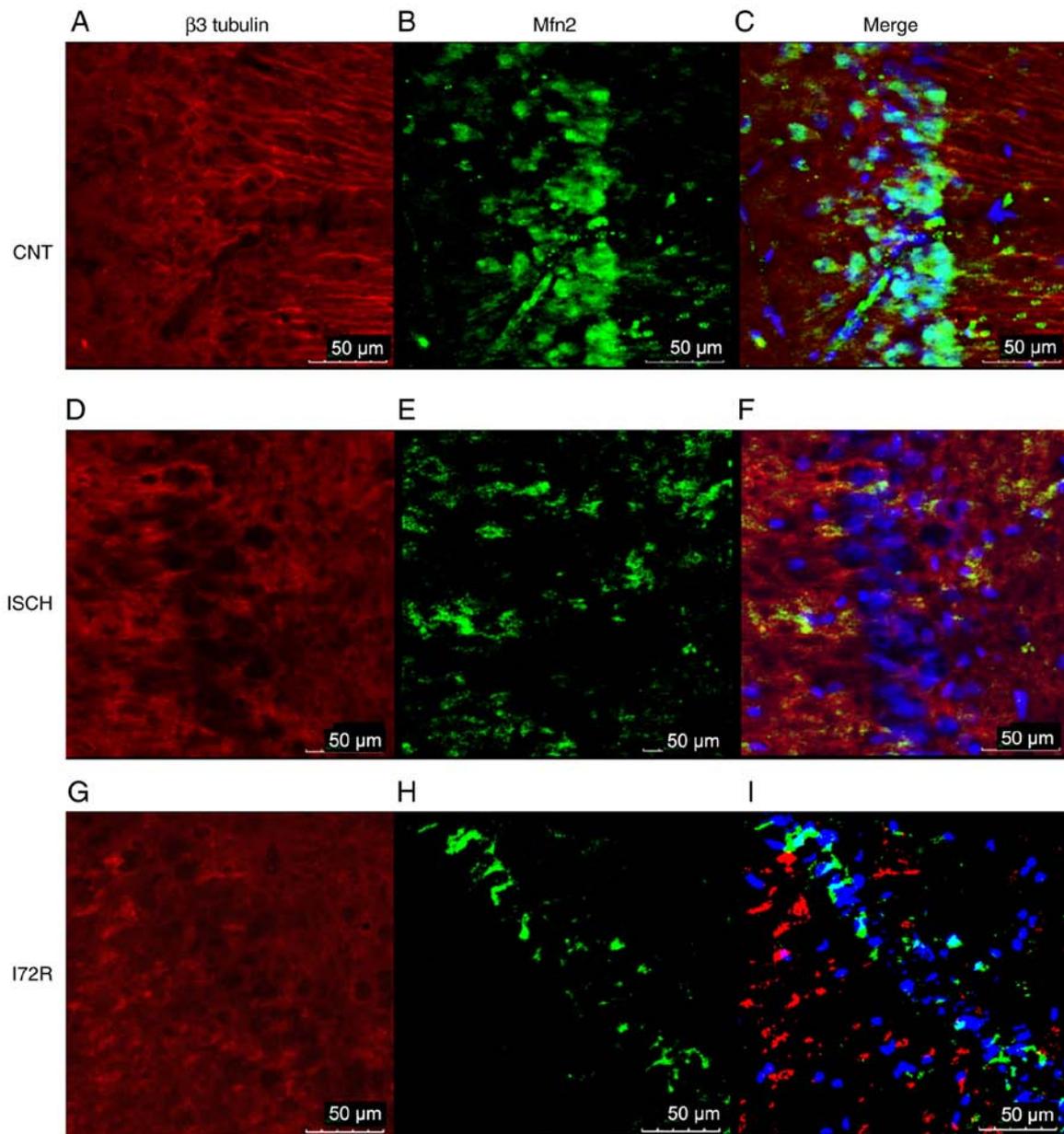
Figure 6. Fluorescence micrographs of cells positive for β3 tubulin (red) and Mfn2 (green) in the CA1 area of rat hippocampus. Nuclei are co-stained with DAPI (blue). (A-C) Micrographs of brain slices of control rats (CNT), (D-F) rats that were subjected to global brain ischemia for 15 min (ISCH), and (G-I) rats that underwent reperfusion for a duration of 72 h after 15 min of global brain ischemia (I72R). Bar, 50 μm. Mfn2, mitofusin 2.

to be upregulated in the mouse hippocampus after 2 and 72 h of reperfusion (26). In the present study, we also observed increased levels of Mfn2 in the rat hippocampus during early reperfusion (1 and 3 h), although the changes were not statistically significant. The upregulation of Mfn2 observed in the mouse hippocampus was paralleled with the upregulation of the inner mitochondrial membrane fusion protein OPA1 during the same reperfusion time (26). Despite the upregulation of the fusion proteins Mfn2 and OPA1, the mitochondria in the CA1 neurons of the hippocampus were fragmented, which has been attributed to the increased phosphorylation of DRP1 observed during late reperfusion (24 and 72 h) (26). In the present study, we did not observe significant changes in either the mitochondrial or total levels of DRP1 in either the hippocampus or the cortex in all experimental groups. However, the results of recent studies (37,38) have clearly

demonstrated that the process of mitochondrial fragmentation is regulated by post-translational modifications of DRP1. In the CA1 neurons of the rat hippocampus, fragmentation of mitochondria during late reperfusion after transient global brain ischemia has also been documented, but has been attributed to the release of OPA1 from the mitochondria (28). The fragmentation of mitochondria, caused by either the disruption of mitochondrial fusion resulting from the downregulation of Mfn2 and OPA1, or the stimulation of mitochondrial fission resulting from post-translation modifications of DRP1, appears to be a common result of ischemic insult.

In addition to the decreased Mfn2 level in cerebral cortex mitochondria, we observed increased total levels of VDAC1 in the hippocampus and cortex at 3 and 72 h after global brain ischemia lasting 15 min. In the hippocampus, increased levels of VDAC1 have also been documented by immunofluorescence

at 72 h after transient global brain ischemia lasting 10 min (29). An increase of VDAC1 in the hippocampus has been observed following the same reperfusion time, resulting in the increased mitochondrial levels of p53 (30,31) and BAD (39) observed after global brain ischemia. The involvement of VDAC1 in ischemic neuronal cell injury is unclear. In general, the involvement of VDAC1 in the mechanisms underlying cell death remains unclear (40,41). With respect to cell death, the results of recent research indicate that post-translational modifications of VDAC1 are even more important than the levels of this protein (42). In accord with this suggestion, the oligomerization of VDCA1 has been shown to play an important role in the process of mitochondrial fragmentation and dysfunction associated with the glutamate excitotoxicity that has often been implicated in the mechanisms underlying brain ischemia-reperfusion injury (43).

In conclusion, the results of the present study indicate that global brain ischemia is associated with the fast release of Mfn2 from mitochondria to the cytoplasm, followed by a decrease of the total Mfn2 level in the cerebral cortex. The release of Mfn2 from mitochondria, as observed during early reperfusion, may represent an important mechanism of mitochondrial dysfunction associated with the neuronal dysfunction or death induced by global brain ischemia.

Availability of materials and data

All the datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

KK, MK, MC and IP performed the experiments; KK and MK analyzed the data; ZT and PK analyzed the data and revised the manuscript for important intellectual content; PR initiated and supervised the study, designed the experiments and wrote the paper. All the authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal studies were performed according to the guideline for Animal Care and Health of the State Veterinary and Food Department of the Slovak Republic (approval no. 2414/06-221/3). The experiments were conducted in accordance with Directive 2010/63/EU of the European Parliament and of the Council for the protection of animals used for scientific purposes.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests to disclose.

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