Gen. Physiol. Biophys. (2011), Special Issue, **30,** S61–S69

doi:10.4149/gpb_2011_SI1_61

Response of secretory pathways Ca^{2+} ATPase gene expression to hyperhomocysteinemia and/or ischemic preconditioning in rat cerebral cortex and hippocampus

Martina Pavlikova, Maria Kovalska, Zuzana Tatarkova, Monika Sivonova-Kmetova, Peter Kaplan and Jan Lehotsky

Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University, Mala Hora 4, 036 01 Martin, Slovak Republic

Abstract. The study determines whether hyperhomocysteinemia (risk factor of brain ischemia) alone or in combination with ischemic preconditioning (IPC) affects the ischemia-induced changes in gene expression of secretory pathways Ca^{2+}-ATPase (SPCA1).

Hyperhomocysteinemia was induced by subcutaneous administration of homocysteine (Hcy; 0.45 μmol/g body weight) twice a day at 8 h intervals for 14 days. Rats were preconditioned by 5 min ischemia and 2 days later, 15 min of global forebrain ischemia was induced by four vessel occlusion. We observed that hyperhomocysteinemia significantly decreased the level of SPCA1 mRNA in the cortex. Pre-ischemic challenge was noticeable in both brain areas. In the cortex, pre-ischemia in Hcy group led to the abrupt stimulation of the mRNA expression by 249% within the Hcy ischemic group and by 321% in the Hcy control. Values further exceeded those observed in the naive control. In the hippocampus, the differences between naive and Hcy groups were not observed. IPC initiated elevation of mRNA expression to 159% ($p < 0.05$) of control with Hcy and to 131% ($p < 0.01$) of ischemia with Hcy, respectively. Documented response of SPCA gene to IPC in hyperhomocysteinemic group might suggest a correlation of SPCA expression consistent with the role of cross-talks between intracellular Ca^{2+} stores including secretory pathways in the tolerance phenomenon.

Key words: Hyperhomocysteinemia — Global brain ischemia — Preconditioning — Secretory pathways — SPCA1 Ca^{2+} pump — mRNA

Abbreviations: ER, endoplasmic reticulum; Hcy, homocysteine; IPC, ischemic preconditioning; SPCA1, secretory pathway Ca^{2+}-ATPase.

Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid, which is derived from methionine metabolism. Hyperhomocysteinemia is a human condition in which, Hcy concentration exceeds 16 μmol/l, as a result of perturbed Hcy metabolism and dietary deficiencies in folic acid, vitamin B6, and/or vitamin B_{12} (Obeid et al. 2007). Hyperhomocysteinemia has been implicated as an independent risk factor for arteriosclerosis and coronary heart disease (Thambyrajah et al. 2000). Severe forms of hyperhomocysteinemia results in convulsions and dementia (van den Berg et al. 1995) with the corresponding multiple participation of Hcy in diverse pathologies that affect the CNS. Hcy has also been associated with the CNS disorders, such as epilepsy (Herrmann et al. 2007), neurodegenerative (Mattson et al. 2002) and neuropsychiatric diseases (Bottiglieri 2005), as well as inborn errors of metabolism (Mudd et al. 2001). Even moderate hyperhomocysteinemia is a factor stimulating the development of dementia and Alzheimer's disease (Seshadri et al. 2002) and incidence of stroke (Obeid et al. 2007).

Correspondence to: Jan Lehotsky, Department of Medical Biochemistry, Jessenius Faculty of Medicine, Comenius University, Mala Hora 4, 036 01 Martin, Slovak Republic
E-mail: lehotsky@jfmed.uniba.sk

Ischemic brain stroke in rodents and in humans is a complex cerebrovascular disease with multiple, parallel, and sequentional pathogenesis. Hyperhomocysteinemia is implicated as an independent risk factor and human patients in acute ischemic phase exhibit higher total Hcy level (Angelova et al. 2008). It has been shown that Hcy induces oxidative stress in the brain through the dual activation of glutamate receptors and the autoxidation to Hcy, with consequent reactive species generation (Jara-Prado et al. 2003; Zieminska and Lazarewicz 2006). Hcy also reduces antioxidant defences and increases lipid peroxidation in the brain (Streck et al. 2002; Wyse et al. 2002; Matte et al. 2007). Finally, selective cell death of vulnerable pyramidal neurons of hippocampal CA1 region and neurons of the cerebral cortex and striatum induced by ischemic event (Endres et al. 2008) are both accompanied by generation of oxygen free radicals. Oxidative stress therefore is an important mechanism of brain injury in both pathologies.

Ischemic preconditioning (IPC) represents an important phenomenon of adaptation of the CNS to sub-lethal short-term ischemia, which results in increased tolerance of the CNS to lethal ischemia (Gidday 2006; Obrenovitch 2008; Dirnagl et al. 2009). The mechanisms underlying ischemic tolerance are rather complex and not yet fully understood. Two windows have been identified in all multiple paradigms for IPC. One window represents very rapid short-duration post-translational changes. The second, which develops slowly (over days) after initial insult as a robust and long lasting transcriptional changes and subsequent cross-talks between subcellar organelles, and eventually culminates in prolonged neuroprotection (Gidday 2006; Obrenovitch 2008; Yenari et al. 2008; Dirnagl et al. 2009).

The Golgi apparatus, a part of secretory pathways in neural cells, represents a newly recognized dynamic Ca^{2+} store which plays a role in secretion processes in the reorganization of neuronal circuits, synaptic transmission, and remodeling of dendrites (Michelangeli et al. 2005). The secretory pathway Ca^{2+}-ATPases (SPCAs) represent a subfamily of P-type ATPases related to the sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) and the plasma-membrane Ca^{2+}ATPase (PMCA) (van Baelen et al. 2004). The isoform SPCA1 plays a pivotal role in normal neural development, neural migration, and morphogenesis (Sepulveda et al. 2007, 2008). Some studies confirmed the presence of SPCA1 isoform in neurons of rat brain (Murin et al. 2006; Sepulveda et al. 2007).

Hyperhomocysteinemia and ischemic/reperfusion injury represent severe metabolic and oxidative stress. As has been established, Golgi apparatus responds to physiological disturbances by a stress repair response analogous to endoplasmic reticulum (ER) stress (Dickhout et al. 2007) and SPCA Ca^{2+}-ATPase and other active components localized in the SP are critical participants in Golgi stress in which Golgi fragmentation and apoptosis could be the final manifestations (Jiang et al. 2011). Such morphological changes are commonly associated with several neurodegenerative diseases: amyotrophic lateral sclerosis, corticobasal degeneration, Alzheimer's, Creutzfeldt-Jacob, and spinocerebelar ataxia type 2 (Gonatas et al. 2006).

Up to now, it has not been shown whether or not hyperhomocysteinemic metabolic stress affects the expression of SPCA1 gene. Hence, we decided to measure changes in mRNA levels of SPCA1 post hyperhomocysteinemia induction. Same study investigates whether ischemic insult and IPC affect the hyperhomocysteinemic-induced changes in gene expression of SPCA1.

In these experiments, we used the chemical experimental model of hyperhomocysteinemia originally developed by Streck et al. (2002), in which the Hcy levels were similar to those found in human homocystinuria (Mudd et al. 2001).

Materials and Methods

Ischemia and ischemic preconditioning

Animal studies were performed under a protocol approved by the State Veterinary and Food Department of the Slovak Republic. Adult male Wistar rats (mean body weight 320 g, total $n = 42$) used for the experiments were housed in a menagerie under standard conditions with a temperature of 22 ± 2°C, and periodical variation in day light at 12 hour intervals. Food and water were provided *ad libitum*.

Global forebrain ischemia was induced by the standard four-vessel occlusion model (Pulsineli et al. 1982) as was described in previous papers from our laboratory (Lehotský et al. 2004; Urikova et al. 2006; Sivonova et al. 2008; Urban et al. 2009). Briefly, on day 1, both *vertebral arteries* were irreversibly occluded for 10 minutes by thermo coagulation through the *alar foramina* after anesthesia with 2.5% halothane in a mixture of oxygen/nitrous oxide (30/70%), without any visible influence on the animals. On day 2, both *common carotid arteries* were occluded for 15 min by small atraumatic clips under anesthesia with 2.5% halothane in a mixture of oxygen/nitrous oxide, same ratio as above. Two minutes before carotid occlusion, the halothane was removed from the mixture. Normothermic conditions (37°C) were monitored by a microthermistor placed in the ear. Temperature was maintained using a homeothermic blanket. Sham control animals were prepared in the same way without carotid occlusion. The rats then underwent 15 min ischemia. Criteria for forebrain ischemia comprised loss of the righting reflex, mydriasis and paw extension. The rats used for the experiment were those that became unresponsive, lost the righting reflex during bilateral carotid artery occlusion and showed no seizures during and after ischemia.The selected animals

therefore met the criteria for adequate ischemia (Pulsinelli et al. 1982). Those that met the criteria for global forebrain ischemia were divided into groups in the same way as non treated animals (each group $n = 6$). IPC was induced by 5 min of sublethal ischemia followed by 2 days of reperfusion. The rats then underwent lethal ischemia for 15 min as described above. After ischemia, animals were sacrificed by decapitation in a mild halothane anesthesia. Cortex and hippocampi were dissected and processed immediately. Control animals for both ischemia group and preconditioned ischemia group underwent the same procedure with the exception of carotid occlusion.

Chemically-induced hyperhomocysteinemia

Hcy (Sigma-Aldrich, Bratislava, Slovak Republic) was dissolved in 0.85% (w/v) NaCl solution (saline) and buffered to pH 7.4. Hcy solution (0.45μmol/g body weight) was administered subcutaneously twice a day at 8 h intervals for 14 days. It is well know that Hcy cross the blood/brain barrier and present a peak in the cerebrum and parietal cortex between 15–60 minutes after subcutaneous injection (Streck et al. 2002; Matté et al. 2007). Doses of Hcy were calculated from pharmacokinetic parameters as previously determined (Streck et al., 2002). Plasma Hcy concentration in rats treated this way achieved levels similar to those found in homocystinuric patients (moderate hyperhomocysteinemia) (Mudd et al. 2001).

Experimental groups of animals

Groups of rats were classified as follows:
1) sham-operated control (naive) animals (C-nai, $n = 6$)
2) sham-operated control (preconditioning) animals (C-IPC, $n = 6$)
3) the animals that underwent 15 min ischemia (naive) (Isch-nai, $n = 6$)
4) the animals with induced IPC following 15 min ischemia (Isch-IPC, $n = 6$)
5) sham-operated hyperhomocysteinemic control animals (C-Hcy, $n = 6$)
6) the hyperhomocysteinemic animals that underwent 15 min ischemia (Isch-Hcy, $n = 6$)
7) the hyperhomocysteinemic animals with induced preconditioning animals following 15 min ischemia (Isch-IPC-Hcy, $n = 6$).

RT-PCR Assays

Cortex and hippocampi from all rats (sham control, ischemic, pre-ischemicand all groups with Hcy were extracted under RNAse-free conditions. RNA was extracted by fenol-chloroform with consequent reverse transcription using Ready-to-Go You Prime first strand beads (Amersham Biosciences). To amplify SPCA1, the forward primer was 5´-AAACTGGAACCCTGACGAAG-3´ and the reverse primer 5´-TTGGCTTTCCCATCAGAGTG-3´. Reverse transcription was performed using Thermoscript RT-PCR system (Invitrogen) with 2 μg of each RNA. Multiplex PCR for SPCA1 and ß actin was carried out with 2 μm of each cDNA in a final volume of 50 μl containing 1 × PCR buffer, 200 μmol/l dNTPs (Roche), 1.5 mmol/l MgCl2, 0.5 μmol/l of each primer, and 2 U Taq polymerase (Invitrogen). To amplify SPCA1, the forward primer 5´-AAACTGGAAC-CCTGACGAAG-3´ and the reverse primer 5´-TTGGCTT-TCCCATCAGAGTG-3´ were used. The forward primer 5´-TCTACAATGAGCTGCGTGTG6-3´ and the reverse primer 5´-TACATGGCTGGGG

TGTTGAA-3´ were used for ß actin amplification as an internal control. The PCR reaction conditions were 3 min denaturation at 94°C, followed by 23 cycles (matching the linear range of amplification) of 1 min denaturation at 94°C, 1 min of annealing at 54°C, and 1 min of extension at 72°C, followed by 10 min of final extension at 72°C. The level of SPCA mRNA represents the optical density of the amplification products expressed as arbitrary units.

Data analysis

Results were presented as mean ± SEM. For statistical analysis, data were analysed using INSTAT software with one-way analysis of variance (ANOVA) followed by Student-Neuman-Keuls test. Comparisons were made between appropriate groups and *p*-values smaller than 0.05 ($p < 0.05$) were considered to be statistically significant.

Results

In our previous paper (Pavlikova et al. 2009) we investigated the response of hippocampal tissue to ischemic reperfusion injury and IPC on the level of mRNA SPCA1 expression.

In order to evaluate whether or not the severe metabolic stress, induced by: 1) hyperhomocysteinemia alone for 14 days, 2) in combination with other stress condition (15 min ischemia), and 3) with induced ischemic tolerance by preconditioning, affects transcription of SPCA1 gene, we have analyzed the respective mRNA levels in rat cerebral cortex and hippocamus by quantitative PCR. Four vessel occlusions in rats (Pulsinelli et al. 1982; Lehotsky et al. 2004) induce global forebrain ischemic attack and affect selective vulnerable neurons in cortical and hippocampal regions. This is the reason why these two brain areas were chosen for molecular analysis.

Initially, we investigated differences between naive control and hyperhomocysteinemic control animals in each group independently. We have shown here for the first time that

experimental 2-weeks hyperhomocysteinemia significantly decreases the level of SPCA1 mRNA gene expression in cerebral cortex (Fig. 1) with no accompanying significant decreases in the expression level in the hippocampal area (Fig. 2).

As seen in cerebral cortex (Fig. 1), ischemic challenge for 15 min (Isch-nai) did not effect any significant changes in the level of mRNA SPCA1 expression in comparison to control (C-nai). On the other hand, the gene response to preischemic challenge (IPC) was evident in Isch-IPC-Hcy group by the abrupt stimulation of the mRNA expression level to 249% ($p < 0.01$) of Isch-Hcy group and to 321% ($p < 0.01$) of control – C-Hcy group. More notably, values exceeded those observed in the naive control. However, there was no effect of IPC challenge in the naive groups.

We determined the differences between controls from all three groups (C-nai, C-IPC, C-Hcy) in the cortical area. The expression level of mRNA SPCA1 in the C-Hcy group is 259% lower ($p < 0.05$) than in the C-nai group and 277% lower ($p < 0.05$) than in the C-IPC group. When we compared changes between all ischemic groups, we observed low level of mRNA expression in Isch-Hcy group (201% and 185%, $p < 0.05$) in compare with Isch-nai group and Isch-IPC group, respectively. But there were no significant differences between C-Hcy and Isch-Hcy groups. Of more interest, however, the preischemic challenge initiated stimulation of the mRNA expression to 249% ($p < 0.01$) of Isch-Hcy group. This response might be part of the protective tolerance phenomenon induced by preconditioning treatment.

Fig. 2 summarizes the results of mRNA SPCA1 expression in the hippocampal area. We were not able to detect any statistically significant changes between C-nai and C-IPC groups here. Hyperhomocysteinemia for 14 days supressed mRNA expression, but again the changes between C-Hcy and Isch-Hcy groups were not statistically significant. Similarly, the preischemic challenge in hippocampal region initiated stimulation of the mRNA expression in Isch-IPC-Hcy group by 159% ($p < 0.01$) of hyperhomocysteinemic control (C-Hcy) group and by to 131% ($p < 0.01$) of hyperhomocysteinemic ischemic (Isch-Hcy) group as shown in the cortex. We suggest that this response might also be part of the protective tolerance phenomenon induced by preconditioning treatment.

Discussion

This study determines whether experimental hyperhomocysteinemia alone (as an example of metabolic stress) and/or in combination with IPC affects ischemia-induced changes in gene expression of secretory pathways (SPCA1). Ischemic brain stroke in humans is a very complex cerebrovascular disease. A number of conventional risk factors for ischemic stroke are known. For instance a history of previous stroke,

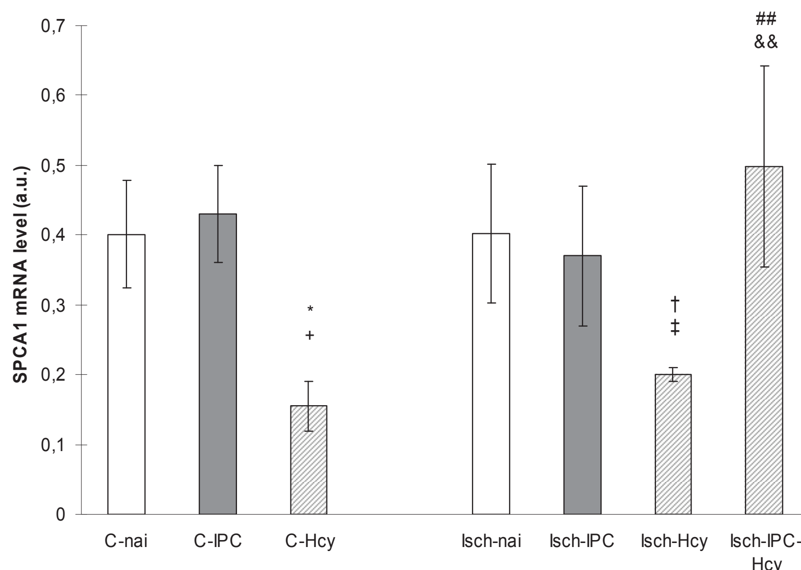

Figure 1. Comparison of mRNA levels of SPCA1 between naive group (C-nai, Isch-nai), IPC group (C-IPC, Isch-IPC) and hyperhomocysteinemic group (C-Hcy, Isch-Hcy, Isch-IPC-Hcy) in rat cortex. Results are presented as mean ± SEM for $n = 6$. * $p < 0.05$ compared to C-nai group, $^{+}$ $p < 0.05$ compared to C-IPC groups, † $p < 0.05$ compared to Isch-IPC group, ‡ $p < 0.01$ compared to C-nai group, $^{\#\#}$ $p < 0.01$ compared to Isch-IPC-Hcy group, $^{\&\&}$ $p < 0.01$ compared to C-Hcy group. C-nai, control naive group; Isch-nai, ischemia naive group, C-IPC, control preischemic group; Isch-IPC, preischemic group; C-Hcy, control Hcy group; Isch-Hcy, ischemia Hcy group; Isch-IPC-Hcy, preischemic Hcy group. (For more details see in Materials and Methods).

previous transient ischemic attack, arterial disease, atrial fibrillation, poor diet, obesity and physical inactivity (Prasad 1999). Hyperhomocysteinemia is implicated as an independent risk factor of human stroke due to pleiotropic activity of Hcy and acceleration of atherosclerotic changes (Refsum et al. 1998; Thambyrajah et al. 2000). At first, Hcy affects normal function of endothelial cells by the suppression of NO production and generation of reactive oxygen species (ROS) by the release of arachidonic acid from platelets (Signorello et al. 2002). It also inhibits glutathione peroxidase (Upchurch et al. 1997), and thus stimulates proliferation of endothelial cells (Domagala et al. 1998; Jeremy et.al. 1999). On the other hand, chronic hyperhomocysteinemia also alters antioxidant defenses and increases DNA damage in the brain as well as in blood and activates low density lipoprotein oxidation (Tagliari et al. 2006; Matté et al. 2007). Furthermore, neurotoxicity of Hcy is based on several mechanisms, including disturbed neuronal calcium homeostasis as results of Hcy-stimulated N-methyl-d-aspartate (NMDA) receptors and several kinases activity (Robert et al. 2005; Obeid et al. 2007). An altered ratio of *S*-adenosyl methionine/*S*-adenosyl Hcy (SAM/SAH) promotes DNA damage (Kruman et al. 2002) and changes phosphorylation of tau protein leading to neurofibrilary tangle formation (Vafai and Stock 2002). These have been associated with neurodegenerative disorders and dementia.

We have demonstrated here for the first time, that chemically-induced 2-week hyperhomocysteinemia under experimental conditions significantly decreases the level of SPCA1 mRNA gene expression in cerebral cortex and leads to decreased expression levels in hippocampal area, even if not so significantly. The expression level of mRNA for SPCA1 in cerebral cortex is evidently also decreased in the Hcy groups in comparison to the naive groups, both in controls and ischemic points.

Hyperhomocysteinemia represents severe metabolic and oxidative stress. There are no literature reports as to how the Hcy might affect the expression profile of the Ca^{2+}-transport proteins in neuronal cells. In addition, no further information is available on the possible influence of Hcy on mRNA stability or translational machinery of Golgi resident genes in the brain. In fact, the general mechanism of transcriptional regulation of SPCA1 gene is not yet fully understood. The transcription factors Sp1 and YY1 were shown to be involved in gene regulation by the *cis*-enhancing elements in 5′-untranslated regions (Kawada et al. 2005; Sepúlveda et al. 2008).

There are only a few recent papers dealing with the possible deregulatory effects of Hcy on gene expression in differents cells. As was observed in hyperhomocysteinemic individuals, peripheral blood mononuclear cells exhibit significantly higher gene expression of RANK ligand and its receptor RANK on mRNA and on protein levels compared to controls. RANK ligand and its receptor RANK modulate matrix degradation and inflammatory cytokines in response to Hcy (Nenseter et al. 2009). Conversely, Hcy influences the formation of a stable bone matrix through the inhibition of the collagen cross-linking enzyme lysyl oxidase and, as has been shown recently, by repressing its mRNA expression (Thaler et al. 2011). In rat brain parenchyma, systemic hyper-

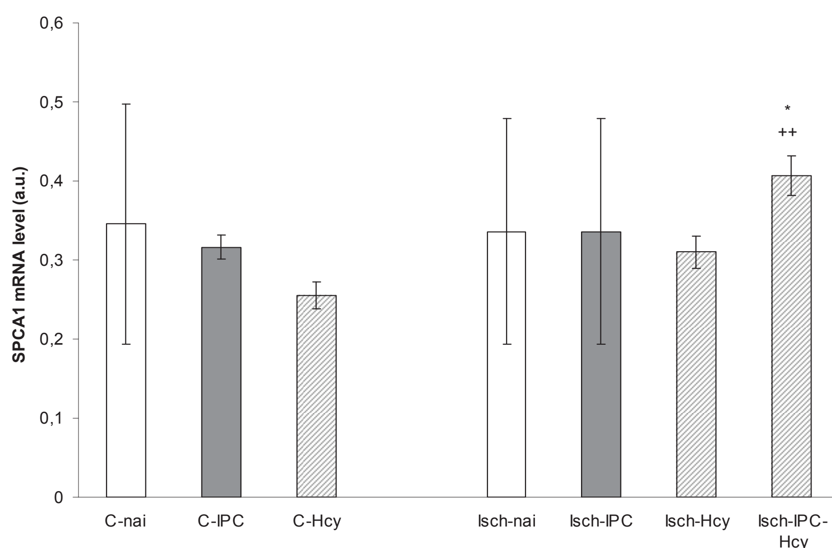

Figure 2. Comparison of mRNA levels of SPCA1 between naive group (C-nai, Isch-nai), IPC group (C-IPC, Isch-IPC) and hyperhomocysteinemic group (C-Hcy, Isch-Hcy, Isch-IPC-Hcy) in rat hippocampus. Results are presented as mean ± SEM for $n = 6$. $^{*}p < 0.05$ compared to Isch-Hcy group, $^{++}p < 0.01$ compared to C-Hcy group. (Abbreviations see Fig. 1).

homocysteinemia increases β-amyloid levels by enhancing expression of γ-secretase and phosphorylation of amyloid precursor protein (Zhang et al. 2009). In addition, Hcy in *in vitro* conditions (neuronal culture) causes protein misfolding in the endoplasmic reticulum and activates the unfolded protein response, leading to increased expression of the ER stress-response genes, such as GRP78/BiP and GADD153 and HERPUDI (Hcy-inducible, ER stress-inducible, ubiquitin-like domain member 1) with the consequent calcium disequilibria and stress of ER (Althausen and Paschen 2000; Dickhout et al. 2007). In fact, hyperhomocysteinemia often results in intracellular Ca^{2+} mobilization, ER stress, and the subsequent development of apoptotic events and remodeling of the extracellular matrix. Hcy has also been reported to induce modulation of the gene expression through alteration of the methylation status (Dionisio 2010). As we found in our experiments, the decreased level of mRNA for SPCA in hyperhomocysteinemic animals can offer additional explanation for the proposed dysregulated Ca^{2+} cellular homeostasis induced by Hcy.

Hyperhomocysteinemia as well as I/R injury represent severe metabolic and oxidative stress. In general, I/R injury initiates suppression of global proteosynthesis, which is practically recovered in the reperfusion period with a few exceptions such as in the most vulnerable neurons, like pyramidal cells of CA1 hippocampal region (Pulsinelli et al. 1982). On the other hand, ischemia is one of the strongest stimuli of gene induction in the brain. Different gene systems related to reperfusion processes of brain injury, repair, and recovery are modulated (Gidday 2006).

In our previous experiments we were able to show that I/R injury alters time expression profiles of SPCA1 on mRNA and protein levels (Pavlikova et al. 2009). In this paper, we have shown that the combination of both stressors (ischemia + Hcy) had considerable detrimental effects on mRNA SPCA expression, mainly in the cortical area. Other studies have also shown that the Golgi apparatus respond to physiological disturbances in cells, by a stress response similar to that of the ER (Hicks and Machamer 2005; Jiang et al. 2011). SPCA Ca^{2+}-ATPase and other active components localized to the SP are critical participants in Golgi stress, with Golgi fragmentation and apoptosis as the apparent final manifestations. Interestingly, during apoptosis, Golgi complex undergoes morphological changes, which represents an early causative step that is very commonly associated with several neurodegenerative diseases. It is indicative in this aspect therefore, that chronic hyperhomocysteinemia alters gene expression of amyloid peptide, a component linked with etiopathogenesis of Alzheimer disease (Hicks and Machamer 2005; Gonatas et al. 2006; Jiang et al. 2011).

In a series of papers from our laboratory (Lehotsky et al. 2009a; Pavlikova et al. 2009; Urban et al. 2009) we have also found that I/R injury initiates time-dependent differences in ER gene expression at both the mRNA and protein levels in rat hippocampus and that gene expression is affected by pre-ischemic treatment. Moreover, there was also a correlation between Golgi gene, the SPCA Ca^{2+}-ATPase expression and the response to the pre-ischemic challenge (induction of tolerance). This maneuver does not only preserve the majority of surviving neurons, but it also initiates partial recovery of the SPCA Ca^{2+}-ATPase activity and earlier induction of SPCA1 mRNA and protein expression ultimately to lethal ischemia in hippocampus.

In this study we found that preischemia had a protective/stimulatory influence on the expression mRNA levels under both stress condition (naive ischemia and/or Hcy ischemia). The gene response to pre-ischemic challenge was demonstrated in Hcy groups in both brain areas (in cortex, by 249% of Isch-Hcy group and by 321% of C-Hcy group). These values exceed those observed in the C-nai and in the somewhat similar tendency in the gene response to preischemia in the hippocampal area. However, the effect of IPC challenge was not observed in the naive groups. The molecular mechanisms underlying ischemic tolerance is yet to be fully understood (Dirnagl et al. 2009; Lehotsky et al. 2009b,c). Significant reductions of oxidative products and reduced protein oxidative changes induced by ischemia are indicative consequences of preischemic treatment in the hippocampal membranes (Lehotský et al. 2009c; Pignataro et al. 2009). A possible explanation of the significant elevation of mRNA expression would seem to be supported from studies describing upregulation of defense mechanisms (antioxidant enzymes) against oxidative stress due to the preconditioning challenge (Danielisova et al. 2005; Gidday 2006; Obrenovitch 2008). Also the results of experiments by Bickler and coworkers (2009) suggested that the attenuation of ER stress response can be an important mediator in the neuroprotective phenomenon of acquired ischemic tolerance. Furthermore, gene polymorphism in the promotor region of ER stress linked XBP1 gene was found to be a significant risk factor for ischemic stroke in humans. The same allele has been shown to correlate with the incidence of hyperhomocysteinemia (Yilmaz et al. 2010).

Similarly to our previous experiments (Pavlikova et al. 2009), we did not see any significant changes in SPCA mRNA level between Isch-nai and Isch-IPC. Instead, IPC initiated the significant elevation of mRNA expression and caused changes that could only be seen in reperfusion time. However, the effect of IPC challenge was not observed in the naive groups (Pavlikova et al. 2009). Although we have no clear explanation at present, it is worth mentioning that, two windows are known in the tolerance phenomenon. These correspond respectively to a very rapid but short-duration post-translational changes while the other develops

more slowly (over days) post initial insult as a robust and long-lasting transcriptional change (Gidday 2006).

In conclusion, our results indicate that chemically-induced *in vivo* hyperhomocysteinemia initiates supression of the SPCA1 gene expression in both rat brain regions, the cerebral cortex and the hippocampus. Documented response of SPCA gene to preischemic challenge in hyperhomocysteinemic group of animals might suggest a correlation of SPCA expression consistent with the role of cross-talks between intracellular Ca^{2+} stores, including secretory pathways, in the proposed phenomenon of ischemic tolerance (Dirnagl et al. 2009; Lehotsky et al. 2009b,c; Pignataro et al. 2009).

Acknowledgements. This study was supported by Grants VEGA 0049/09 from the Ministry of Education of the Slovak Republic, UK-55-15/07 from Ministry of Health of the Slovak Republic, and APVV VVCE 0064-07. M. P., M. K., Z. T., M. S-K., P. K. and J. L. have no conflict of interest and no financial interest in the publication of this manuscript.

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Received: December 9, 2010
Final version accepted: March 28, 2011