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ORIGINAL RESEARCH

Ischaemia-Induced Protein Ubiquitinylation is Differentially Accompanied with Heat-Shock Protein 70 Expression After Naïve and Preconditioned Ischaemia

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Abstract The aim of this study was to investigate the effect of transient global brain ischaemia, both naïve and preconditioned, on accumulation of ubiquitinylated proteins and induction of stress/chaperone proteins specific to cytoplasm and endoplasmic reticulum. In addition, possible correlation between stress response and ischaemia/induced translocation of p53 to mitochondria was investigated. Rats were subjected to 15-min forebrain ischaemia followed by 1, 3, 24 and 72 h of reperfusion. Transient cerebral ischaemia induced a massive increase in protein ubiquitinylation in the hippocampus as well as in both cerebral and cerebellar cortex. Enhanced ubiquitinylation of proteins was paralleled with transcriptional activation of *hsp70.1* gene but not *hsp70.3* gene. However, HSP70 protein level was significantly elevated 24 and 72 h after ischaemia. Neither ischaemia nor ischaemia followed by reperfusion was associated with significant changes of GRP78, GADD34 and GADD153 levels. Ubiquitinylated protein level was elevated 1 and 48 h after sub-lethal 5 min ischaemia. Preconditioned ischaemia (15 min ischaemia followed 48 h after sub-lethal ischaemia) was associated with even enhanced accumulation of ubiquitinylated proteins of molecular mass higher than 110 kDa. HSP70 protein was significantly elevated 48 h after sub-lethal ischaemia as well as after preconditioned ischaemia and all investigated time intervals of reperfusion. The elevated level of HSP70 might represent plausible explanation of inhibition of both translocation of p53 to mitochondria and ischaemia-induced apoptosis observed after preconditioned ischaemia.

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Keywords Global brain ischaemia · Ubiquitin · Heat-shock protein · Mitochondria · Endoplasmic reticulum

Abbreviations

ER	Endoplasmic reticulum
DG	Dentate gyrus
GADD34	Growth arrest and DNA damage protein 34 (also known as MYD116)
GADD153	Growth arrest and DNA damage protein 153 (also known as CHOP)
GRP78	Glucose-regulated protein of 78 kDa (also referred to as BiP or immunoglobulin binding protein)
HSP70	Heat-shock protein 70
OHSCs	Organotypic hippocampal slice cultures
IPC	Ischaemic preconditioning
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
UPR	Unfolded protein response

Introduction

Transient global brain ischaemia represents a form of severe metabolic stress that has impact on all principal cellular molecular pathways including both synthesis and post-translational modifications of proteins (Lipton 1999). Post-translational modification of proteins by mono- or poly-ubiquitinylation is a central mechanism to modulate a wide range of cellular functions like protein stability, intracellular localisation of proteins, protein–protein interactions and transcriptional activity (Glickman and Ciechanover 2002). However, the most prominent function of

ubiquitin is labelling of aged, damaged and misfolded proteins for 26S proteasomal degradation. In addition, proteins involved in regulation of cell cycle, apoptosis initiation and execution as well as proteins involved in signal transduction are often labelled with ubiquitin and further eliminated by 26S proteasomal complex (Schrader et al. 2009; Shabek and Ciechanover 2010). An insufficient proteasome degradation capability to cope with overproduced abnormal proteins has been implicated in numerous neuropathologic conditions (Glickman and Ciechanover 2002; Muchowski and Wacker 2005; Meredith 2005) including ischaemic brain injury (Asai et al. 2002). Previous studies have documented massive accumulation of ubiquitin-conjugated protein aggregates, which takes place mainly in vulnerable ischaemic CA1 pyramidal neurons from the onset of reperfusion onward until delayed neuronal death occurs after 2–3 days of reperfusion (Hayashi et al. 1991; Gubellini et al. 1997; Hu et al. 2000; Liu et al. 2005a, b; Ge et al. 2007). Recent study has also documented similar global ischaemia-induced massive increase in SUMO-2/3 conjugation in the cortex and hippocampus of mouse brain observed after 3 and 6 h of reperfusion (Yang et al. 2008). Inhibition of 26S proteasome observed after global brain ischaemia (Kamikubo and Hayashi 1996; Asai et al. 2002; Ge et al. 2007) has been considered to be main mechanism responsible for ubiquitin-protein aggregates accumulation. In addition, ischaemia led to depletion of free ubiquitin in pyramidal neurones of hippocampus which was irreversible in CA1 hippocampal neurons (Magnusson and Wieloch 1989; Hayashi et al. 1991; Kato et al. 1993; Morimoto et al. 1996) whilst activities of ubiquitin conjugating enzymes were not affected (Kamikubo and Hayashi 1996). Ischaemia-induced accumulation of ubiquitinylated proteins is considered as possible cause of ischaemic-delayed neuronal death (Magnusson and Wieloch 1989). Recent experiments have supported the involvement of proteasomal stress in neuronal death. Stereotactic microinjection of the selective proteasome inhibitor epoxomicin into mouse hippocampus induced a delayed apoptosis within only the CA1 hippocampal neurons and not neurons within the CA3 or dentate gyrus (DG) regions, a selective vulnerability similar to that seen after transient global ischaemia (Tsuchiya et al. 2011). Finally, incubation of organotypic hippocampal slice cultures (OHSCs) with epoxomicin led to a selective injury of the CA1 pyramidal neurons, although similarly increased levels of poly-ubiquitinylated proteins were detected throughout all regions of the hippocampus (Bonner et al. 2010). However, accumulation of ubiquitinylated protein aggregates has also been observed after ischaemia using different protocols of ischaemic tolerance associated with significant neuroprotection (Kato et al. 1993; Ide et al. 1999; Liu et al. 2005a). This apparent discrepancy was explained by reversibility of proteasomal stress in vulnerable neurones, however, another factors contributing to elimination of toxic effects of ubiquitin-protein aggregates cannot be completely excluded.

Disturbances in ubiquitin–proteasome system are associated with activation of heat-shock response and induction of endoplasmic reticulum (ER) chaperones (Bush et al. 1997). Molecular chaperones are considered to be a first line of cellular defence against misfolded, aggregation-prone proteins and are amongst the most potent suppressors of neurodegeneration known for animal models of human diseases (Muchowski and Wacker 2005). It has been proposed that molecular chaperones are neuroprotective because of their ability to modulate the earliest aberrant protein interactions that trigger pathogenic cascades. Expression of HSP70 after global brain ischaemia has already been documented in several studies using different models of brain ischaemia and ischaemic tolerance (Liu et al. 1993; Nishi et al. 1993; Tanaka et al. 2004; García et al. 2004). In contrast, controversial results documenting expression of GRP78 after brain ischaemia have been obtained (Hayashi et al. 2003; García et al. 2004; Truettner et al. 2009).

The aim of this study was to investigate the relationship between accumulation of ubiquitinylated proteins induced by transient global brain ischaemia and possible induction of stress/chaperone proteins specific to cytoplasm and ER. Since the rapid and selective degradation of proteins following brief ischaemia can results in endogenous protection against ischaemia (Meller 2009), effect of ischaemic preconditioning (IPC), representing important phenomenon of neuroprotection induced by sub-lethal ischaemia (Dirnagl et al. 2003; Gidday 2006), on ischaemia-induced accumulation of ubiquitinylated proteins, expression of stress/chaperone proteins and initiation of mitochondrial apoptosis at the level of translocation of p53 to mitochondria was also investigated.

Materials and Methods

Ischaemia–Reperfusion and IPC

Animal studies were performed under a protocol approved by the State Veterinary and Food Department of Slovak Republic. A total of 90 adult male Wistar rats from the breeding house of the Institute of Experimental Pharmacology of Slovak Academy of Science (Dobra voda, Slovak Republic) 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 experiment. Transient global cerebral ischaemia was produced using the four-vessel occlusion model. Briefly, on day 1, both vertebral

arteries were irreversibly occluded by coagulation through the alar foramina after anaesthesia with 2% halothane, 30% O_2 and 68% N_2O mixture. On day 2, both common carotid arteries were occluded for 15 min by small clips under anaesthesia with 2% halothane, 30% O_2 and 68% N_2O mixture. Two minutes before carotid occlusion, the halothane was removed from the mixture. Body temperature was maintained using a homoeothermic blanket. Global ischaemia was followed by 1, 3, 24 and 72 h of reperfusion. IPC was induced by 5 min of sub-lethal global ischaemia followed by 48 h of reperfusion. The rats then underwent lethal ischaemia in duration of 15 min as above, followed by 1, 3, 24 and 72 h of reperfusion. After ischaemia and particular time of reperfusion, animals were sacrificed by decapitation under deep anaesthesia with 2% halothane, 30% O_2 and 68% N_2O mixture. Both hippocampi, cerebral and cerebellar cortex were dissected and processed immediately. Control animals for naïve ischaemia group underwent the same procedure except of carotid occlusion. Control animals of preconditioned ischaemia experimental group underwent 5 min of sub-lethal global ischaemia and they were sacrificed 48 h later.

Preparation of Protein Extracts and Isolation of Mitochondria

Protein extracts were prepared by homogenisation of either both hippocampi or cerebral and cerebellar cortex in homogenisation buffer (10 mM Tris–HCl pH = 7.4, 1 mM EDTA, 0.24 M sucrose) using a Potter Teflon-glass homogeniser. Total cell extract were prepared by addition of appropriate volume of 6× RIPA buffer (6× phosphate buffered saline, 6% (v/v) Nonidet P-40, 3% (w/v) sodium deoxycholate, 0.6% (w/v) sodium dodecyl sulphate (SDS) to homogenate.

Mitochondria were isolated by differential centrifugation. Homogenate was first centrifuged at 400*g* for 5 min and supernatant was then centrifuged at 12,000*g* for 10 min. Resulting sediment was resuspended in homogenisation buffer and centrifuged at 12,000*g* for 10 min. Final sediment was resuspended in homogenisation buffer and proteins were solubilised by addition of 20% SDS solution to final concentration of SDS 10%. Protein concentrations were determined by protein Dc assay kit (Bio-Rad) using BSA as standard.

Isolation of Total RNA and Semi-Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from dissected hippocampi using Trizol reagent (Invitrogen) following the manufacturer's protocol. Total RNA (5 μg) was reversely transcribed to cDNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas) kit according to protocol supplied by manufacturer. Aliquots of resulted cDNA corresponding to 0.35 μg of total RNA were used in PCR reaction. Sequences of primers used for amplification of particular mRNA were designed and verified using nucleotide database of National Centre for Biotechnology Information. Sequences of all primers used in this study are shown in Table 1. Amplification of the cDNAs was initiated by denaturation at 95 C for 2 min, followed by PCR cycles (denaturing at 95 C for 20 s, annealing at 60°C for 40 s and extension at 72°C for 40 s) and final extension at 72°C for 5 min in a DNA thermal cycler (Biometra). Number of cycles (Table 1) for each reaction was set up experimentally to observe a linear increase in the intensity level of PCR amplicons. The PCR products were electrophoresed through a 2% agarose gel and then stained with ethidium bromide. Semi-quantification of the photographic signals was performed using GeneTools image analysis system (SynGene). The entire width of the lane was analyzed with appropriate background subtraction. The relative mRNA level was defined as the ratio of the particular amplicon signal to that of GAPDH. The ratio of the signal of particular amplicon to signal of GAPDH of control sample has been considered to be 100%.

Quantitative Western Blot Analysis

Isolated proteins were separated by SDS-PAGE. After electrophoresis, separated proteins were transferred on 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 solution (Candor), membranes were first incubated for 90 min with primary antibodies against ubiquitin (1:500, P4D1: sc-8017, Santa Cruz), β-actin (1:2000, C-4: sc-47778, Santa Cruz), GRP78 (1:200, C-20: sc-1054, Santa Cruz), HSP70 (1:200, C92F3A-5: sc-66048, Santa Cruz), GADD34

Table 1 Primer sequences, amplicon size (base pairs) and number of amplification cycles

Gene	Sequence of front primer	Sequence of reverse primer	Amplicon size	Number of cycles
Hsp70.1	CGA CCT GAA CAA GAG CAT CAA TC	CTT GTC GTT GGT GAT GGT GAT CT	451	40
Hsp70.3	GTG ATG ACT GTT CTG ATC AAG CG	CTT GTC GTT GGT GAT GGT GAT CT	297	35
Gapdh	GAG CTG AAC GGG AAG CTC ACT GG	GTG AGG GAG ATG CTC AGT GTT GG	430	20

(1:200, S-20: sc-824, Santa Cruz) GADD153 (1:200, F-168: sc-575, Santa Cruz), p53 (1:200, FL-393: sc-6243, Santa Cruz) and COXI (1 μg/ml, 1D6, Molecular Probes) dissolved in BSA blocking solution (Candor). In the case of HSP70 or GRP78, membranes incubated with primary antibodies were washed using washing solution (Candor) and then incubated with secondary antibodies conjugated with horse radish peroxidase (1:5000, Santa Cruz). Incubation of membranes with other primary antibodies was followed by extensive washing and consequent incubation of membranes with particular biotinylated secondary antibodies (1:10 000, Vector Laboratories). Membranes incubated with biotinylated secondary antibodies were washed 3 times 10 min and then incubated with avidin–biotin-conjugated peroxidase (Vector Laboratories). After extensive washing (4 times 15 min), membranes incubated either with peroxidase-conjugated secondary antibodies or with avidin–biotin-conjugated peroxidase (Vector Laboratories) solution were incubated in SuperSignal West Pico Chemiluminescent Substrate (Pierce) solution for 3 min. After exposition of membranes on Chemidoc XRS (Bio-Rad), the intensities of corresponding bands were quantified using Quantity One software (BioRad). Intensities of bands of interest were normalised by corresponding intensities of bands of β-actin.

Statistical Analysis

All statistical analyses were done using GrafPhad InStat V2.04a (GrafPhad Software). For the comparison of ischaemia-induced changes amongst all groups, a one-way ANOVA test was first carried out to test for differences amongst all experimental groups. In addition, the unpaired Tukey's test was used to determine differences between individual groups. Significance level was set at $P < 0.05$.

Results

Ischaemia-Induced Accumulation of Ubiquitinylated Proteins in Hippocampus is Accompanied with Delayed Expression of HSP70 and But is Not Associated with Significant Changes in Expression of ER Chaperone GRP78 and ER Stress Effector Proteins GADD34 and GADD153

The pattern of ubiquitinylated proteins in total cell extracts of samples derived from hippocampus of control animals and experimental animals subjected to 15 min of transient global brain ischaemia and 1, 3, 24 or 72 h of reperfusion is shown in Fig. 1. Massive accumulation of ubiquitinylated

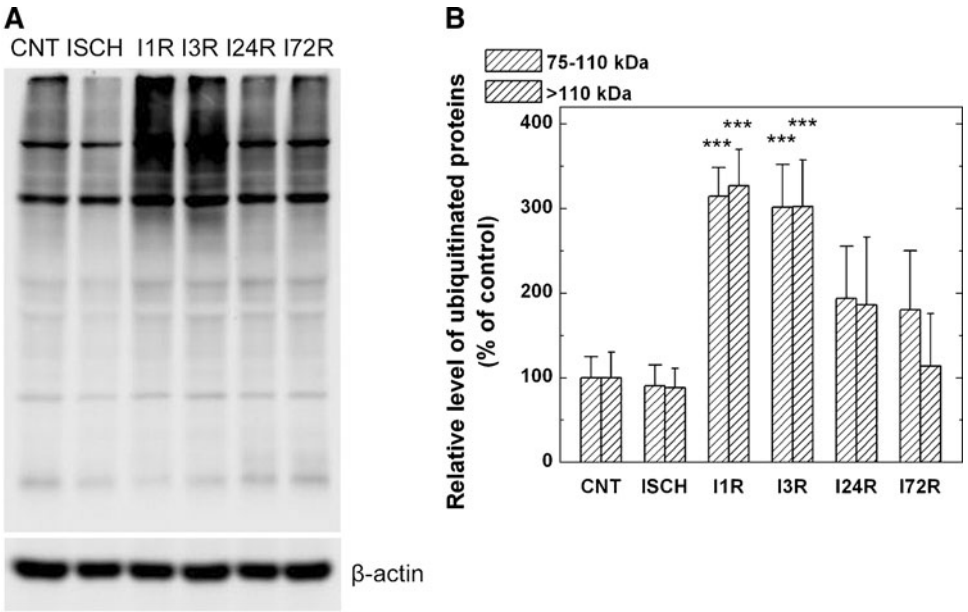

Fig. 1 **a** Effect of transient global brain ischaemia on protein modification with ubiquitin. Rats were subjected to 15 min of transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. The pattern of protein ubiqitinylation was evaluated by Western blot analysis of total cell extracts prepared from the hippocampus of control and experimental rats, as described in 'Materials and Methods'. Bands of molecular mass 75 and 110 kDa represent endogenous biotinylated proteins. **b** Quantification of the post-ischaemic rise in ubiquitinylated proteins in the hippocampus. For analysis of ubiqitinylation of hippocampal proteins, two higher molecular weight areas, between 75 and 110 kDa (determined by signal of endogenous biotinylated proteins) as well as above 110 kDa in each lane were cropped and analyzed. The data were normalised to β-actin level and expressed relative to controls. Data are presented as means ± SD ($n = 5$ per group). ***$P < 0.001$ (ANOVA, followed by Tukey's test to determine differences between individual groups)

proteins was observed in hippocampus 1 and 3 h after ischaemia (Fig. 1). Since, hippocampal CA1 layer of pyramidal neurones represents the selective vulnerable population, the quantitative analysis of the levels of ubiquitinylated proteins in hippocampal total cell extracts was performed. Each lane was cropped to the two higher molecular weight areas (between 75 and 110 kDa, determined by signals of endogenous biotinylated proteins, and above 110 kDa) for analysis of ischaemia-induced changes of the levels of ubiquitin-conjugated proteins. Ischaemia did not significantly affect the levels of ubiquitinylated proteins. Significant increase of ubiquitinylated protein levels was observed in the hippocampus 1 and 3 h after 15 min of ischaemia (Fig. 1). Amount of ubiquitinylated proteins of molecular mass between 75 and 110 kDa was elevated to 314.3% ($P < 0.001$) and 301.4% ($P < 0.001$) of control after 1 and 3 h of reperfusion, respectively. Proteins of molecular mass higher than 110 kDa were elevated to 326.8% ($P < 0.001$) and 302.3% ($P < 0.001$) of control after 1 and 3 h of reperfusion, respectively.

Since accumulation of ubiquitinylated proteins due to disturbances in ubiquitin–proteasome system is associated with activation of heat-shock response and induction of ER chaperones (Bush et al. 1997), the time course of expression of the main cytoplasmic stress protein HSP70 as well as ER-specific chaperone GRP78 and ER-stress effector proteins GADD34 and GADD153 was investigated.

Unlike accumulation of ubiquitinylated proteins, different time course of HSP70 expression was observed (Fig. 2). Whilst the level of HSP70 protein in total cell extracts from hippocampi of control, ischaemia and early reperfusion was close to Western blot detection limit, significant increase of HSP70 protein amount was documented 24 and 72 h after 15 min of global ischaemia [to 524.6% ($P < 0.001$) and

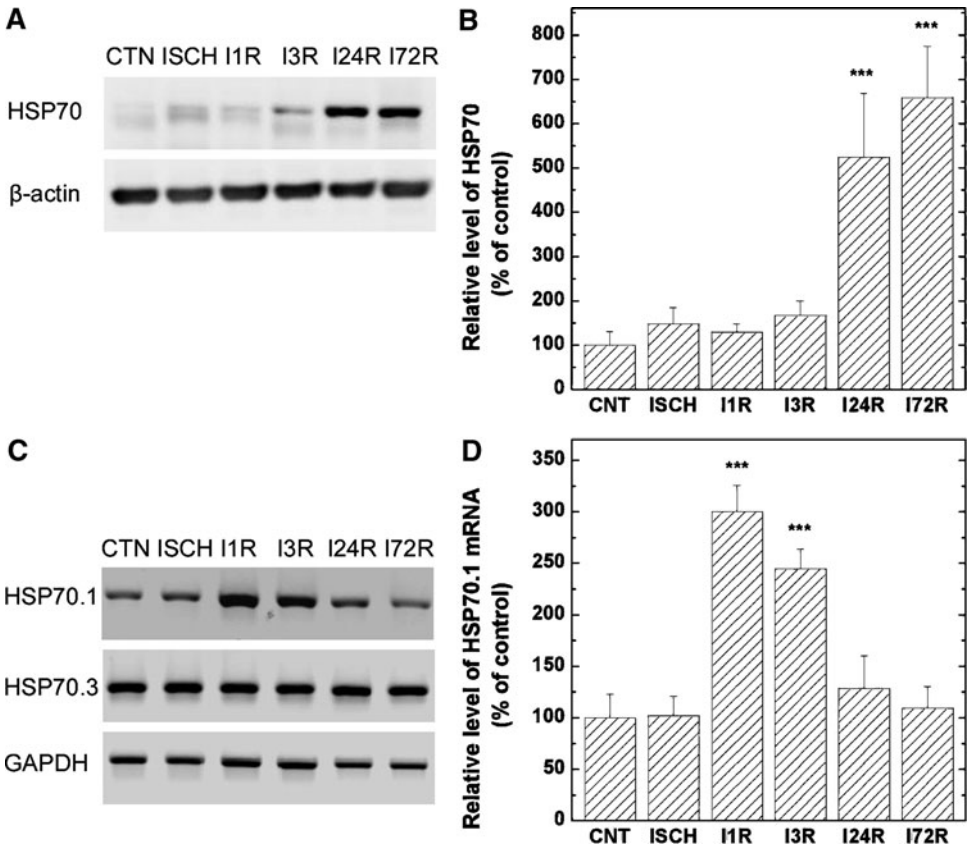

Fig. 2 **a** Effect of transient global brain ischaemia on HSP70 protein level. Rats were subjected to 15 min of transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. The pattern of protein ubiqitination was evaluated by Western blot analysis of total cell extracts prepared from both hippocampi of control and experimental rats as described in 'Materials and Methods'. **b** Quantification of the post-ischaemic rise in HSP70 protein in the hippocampus. The data were normalised to β-actin level and expressed relative to control. Data are presented as means ± SD ($n = 5$ per group). *$P < 0.05$ (ANOVA, followed by Tukey's test to determine differences between individual groups). **c** Effect of transient global brain ischaemia on transcription of *hsp70.1* and *hsp70.3* genes. Rats were subjected to 15 min of transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. The level of HSP70.1 and HSP70.3 mRNA was evaluated by RT-PCR analysis of total RNA isolated from hippocampi of control and experimental rats, as described in 'Materials and Methods'. GAPDH served as loading control. **d** Quantification of the post-ischaemic rise in HSP70.1 mRNA in the hippocampus. The data were normalised to GAPDH mRNA level and expressed relative to controls. Data are presented as means ± SD ($n = 3$ per group). ***$P < 0.001$ (ANOVA, followed by Tukey's test to determine differences between individual groups)

658.1% ($P < 0.001$) of control, respectively]. The expression of protein HSP70 after transient global ischaemia was also investigated at the level of transcription. RT-PCR determination of mRNA level revealed that HSP70.1 mRNA was significantly elevated 1 and 3 h after 15 min of ischaemia [to 300.3% ($P < 0.001$) and 244.5% ($P < 0.001$) of control, respectively] (Fig. 2). However, transient global brain ischaemia as well as consequent reperfusion did not alter amount of HSP70.3 mRNA.

Unlike HSP70, GRP78 has been already detected in total cell extracts from hippocampi of control animals and neither ischaemia nor ischaemia followed by reperfusion has been associated with significant changes of GRP78 level (Fig. 3). Similar to GRP78, both GADD34 and GADD153 have been already detected in total cell extracts from hippocampi of control animals and their levels have not been significantly affected by ischaemia and ischaemia followed by reperfusion (Fig. 3).

Sub-lethal Ischaemia Increases Both Level of Ubiquitinylated Proteins and HSP70 48 h After Ischaemia

Since the rapid and selective degradation of proteins following brief ischaemia can result in endogenous protection against ischaemia (Meller 2009), the impact of brief sub-lethal ischaemia on accumulation of ubiquitinylated proteins and expression of HSP70 was also investigated. As shown on Fig. 4, the level of ubiquitinylated proteins of molecular mass 75–110 kDa has been significantly increased 1 and 48 h after sub-lethal global brain ischaemia in duration of 5 min (to 141% ($P < 0.05$) and 139.8% ($P < 0.05$) of control, respectively). Similarly, ubiquitinylated proteins of molecular mass higher than 110 kDa were

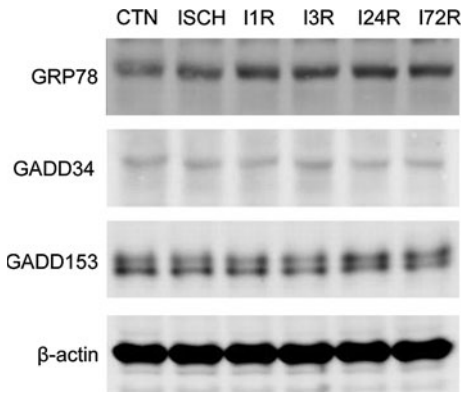

Fig. 3 Effect of transient global brain ischaemia on GRP78, GADD34 and GADD153 protein level. Rats were subjected to 15 min of transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. The pattern of protein expression was evaluated by Western blot analysis of total cell extracts prepared from both hippocampi of control and experimental rats as described in 'Materials and Methods'. β-actin served as loading control

significantly elevated 1 and 48 h after 5 min of ischaemia [to 173.6% ($P<0.001$) and 159.8% ($P<0.001$) of control, respectively]. However, HSP70 protein level has been significantly elevated only 48 h after 5 min of ischaemia [to 347.9% ($P < 0.05$) of control]. Sub-lethal ischaemia followed by 1 and 48 h of reperfusion was not associated with translocation of p53 to mitochondria (Fig. 4).

Lethal Ischaemia Followed 48 h After Sub-lethal Ischaemia is Associated with Increased Accumulation of Ubiquitinylated Proteins of Molecular Mass Higher Than 110 kDa

Sub-lethal ischaemia is associated with significant neuroprotection to consequent lethal ischaemia. This phenomenon known as IPC represents important mechanism of neuroprotection (Dirnagl et al. 2003; Gidday 2006). The effect of IPC on the level of ubiquitinylated proteins has also been investigated. IPC has been induced by sub-lethal ischaemia in duration of 5 min and 48 h latter lethal global brain ischaemia in duration of 15 min was induced. Such protocol is associated with significant resistance of vulnerable pyramidal neurones of hippocampal CA1 layer to ischaemic insult (Racay et al. 2009) as well as inhibition of p53 translocation to mitochondria (Racay et al. 2007, 2009). Likewise naïve ischaemia, preconditioned ischaemia is associated with increased accumulation of ubiquitinylated proteins after 1 and 3 h of reperfusion (Fig. 5). Amount of ubiquitinylated proteins of molecular mass between 75 and 110 kDa was elevated to 379.5% ($P < 0.001$) and 377% ($P < 0.001$) of naïve control after 1 and 3 h of reperfusion, respectively. Although, these values were higher than those observed after naïve ischaemia and consequent reperfusion in duration 1 and 3 h [310.9% ($P < 0.01$) and 293.8% ($P < 0.05$) of naïve control, respectively], the difference was not statistically significant. However, ubiquitinylated proteins of molecular mass higher than 110 kDa were elevated to 517% ($P < 0.001$) and 360.4% ($P < 0.001$) of naïve control after preconditioned ischaemia followed by 1 and 3 h of reperfusion, respectively. The value obtained after preconditioned ischaemia followed by 1 h of reperfusion (517% of naïve control) was significantly higher, ($P < 0.001$), than those obtained after naïve ischaemia followed by 1 h of reperfusion (322.5% of naïve control).

Lethal Ischaemia Followed 48 h After Sub-lethal Ischaemia is Associated with Elevated Levels of HSP70 But Not GRP78 as well as with Inhibition of Ischaemia-Induced Translocation of p53 to Mitochondria

The impact of 15 min of global brain ischaemia 48 h after sub-lethal ischaemia in duration of 5 min on the levels of

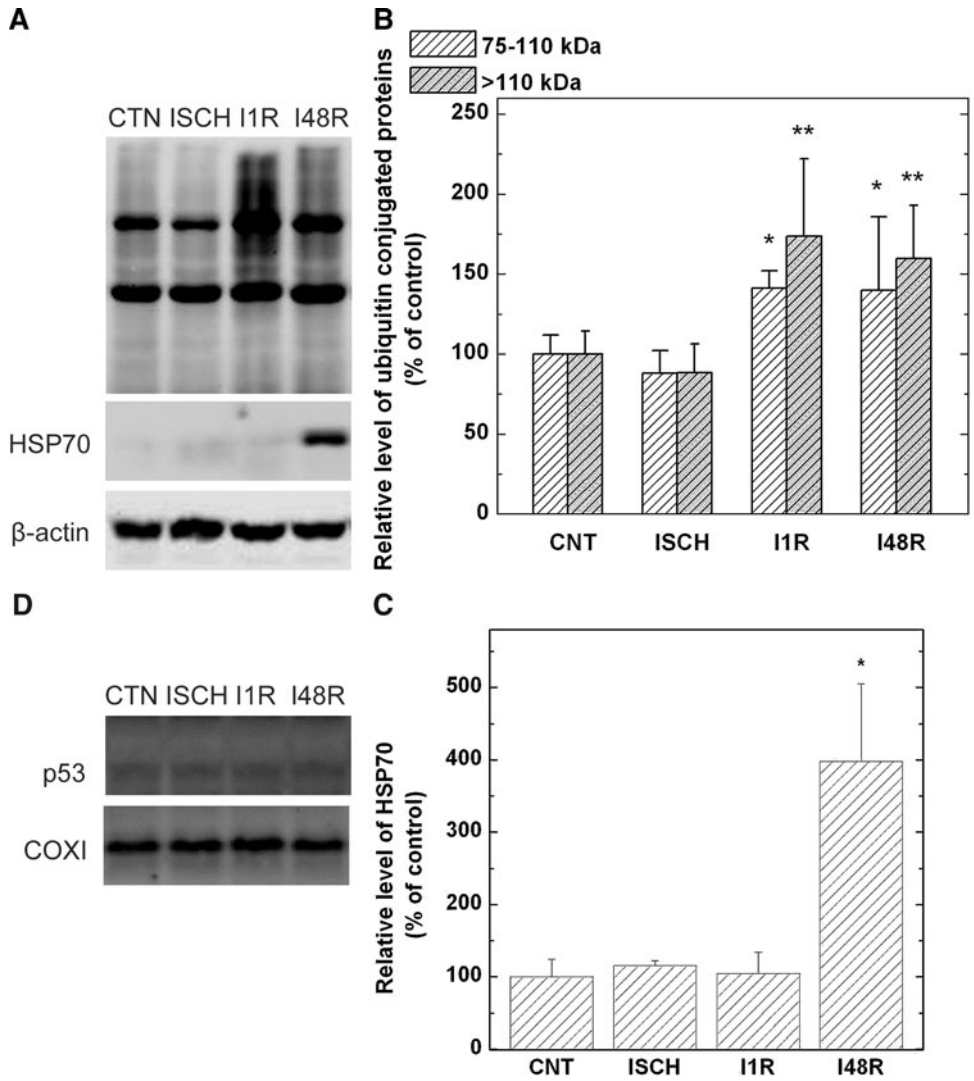

Fig. 4 **a** Effect of transient sub-lethal global brain ischaemia on protein modification with ubiqitin and expression of HSP70. Rats were subjected to 5 min of transient global brain ischaemia and 1 and 48 h of reperfusion. The pattern of protein ubiqitinylation as well as HSP70 protein level was evaluated by Western blot analysis of total cell extracts prepared from both hippocampi of control and experimental rats, as described in 'Materials and Methods'. **b** Quantification of the post-ischaemic rise in ubiquitinylated proteins in the hippocampus. For analysis of ubiqitinylation of hippocampal proteins, two higher molecular weight areas, between 75 and 110 kDa (determined by signal of endogenous biotinylated proteins) as well as above 110 kDa in each lane were cropped and analyzed. Data are presented as mean ± SD ($n = 4$ per group). *$P < 0.05$, **$P < 0.01$ (ANOVA, followed by Tukey's test to determine differences between individual groups). **c** Quantification of the post-ischaemic rise in HSP70 protein level in the hippocampus. The data were normalised to β-actin level and expressed relative to controls. Data are presented as mean ± SD ($n = 4$ per group). *$P < 0.05$ (ANOVA, followed by Tukey's test to determine differences between individual groups). **d** Effect of sublethal global brain ischaemia on mitochondrial level of p53. Rats were subjected to 5 min of transient global brain ischaemia and 1 and 48 h of reperfusion. The mitochondrial level of p53 was determined by Western blot analysis of mitochondria isolated from control and experimental animals as described in 'Materials and Methods'

stress proteins HSP70 and GRP78 has been investigated as well (Fig. 6). The level of HSP70 was already elevated in preconditioned control (i.e., after sub-lethal ischaemia in duration of 5 min and consequent 48 h of reperfusion) to 578% of naïve control ($P < 0.05$). The level of HSP70 was significantly elevated after preconditioned ischaemia [837% of naïve control ($P < 0.001$)] as well as after preconditioned ischaemia followed by 1, 3, 24 and 72 h of reperfusion [632% ($P < 0.05$), 732.7% ($P < 0.01$), 588.5% ($P < 0.05$) and 805.1% ($P < 0.001$) of naïve control].

Similar to the results obtained after naïve ischaemia, neither preconditioned ischaemia nor preconditioned ischaemia followed by all investigated intervals of reperfusion has been associated with significant changes of GRP78 level (Fig. 6).

Finally, mitochondrial level of p53 protein after both naïve and preconditioned ischaemia has been investigated

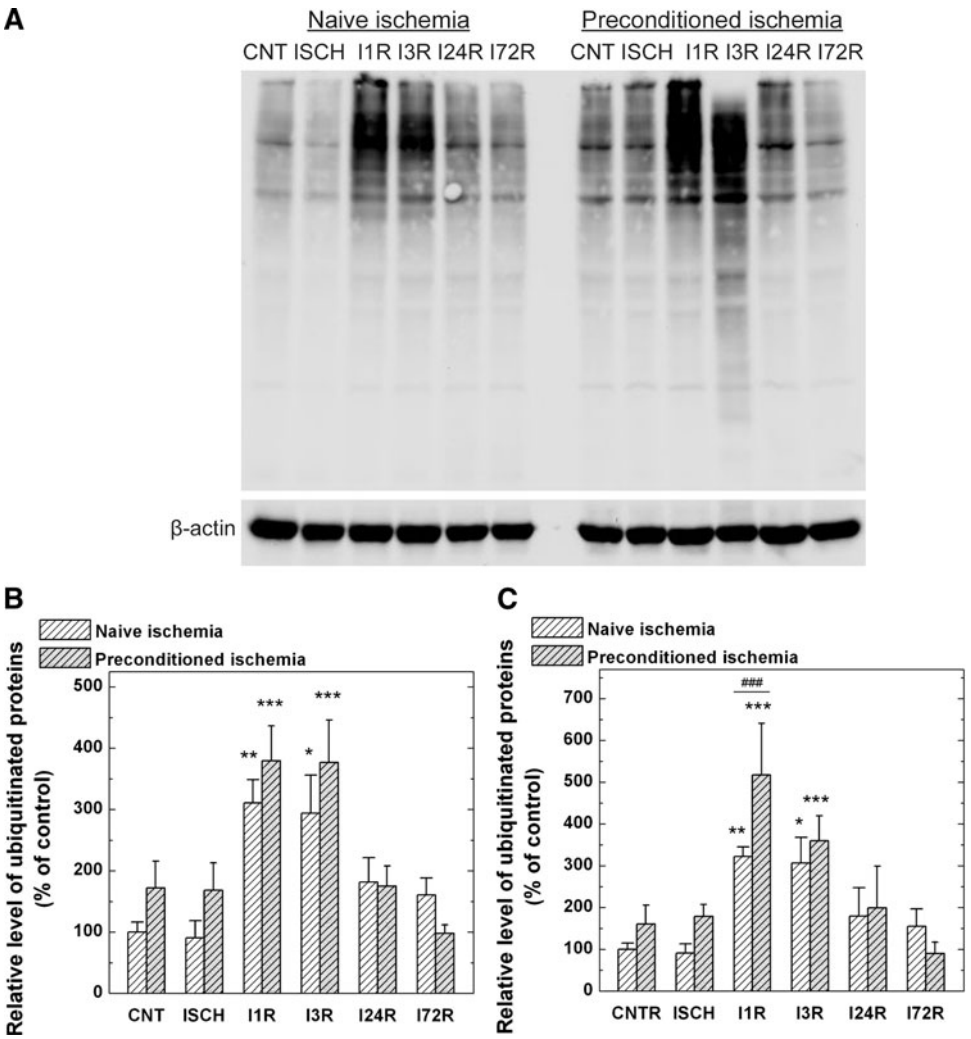

Fig. 5 **a** Effect of naïve and preconditioned global brain ischaemia on protein modification with ubiqitin in the hippocampus. Naïve rats were subjected to 15 min of transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. Preconditioned rats were first subjected to 5 min of transient global brain ischaemia. 48 h later, they were subjected to 15 min of transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. The pattern of protein ubiqitinylation was evaluated by Western blot analysis of total cell extracts prepared from both hippocampi of experimental animals, as described in 'Materials and Methods'. **b** Quantification of the post-ischaemic rise in ubiquitinated proteins of molecular weight between 75 and 110 kDa. For analysis of ubiqitinylation of hippocampal proteins, molecular weight area between 75 and 110 kDa (determined by signal of endogenous biotinylated proteins) in each lane was cropped and analyzed. The data were normalised to β-actin level and expressed relative to controls. Data are presented as mean ± SD ($n = 5$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ statistically significantly different from control (ANOVA, followed by Tukey's test to determine differences between individual groups). **c** Quantification of the post-ischaemic rise in ubiquitinated proteins of molecular weight above 110 kDa. For analysis of ubiqitinylation of hippocampal proteins, molecular weight area above 110 kDa in each lane was cropped and analyzed. The data were normalised to β-actin level and expressed relative to controls. Data are presented as mean ± SD ($n = 5$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ statistically significantly different from control; $^{\#\#\#}P < 0.001$ statistically significantly different from naïve ischaemia followed by 1 h of reperfusion (ANOVA, followed by Tukey's test to determine differences between individual groups)

to correlate possible differences in translocation of p53 to mitochondria with the observed changes at the level of ubiquitinylated proteins and HSP70. Similar to previous results (Racay et al. 2007, 2009), naïve ischaemia is associated with significantly elevated levels of p53 in mitochondria isolated from hippocampi of rats exposed to 15 min of ischaemia followed by 3, 24 and 72 h of reperfusion [225.4% ($P < 0.05$), 326.2% ($P < 0.001$) and 265% ($P < 0.05$) of naïve control, respectively] (Fig. 6). Translocation of p53 to mitochondria was significantly attenuated by IPC since nonsignificant changes of mitochondrial p53 were documented after preconditioned ischaemia and all investigated intervals of reperfusion (Fig. 6).

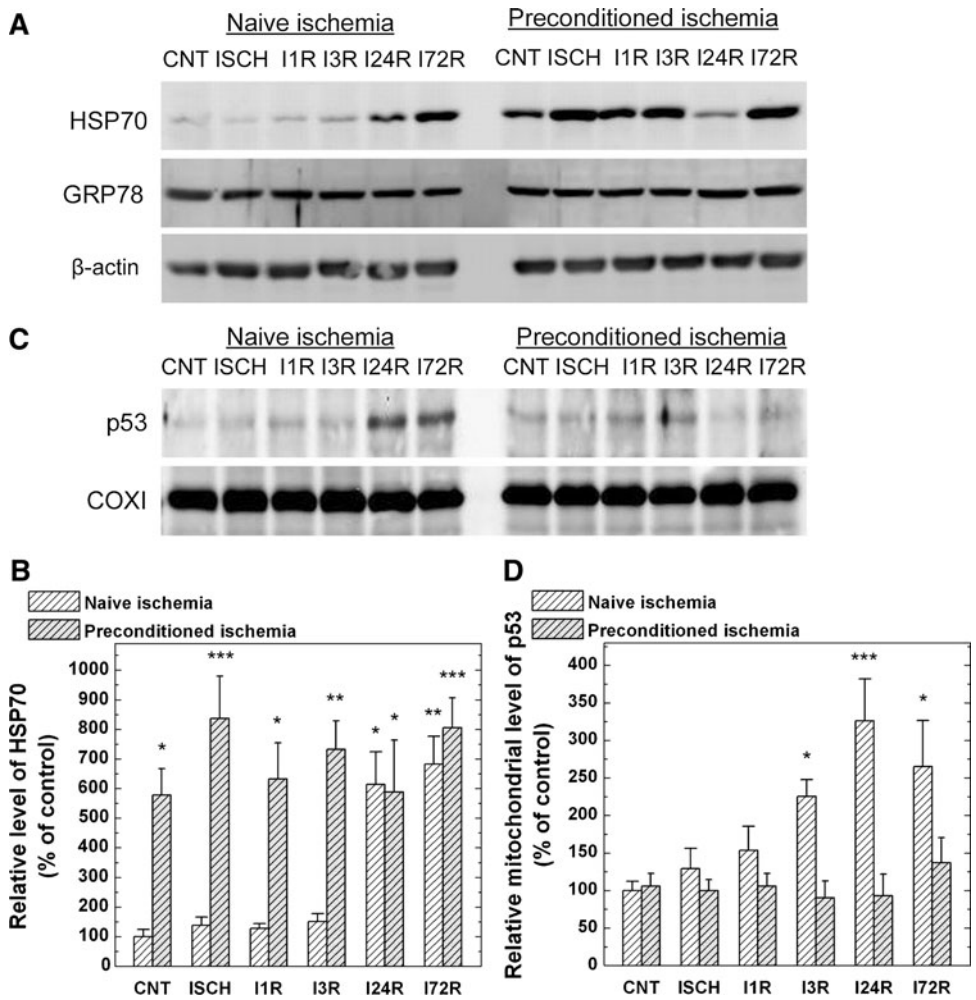

Fig. 6 **a** Effect of naïve and preconditioned global brain ischaemia on expression of HSP70 and GRP78. Naïve animals were subjected to 15 min of transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. Preconditioned rats were first subjected to 5 min of transient global brain ischaemia. 48 h later, they were subjected to 15 min transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. The level of HSP70 and GRP78 was evaluated by Western blot analysis in total cell extracts prepared from the hippocampus of experimental animals, as described in 'Materials and Methods'. **b** Quantification of the post-ischaemic rise in HSP70 protein level in the hippocampus. The data were normalised to β-actin level and expressed relative to controls. Data are presented as mean ± SD ($n = 5$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA, followed by Tukey's test to determine differences between individual groups). **c** Effect of naïve and preconditioned global brain ischaemia on mitochondrial level of p53. Naïve rats were subjected to 15 min of transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. Preconditioned rats were first subjected to 5 min of transient global brain ischaemia. 48 h later, they were subjected to 15 min transient global brain ischaemia and 1, 3, 24 and 72 h of reperfusion. The mitochondrial level of p53 was determined by Western blot analysis of mitochondria isolated from control and experimental animals as described in 'Materials and Methods'. **d** Quantification of the post-ischaemic changes in p53 protein level in the hippocampal mitochondria. The data were normalised to COXI level and expressed relative to controls. Data are presented as mean ± SD ($n = 5$ per group). $^{*}P < 0.05$, $^{***}P < 0.001$ (ANOVA, followed by Tukey's test to determine differences between individual groups)

Discussion

In this study, it has been demonstrated that ischaemia-induced massive accumulation of ubiquitinylated proteins in hippocampus, observed after both sub-lethal and lethal ischaemia, is accompanied with delayed expression of HSP70 and but is not associated with significant changes in expression of ER-specific chaperone GRP78 and ER-stress effector proteins GADD34 and GADD153. However, preconditioned ischaemia was associated with simultaneous accumulation of ubiquitinylated proteins and expression of HSP70. Massive accumulation of ubiquitin-conjugated protein aggregates after transient global brain ischaemia has already been documented in several previous studies (Hayashi et al. 1991; Gubellini et al. 1997; Hu et al. 2000; Liu et al. 2005a, b; Ge et al. 2007). Expression of cytoplasmic molecular chaperone HSP70 after global brain ischaemia has also been documented using different

models of transient global brain ischaemia and ischaemic tolerance (Liu et al. 1993; Nishi et al. 1993; Tanaka et al. 2004; García et al. 2004; Truettner et al. 2009). In contrast, controversial results documenting expression of ER-specific chaperone GRP78 after brain ischaemia have been obtained (Hayashi et al. 2003; García et al. 2004; Truettner et al. 2009).

The aggregation of ubiquitinylated proteins after brain ischaemia reflects a quantitative imbalance between the amounts of toxic unfolded proteins and the capacity of proteasomal system to eliminate them. Molecular chaperones are considered to be a first line of cellular defence against misfolded, aggregation-prone proteins and are amongst the most potent suppressors of neurodegeneration known for animal models of human diseases (Muchowski and Wacker 2005). In fact, disturbances in ubiquitin–proteasome system are associated with activation of heat-shock response and induction of ER chaperones (Bush et al. 1997). Upregulation of both HSP70 mRNA and protein level in primary cortical neurones was documented after partial inhibition of 26S proteasome by lactacystin (Yew et al. 2005). The results presented in this study showed specific transcriptional activation of *hsp70.1* gene, coding for HSP70, within the same period of reperfusion as accumulation of ubiquitinylated proteins. Whilst significant increase of the level of HSP70.1 mRNA was observed 1 and 3 h after ischaemia, the level of HSP70 protein was significantly elevated 24 and 72 h after ischaemia. This fact might indicate that abundance of HSP70 protein is controlled by an unknown post-transcriptional regulation as it has also been suggested recently studying the neuroprotective effect of moderate hypothermia on focal ischaemia-induced brain injury (Terao et al. 2009). Finally, specific activation of *hsp70.1* transcription is in hand with recent studies documenting enhanced initiation of mitochondrial apoptosis after focal brain ischaemia in *hsp70.1* knockout mice (Lee et al. 2004) and the enhanced transcription of *hsp70.1* after infusion of geldanamycin into the mouse brain (Kwon et al. 2009). In addition to cytoplasmic stress response, proteasomal stress is accompanied by activation of ER stress signalling pathway. Unfolded protein response (UPR), cellular stress response related to ER, is often implicated amongst mechanisms activated after global brain ischaemia due to inhibition of protein synthesis (Paschen 2003; Degracia and Hu 2007). In addition, GRP78 represents the main ER chaperone/stress protein with strong impact on survival of Purkinje neurones (Wang et al. 2010). Similar to cytoplasmic stress response, treatment of cultured cortical neurons with the proteasomal inhibitor lactacystin is also associated with up-regulation of ER stress-related genes (Choy et al. 2011). ER stress is also considered as the main trigger of protein ubiquitinylation since exposure of cerebellar granule neurons to the ER stressor tunicamycin induced strong ER stress response accompanied with massive ubiquitinylation and accumulation of ubiquitin-protein aggregates (Concannon et al. 2008). In hand with previous studies showing that ischaemia–reperfusion does not induce expression of the ER stress effector proteins 55-kd XBP-1 and GADD34 (Kumar et al. 2003) as well as GRP78 (García et al. 2004) enhanced expression of ER-specific chaperone, GRP78, as well as expression of two other ER-stress effector proteins, GADD34 and GADD153, was not observed after ischaemia–reperfusion in this study. In hand with Truettner et al. (2009), the results present in this study also indicate that ischaemia-induced proteasomal stress is associated with specific induction of cytoplasmic stress proteins but does not induce ER-specific stress proteins.

The key question whether proteasomal dysfunction and aggregation of ubiquitinylated proteins during the early period of reperfusion can eventually lead to delayed neuronal death after brain ischaemia is not yet clear. Although, glutamate excitotoxicity represents the most prominent pathway connecting cerebral blood flow arrest to delayed neuronal death (Lau and Tymianski 2010), neither ubiquitinylation nor ER stress response was observed in the in vitro models of glutamate- and NMDA-induced excitotoxic apoptosis (Concannon et al. 2008). In addition, expression of both pro-survival and pro-apoptotic proteins have been documented after treatment of neuronal cell lines with proteasomal inhibitors (Yew et al. 2005; Butts et al. 2005; Suh et al. 2005; Choy et al. 2011). However, there are consistent evidences demonstrating that defects in the ubiquitin–proteasomal pathway induce neuronal death virtually in all pathologic conditions (Lang-Rollin et al. 2004; Mytilineou et al. 2004). Conversely, enhancing proteasomal function by overexpression of proteasomal proteolytic subunits or ubiquitin ligases protects cells from lethal stress (Chondrogianni et al. 2005). Stereotactic intrahippocampal microinjection of the selective proteasome inhibitor epoxomicin induced a delayed apoptosis within only the CA1 hippocampal neurons and not neurons within the CA3 or DG regions, a selective vulnerability similar to that seen during ischaemia (Tsuchiya et al. 2011). Finally, incubation of OHSCs with the specific proteasome inhibitors, epoxomicin or bortezomib, led to a selective injury of the CA1 pyramidal neurons although similarly increased levels of poly-ubiquitinylated proteins were detected throughout all regions of the hippocampus (Bonner et al. 2010). Based on the results of Marchenko et al. (2007), prolonged life time of monoubiqitinylated p53 due to ischaemia-induced dysfunction of ubiquitin–proteasomal system could be associated with increased probability of p53 translocation to mitochondria. Such mechanism might represent one plausible explanation of p53-dependent mitochondrial apoptosis initiation observed

after transient global brain ischaemia (Endo et al. 2006; Racay et al. 2007, 2009). The involvement of proteasomal stress in delayed neuronal death is apparently puzzled by effect of sub-lethal and preconditioned ischaemia on accumulation of ubiquitinylated protein aggregates. With respect to sub-lethal ischaemia, accumulation of ubiquitinylated proteins also precede expression of HSP70, however, it seems that amount of ubiquitinylated protein aggregates is not high enough to induce translocation of p53 to mitochondria. In contrast to Liu et al. (2005a), massive accumulation of ubiquitinylated protein aggregates, for the aggregates of molecular mass above 110 kDa even significantly higher, has been observed in this study after preconditioned ischaemia. This indicates that despite accumulation of ubiquitinylated protein aggregates, simultaneous expression of HSP70 might prevent consequent induction of delayed cell death since the protocol of IPC used in this study is associated with significant neuroprotection (Racay et al. 2009). Thus, p53 activated due to ischaemia-induced proteasomal stress might be neutralized by binding to HSP70. The binding of active p53 to HSP70 has already been documented in some types of cancer cell lines (Matsumoto et al. 1994; Esser et al. 2005; Sherman et al. 2007; Muller et al. 2008) with disruption of this interaction demonstrated to induce cell death (Leu et al. 2009). The results presented here are consistent with such view as well as with recent knowledge about anti-apoptotic properties of HSP70 (Steel et al. 2004; Stankiewicz et al. 2005). Whilst after naïve ischaemia translocation of p53 to mitochondria precedes expression of HSP70 protein (Fig. 6), the level of HSP70 protein was significantly elevated 48 h after sub-lethal ischaemia as well as after preconditioned ischaemia and all investigated time intervals of reperfusion. Thus, expression of HSP70 during preconditioned ischaemia and reperfusion possibly precedes activation of p53 and consequently inhibits translocation of active p53 to mitochondria. HSP70 protein expression was observed in CA1, CA3 and dentate hilar neurons at the time intervals of reperfusion after ischaemia, both naïve and preconditioned (Liu et al. 1993; Nishi et al. 1993), comparable to reperfusion intervals investigated in this study. In addition, involvement of HSP70 in IPC-induced neuroprotection has already been proposed (Nishi et al. 1993; Tanaka et al. 2004). As HSP70 exerts a protective role against ischaemic damage, the specific increase in HSP70 production before activation of p53 may contribute to the neuroprotective effect of IPC. In addition, the results presented here are also consistent with recent studies. Bonner et al. (2010) have demonstrated that induction of HSP70 by treatment of OHCSs with geldanamycin inhibited the selective activation of p53 signalling within the CA1 neurons and protected CA1 neurons from epoxomicin-induced cell death. Finally, the treatment of OHSCs with geldanamycin 24 h before oxygen glucose deprivation induced HSP70 and significantly reduced delayed neuronal death of CA1 neurons (Ouyang et al. 2005).

In conclusion, this study has documented massive accumulation of ubiquitinylated proteins in rat hippocampus 1 and 3 h after transient global ischaemia that is not associated with induction of proteins of ER stress signalling pathways. It seems that it represents separate form of unfolded proteins response independent from those associated with activation of ER stress signalling. In favour of this, transcriptional activation of *hsp70.1* gene as well as post-transcriptional activation of expression of HSP70 has been documented. Massive accumulation of ubiquitinylated protein aggregates, for the aggregates of molecular mass above 110 kDa even enhanced, accompanied with elevated level of HSP70 was also observed using protocol of IPC which was associated with significant neuroprotection. Finally, elevated level of HSP70 after preconditioned ischaemia and all periods of reperfusion might represent plausible explanation of IPC-induced inhibition of both translocation of p53 to mitochondria and ischaemia-induced apoptosis.

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