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Mechanisms of Ischemic Induced Neuronal Death and Ischemic Tolerance

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1. Introduction

Stroke is the second leading cause of death and the primary cause of disability in humans. The phenomenon of ischemic tolerance perfectly describes the quote: "What does not kill you makes you stronger.'' Ischemic pre- or post- conditioning is actually the strongest known procedure to prevent or reverse delayed neuronal death. It works specifically in sensitive vulnerable neuronal populations, which are represented by pyramidal neurons in the hippocampal CA1 region. However, tolerance is effective in other brain cell populations as well. Although, its nomenclature is **''ischemic'' tolerance (IT)** '', the tolerant phenotype can also be induced by other stimuli that lead to delayed neuronal death (intoxication). Recent data have proven further that this phenomenon is not only limited to application of sublethal stimuli before the lethal stress (**preconditioning)** but also that reversed arrangement of events, sublethal stress after lethal insult (**postconditioning),** are equally effective. Another very important term is **''cross conditioning,''** or the capability of one stressor to induce tolerance against another. Delayed neuronal death is the slow development of post-ischemic neuro-degeneration. This delay allows a therapeutic window of opportunity lasting 2–3 days to reverse the cellular death process. It seems therefore that the mechanisms of ischemic tolerance-delayed post-conditioning could be of use not only after ischemia but also in some other processes leading up to apoptosis.

This paper summarizes results of experimental studies which have shown that acute *in vivo* forebrain ischemia as well as ischemic/reperfusion injury (IRI) both alter, the expression, function and kinetic parameters of Ca^{2+} transporters as well as the physical membrane environment. Furthermore, that IRI leads to the inhibition of mitochondrial respiratory complexes I and IV. Also, that conversely, ischemic preconditioning (IPC) acts at the level of both initiation and execution of IRI-induced mitochondrial apoptosis and activates inhibition of p53 translocation to mitochondria.

Evidence is presented to show that endoplasmic reticulum (ER) is the site of complex processes such as calcium storage, synthesis and folding of proteins as well as cell response to stress. ER function is impaired in IRI which in turn induces depletion of stored calcium, the conserved stress responses linked with delayed neuronal death. In addition, IRI initiates time dependent differences in endoplasmic reticular (ER) gene expression of the key

unfolded protein response (UPR), or proteins at both the mRNA and protein levels. Moreover, gene expression of the UPR proteins is affected by pre-ischemic (IPC) treatment caused by the increased expression of Ca^{2+} binding protein, GRP 78 and transcriptional factor ATF6 in reperfusion times. Thus, IPC exerts a role in the attenuation of ER stress response, which might, in turn, be involved in the neuroprotective phenomenon of ischemic tolerance. Hippocampal cells respond to the IRI by the specific expression pattern of the secretory pathways Ca^{2+} pump (SPCA1) and this pattern is affected by preischemic challenge. IPC also incompletely suppresses lipid and protein oxidation of hippocampal membranes and leads to partial recovery of the ischemic-induced depression of SPCA activity. The data suggests a correlation of SPCA function with the role of secretory pathways (Golgi apparatus) in response to preischemic challenge.

2. Ischemic stroke

Ischemic stroke arises in humans as a consequence of a cardiac arrest, the stoppage of blood flow to the brain due to embolic or thrombic occlusion of arteries. Global or focal ischemia is very severe pathogenic event with multiple, parallel, and sequential pathogenesis. Global forebrain ischemia leads to selective cell death of vulnerable pyramidal neurons in the hippocampal CA1 region. It also leads to death of cerebral cortex neurons (layers 3, 5, and 6) and the dorsolateral striatum. When blood flow decreases during focal ischemia, the area surrounding the necrotic core of ischemia, also known as ''penumbra'' is perfused by collateral vessels. It also undergoes fatal apoptosis of neurons (Endres et al., 2008).
Despite decades of intense research, no effective neuroprotective drugs are available to treat acute stroke or cardiac arrest. For this reason, recent attention has shifted to defining the brain's own evolutionarily conserved endogenous neuroprotective mechanisms, which occurs in **ischemic tolerance (IT) or after ischemic preconditioning (IPC).** IT induced by several paradigms represents an important phenomenon of the central nervous system (CNS) including adaptation to sublethal short-term ischemia. This results in increased tolerance of CNS to lethal ischemia (Kirino, 2002; Dirnagl et al., 2003; Gidday, 2006). The molecular mechanisms underlying IT are not yet fully understood because of its extreme complexity, involving many signaling pathways and alterations in gene expression. Additionally, a metabolic depression has also been suggested to play an important role in IT (Yenari et al., 2008).

2.1 Ischemic tolerance as a possible neuroprotective strategy

A transient, ischemia-resistant phenotype known as **"ischemic tolerance (IT)"** can be established in brain in a rapid or delayed fashion by a preceding non-injurious "preconditioning" stimulus. Thus, **ischemic preconditioning (IPC)** as one of the inducers, represents a phenomenon which eventually leads to an increase in the **tolerance** of CNS to the lethal ischemia (Dirnagl et al., 2009; Obrenovitch, 2008). Initial pre-clinical studies of this phenomenon relied primarily on brief periods of ischemia or hypoxia as the IPC stimuli, but it was later realized that many other stressors, including pharmacological agents, are also effective. Although considerably more experimentation is needed to thoroughly validate the efficacy of any already identified preconditioning agent to protect ischemic brain, the fact that some of these agents are already clinically used implies that the growing enthusiasm for translational success in the field of pharmacologic pre-conditioning may be well justified.

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 that represents very rapid and short-lasting post-translational changes and a second, which develops slowly (over days) after initial insult as a robust and long lasting transcriptional changes which culminate in prolonged neuroprotection (Dirnagl et al., 2009; Obrenovitch, 2008; Yenari et al., 2008). Differences in intensity, duration, and frequency of specific inducer/stressor determine the spectrum of responses to noxious stimuli. In other words, when the stimulus is too weak to induce any response, when it is sufficient to serve as a tolerance trigger, or when it is too strong and harmful, resulting in apoptotic or necrotic damage.

It is symptomatic that there are no clear boundaries between acquisition of tolerance and cellular apoptosis/necrosis (Dirnagl et al., 2009). Rodent and cell culture models serve as a basis for the study of the tolerance phenomenon. Mother nature presents the perfect model to help understand this better. In nature, we ubiquitously find adaptation to extreme environmental conditions, for example, the hypoxic or anoxic tolerance. Hibernation is another example of inherent adaptation to extreme low-blood perfusion in animals. As such, ischemic tolerance can be conceived as an evolutionary conserved form of cerebral plasticity (Dirnagl et al., 2009,). It is not surprising therefore that different animal species have evolved different molecular strategies to cope with anoxia and severe metabolic stress. This leads to the trigger of the neuroprotective tolerance state.

A number of common mechanisms with different relevance features can be recognized (Lehotsky et al., 2009b):

- depression of metabolic rate,
- modulation of glycolytic enzymes,
- reduction of ion channel fluxes,
- suppression of neural activity,
- expression of chaperones and heat shock proteins (Hsp),
- activation of antioxidant defense systems,
- adaptation of blood rheology and others.

At first pass, the patient population that suffers from cerebral ischemic injury due to unpredictable focal stroke, cardiac arrest, or subarachnoid hemorrhage represents, by definition, one that is unlikely to derive benefit from preconditioning research. However, the novel endogenous survival pathways identified in preclinical IT studies may ultimately become **targets for drugs** that protect the brain even when acutely administered after the precipitating event. Importantly, a significant number of other patients—those in which we can anticipate a period of cerebral ischemia following transient ischemic attack, aneurysm clipping, subarachnoid hemorrhage, carotid endarterectomy or stenting, asymptomatic carotid stenosis, coronary bypass, and cardiac valve replacement—represent defined at-risk populations ideally suited for translational **therapeutic preconditioning.** The candidate drugs that might underpin clinical trials for this latter group of patients actually comprise a relatively long and therefore promising list, particularly if the current foundation of preclinical studies is expanded with intention.

The concept of IPC in the heart was introduced in the late 80s by Murry et al. (1986) and later on in the brain by Schurr et al. (1986) and Kitagawa et al. (1991). Most stressors, including preischemia/hypoxia, induce both rapid and delayed tolerance phenotypes (Gidday, 2006). Mechanisms that are prominent during the first phases of acute ischemic insults such as excitoxicity are presumed to be induced during rapid IT. In particular,

elevation of adenosine and activation of adenosine receptor with the modulation of ATP sensitive K^+ channel are paralleled by the activation of protein kinase C and other kinases in rapid tolerance. A critical role for nitric oxide signaling pathways in IPC and tolerance was also suggested (Nandagopal et al., 2001). As was recently shown by Meller et al. (2008), the selective ubiquitin–proteasome degradation of a cell death-associated protein, Bcl-2-interacting mediator of cell death (Bim) with the reduced activation of programmed cell death-associated caspases (caspase 3) could play an important role in rapid tolerance to ischemia. As mentioned earlier, IT can be induced by various stimuli that are not necessarily ischemic or hypoxic.

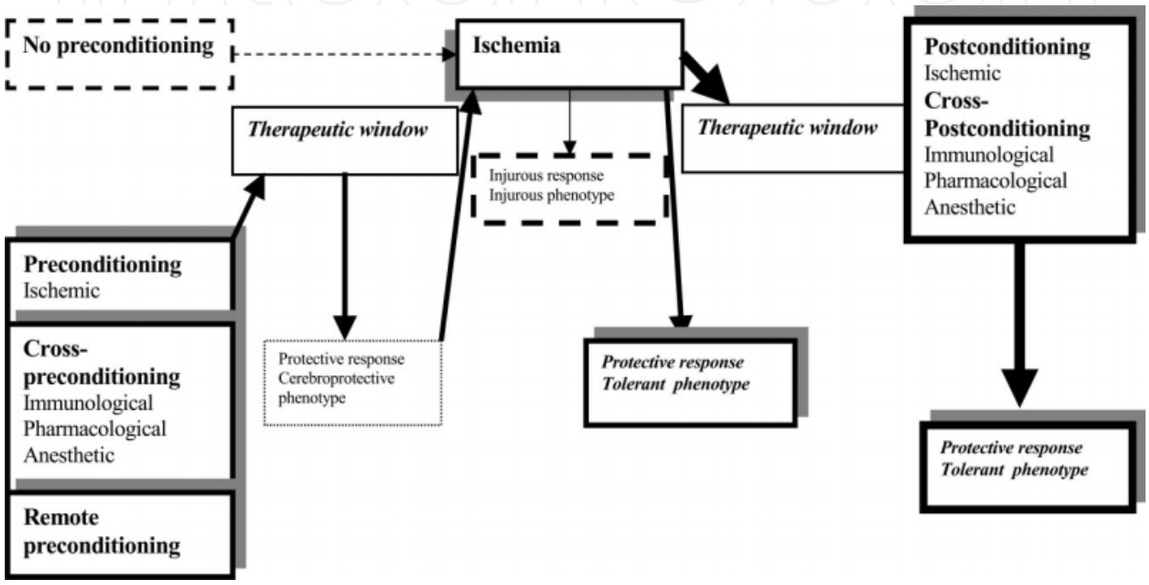

Fig. 1. Ischemic insult without any maneuvers leads to ischemic/reperfusion injured phenotype. Cerebroprotection can be induced by different types of preconditioning or postconditioning maneuvers/stimuli (ischemic, immunological, pharmacological and anesthetic). Temporary defined responses during therapeutical window may induce protective response with which subsequent ischemia serve as basis of the ischemic-tolerant phenotype. Adapted from Lehotsky et al. (2009b).

Thus, the phenomenon of cross-tolerance implies that noxious stress can initiate cellular tolerance to subsequent stress that is different in nature from the first one. Therefore, one stressor can promote cross-tolerance to another; however, the efficacy of this tolerance may be more modest, and it appears to vary with the nature and intensity of the first challenge. Additionally, the window of evolved IT may also be shifted. However, the nature of the stimulus may determine the specific protective or in worse meaning the reduced damage epiphenotype.

3. Prophylactic treatment with statins: Effect on ischemic damage

Neuronal ischemic/reperfusion damage in the brain occurs rapidly. However, significant structural changes are observed over a course of hours or days in the form of delayed neuronal death. Interruption of blood flow initiates high-energy metabolism failure, ATP

depletion, ion imbalance, as well as other biochemical changes, such as an increase of free radicals, mitochondrial dysfunction, lactic acidosis, and inhibition of proteosynthesis as a consequence of endoplasmic reticular (ER) stress (DeGracia et al., 2002).

The endoplasmic reticulum of eukaryotic cell reacts to ischemic injury by **the unfolded protein response (UPR),** which can be highly variable, depending on dosage and duration of ischemic treatment (Imaizumi et al., 2001), and intensity of UPR signals (Yoshida et al., 2003). However, when ER stress is too severe and prolonged, apoptosis is induced. Various enzymes and transcription factors including the double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK) (Harding et al., 1999), the transcription factors ATF4 and ATF6 (activating transcription factor 6) and the inositol-requiring enzyme IRE1 (Shen et al., 2001) are involved in the UPR. In the physiological state, PERK, ATF6, and IRE1 activity is suppressed by binding of the ER chaperone: glucose regulated protein 78 (GRP78). Morimoto et al. (2007) reported that induction of GRP78 prevents neuronal damage induced by ER stress, and the increase in GRP78 (BiP) expression may correlate with the degree of neuroprotection.

Statins, inhibitors of sterol synthesis, have been shown to reduce cerebrovascular events by their pleiotropic effects independent of the cholesterol lowering mechanism. Nagotani et al. (2005) found that simvastatin was the most effective statin against spontaneous stroke in human and animals. Strong liposolubility of statins may result in high permeability through the blood–brain barrier to the parenchyma, thereby protecting the neurons against ROS-induced lipid peroxidation and DNA oxidation. The neuroprotective properties of **simvastatin** in experimental stroke have been evaluated by using several rodent-simulated models of cerebral ischemia (Shabanzadeh et al., 2005; Hayashi et al., 2005). As shown by previous studies, the changes of the UPR gene expression induced by transient ischemia occur mostly during the first 24 h (Paschen 2003b) or the first few days after the insult (Qi et al., 2004). In line with this, Urban et al. (2009) have decided to measure changes in mRNA and protein levels of GRP78, ATF6, and XBP1 after 15 min of global ischemia and 1, 3, and 24 h reperfusion (UPR reaction). In addition, they have focused their attention on the effect of simvastatin pretreatment on the stress reaction of endoplasmic reticulum induced by ischemic/reperfusion insult.

Adult male Wistar rats were used as animal model for the experiment. Global forebrain ischemia was induced by the standard four-vessel occlusion model (Lehotský et al., 2004; Sivonova et al., 2008; Uríkova et al., 2006). For maximal proof of changes in mRNA levels, authors used real-time PCR. Cortexes from sham control, ischemic and simvastatin-treated animals were homogenized, and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and level of levels of ER stress gene proteins was analyzed by Western blotting after ischemic/ reperfusion damage (I/R) in naive rats and rats pretreated with simvastatin (20 mg/kg for 14 days). In the non-treated I/R animals, the mRNA level was significantly maximal in ischemic period (43 ± 3.2% in comparison to control), followed by rapid significant decrease from the first hour of reperfusion to a minimum value reached at the third hour (57 ± 7.8% lower than control). The mRNA level at 24 h of reperfusion reached control values. The level of XBP1 protein in non-treated animals showed only slight, not significant, differences compared to controls, mainly at later reperfusion periods (3 and 24 h).

The influence of simvastatin on mRNA level was significant only in the first and the third hours of reperfusion compared to control I/R animals (about 32.8 ± 4.1% lower and two times higher in I3R, respectively). The changes in mRNA levels were not projected onto

protein levels, which, in contrast to control I/R animals, was found to be significantly lower (about 37.4 ± 2.2% in ischemic phase and about 36.3 ± 5.7% lower in first hour of reperfusion). In this paper, Urban et al (2009) were interested in finding whether global ischemia induced by four vessel occlusion followed by reperfusion at different time points would initiate the unfolded protein response of ER in cortical neurons. In addition, they have proved that prophylactic simvastatin therapy affects expression of gene coding for the main proteins involved in UPR.

Clinical trials demonstrated that 3-hydroxy-3- methylglutaryl coenzyme A reductase inhibitors or statins exert beneficial effects when used as stroke prophylactic agents (Byington et al., 2001; Vaughan et al., 2001). These studies showed that statins reduce the incidence of both first and secondary events by 25– 30% and prevention is believed to be achieved mainly through their activity on blood vessel wall function. However, in addition to exerting anti-atherosclerotic and anti-thrombotic effects, statins also possess antiinflammatory and neuroprotective actions, which have been identified as cholesterol-independent or pleiotropic effects (Vaughan and Delanty, 1999; Takemoto and Liao, 2001). The findings indicated that administration of simvastatin or other statins reduced the size of brain damage (Sironi et al., 2003; Amin-Hanjan et al., 2001). The beneficial effect of simvastatin is achieved only when the drug is administered before the ischemic insult; therefore, acting as a prophylactic agent (Balduini et al., 2003). In the model of focal ischemia induced by middle carotid artery occlusion (MCAO), the size of the damaged tissue increased by 47% after 24 h and by 83% after 48 h as compared to the infarct size detected at 2 h. This time-dependent enhancement of the damage was abolished in animals pre-treated with simvastatin, as the volume of infarct was never larger than the volume reported 2 h after MCAO. (Cimino et al., 2005).

In general, I/R injury initiates suppression of global proteosynthesis (de la Vega et al., 2001; Paschen, 2003a). 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 up-regulated (Gidday, 2006). Focal ischemia shorter than 3,5 minutes and seven days of reperfusion usually causes degeneration of 75% of the neurons in the hippocampal CA1 region (Ohtsuki et al., 1996). On the other hand 6–10-minutes long global ischemia and three days of reperfusion caused death of almost all pyramidal neurons in the same hippocampal area (Coimbra and Wieloch, 1994). Urban et al., (2009) showed a significant increase of XBP1 mRNA level in ischemic phase in comparison to control (about 43% more). These findings are similar to those observed by Paschen (2003a), which showed a marked increase in processed XBP1 mRNA levels using semi-quantitative RT-PCR after focal ischemia. These changes were most pronounced in the cerebral cortex, where high levels were found throughout the entire observation period. Urban et al (2009) obtained similar results; however, the differences were smaller probably due to the different ischemic model. The rapid increase of mRNA level of XBP1 along with other genes in ischemic phase of non-treated animals was probably due to forthcoming dissociation of protein GRP78, which reached a maximum at ischemic phase and first hour of reperfusion, from bounds with sensors of UPR which quickly (ATF6) or slowly (XBP1) started transcription of effector's genes.

In simvastatin-treated animals, rapid increase of mRNA in ischemic phase was mainly a consequence of transcription factor ATF6. It has been proposed that the strong inhibition of translation induced after transient cerebral ischemia prevents the expression of key effector UPR proteins such as the XBP1, GRP78, or ATF4, thereby hindering recovery from ischemia-

induced ER dysfunction (Kumar et al., 2001; Paschen, 2003a) and possibly leading to a pro apoptotic phenotype (De-Gracia and Montie, 2004). Similarly, in experiments of Urban et al. (2009), authors did not detected any significant changes in the protein level of XBP1 neither in ischemic period nor in the first 24 h of reperfusion. The results from measurements of XBP1 mRNA in simvastatin-treated animals did not show any significant changes in comparison to naive ischemic animals, i.e., the maximal differences were detected in the first and third hour of reperfusion (about 32.8 ± 4.1% lower in I1R and two times higher in I3R, respectively). A bit surprisingly, the protein level of XBP1 was generally decreased in pre-treated animals (mainly in ischemic and I1R phase than non treated group), and did not reach control levels. Recently, a novel action of statins was proven in neurons, involving cell growth and signaling as well as down-regulation of proinflammatory gene expression attenuating neurogenic inflammation (Johnson-Anuna et al., 2005; Bucelli et al., 2008).

The results of real-time PCR measurement showed an increased mRNA level of GRP78 in ischemic time and at later phases of reperfusion in non-treated animals. Probable reason is that GRP78 is a member of the 70-kDa heat shock protein family that acts as a molecular chaperone in the folding and assembly of newly synthesized proteins within the ER. Yu et al. (1999) reported that suppression of GRP78 expression enhanced apoptosis and disruption of cellular calcium homeostasis in hippocampal neurons exposed to excitotoxic and oxidative insults. This indicates that a raised level of GRP78 makes cells more resistant to the stressful conditions (Aoki et al., 2001).

In experiments of Urban et al. (2009), authors did not find any significant changes in protein levels of GRP78 neither in simvastatin-treated nor in non-treated group of animals. They have just found maximum at third hour of reperfusion in statin group and small decrease at 24 h of reperfusion in both groups. Those results are similar to the findings of Burda et al. (2003), who failed to find any differences in GRP78 protein levels at any of the reperfusion times considered (max 4 h), either in rats with or without acquired ischemic tolerance. However, in a model of ischemic preconditioning in rats (Hayashi et al., 2003; Garcıa et al., 2004) an increase in GRP78 expression was detected after 2 days of preconditioning. Authors proposed that development of tolerance includes changes in PERK/GRP78 association, which were responsible for the decrease in eIF2a phosphorylation induced by preconditioning. Other studies using distinct ischemic models also failed to detect increased levels of GRP78 protein (Paschen 2003a).

The results of Urban et al, (2009) also showed an increased mRNA expression of ATF6, however, only in ischemic time. Consequently levels of mRNA for GRP78 were increased only slightly compared to controls. The minimum level of mRNA for ATF6 was observed at third hour of reperfusion followed by increase till 24 h of reperfusion. This minimum was probably due to pro-survival mechanism through inhibition of proapoptotic protein GADD153, which usually acts as a transcription factor of UPR genes. GADD153 protein decreased during reperfusion, until the minimum was reached at the third hour of reperfusion (Kumar et al., 2003). Urban et al. (2009) also showed significant higher levels of ATF6 mRNA in statin-treated animals in comparison to non-statin animals at ischemic period and at third hour of reperfusion (about 35.2 ± 6.6% and 42 ± 2.6% higher level), which was also translated into the higher protein level, whose values had significant maximum at third hour of reperfusion (about 60% higher level than in non-treated animals).

The experimental results altogether indicate that global ischemia/reperfusion initiates time-dependent differences in endoplasmic reticular gene expression at both the mRNA and

protein levels and these authors also found the generally enhanced level of mRNA in simvastatin pre-treated animals. The maximal differences between naive ischemic and pre-treated ischemic animals authors detected in protein levels of proteins ATF6 and XBP1. The level of ATF6 was 60% higher in simvastatin pre-treated animals, which might suggest that ATF6 is one of the main proteins targeted to enhance neuroprotective effect at the ER gene level during first two hours of reperfusion.
In conclusion, these data indicate that statins, in addition to their cholesterol-lowering effect may exert a neuroprotective role in the attenuation of ER stress response after acute ischemic/reperfusion insult.

4. Impact of IRI and IPC on mitochondrial calcium transport, p53 translocation and neuronal apoptosis

Mitochondria are important regulators of neuronal cell life and death through their role in metabolic energy production and involvement in apoptosis (Yuan and Yanker, 2000). Remarkably, mitochondrial dysfunction is considered to be one of the key events linking ischemic/recirculation insult with neuronal cell death (Berridge et al., 2003). In addition, mitochondria play a dual role in intracellular calcium. They are involved in the normal control of neuronal Ca^{2+} homeostasis (Berridge et al., 2003), such as Ca^{2+} signaling, Ca^{2+} - dependent exocytosis and stimulation of oxidative metabolism and ATP production (Rizzuto, 2001; Gunter et al., 2004).
Conversely, mitochondrial Ca^{2+} overload and dysfunction, due to excitotoxic activation of glutamate receptors, is a crucial early event which follows ischemic or traumatic brain injury (Nicholls et al., 2007). Evidence for mitochondrial Ca^{2+} accumulation after excitotoxic stimulation comes from experimental studies which support the idea that mitochondrial depolarization during glutamate exposure is neuroprotective (Pivovarova et al., 2004), while its reduction correlates with excitotoxicity (Ward et al., 2007). In addition, activation of apoptosis has been documented after brain ischemia in several studies (Cao et al., 2003; Endo et al., 2006), and that this phenomenon might be closely linked to mitochondrial dysfunction. In fact, mitochondrial dysfunction provoked activation of apoptotic machinery by direct triggering of cytochrome c release (Clayton et al., 2005), or induction of Bax-dependent neuronal apoptosis through mitochondrial oxidative damage (Endo et al., 2006).
Mitochondria are involved in the control of neuronal Ca^{2+} homeostasis and neuronal Ca^{2+} signaling. In a series of recent papers (Racay et al., 2007, 2009a,b,c), authors have studied the effect of global cerebral ischemia/reperfusion injury (IRI) and ischemic tolerance developed by prior ischemic non-injurious stimulus – preconditioning- ischemic preconditioning (IPC) on mitochondrial **Ca^{2+}homeostasis** and mitochondrial way of **apoptosis**. As documented by Racay et al. (2007, 2009a), global ischemia led to progressive decrease of complex I activity after IRI to 65.7% of control at 24 h after reperfusion. In preconditioned animals, the activity of complex I was also significantly inhibited after ischemia (to 65.4% of control) and ischemia/reperfusion for 1, 3, and 24 h (62-78% of control). Although the values in preconditioned animals were significantly smaller compared to naive ischemia, IPC did not protect complex I from ischemia induced inhibition. On the other hand, activity of the terminal enzyme complex of respiratory chain, complex IV were slightly protected by IPC and the net effect of IPC was the shift of its minimal activity from 1 h to 3 h after reperfusion (Racay et al., 2009c).

Mitochondrial dysfunction and oxidative stress were often implicated in pathophysiology of neurodegenerative diseases, including cerebral ischemia (Lin and Beal, 2006). Inhibition of complex I itself or in combination with elevated Ca^{2+} led to enhanced ROS production in different *in vitro* and *in vivo* systems (Yadava and Nicholls, 2007). Importantly, an enhanced production of ROS and consequent induction of p53-dependent apoptosis due to damage to neuronal DNA has also been documented after inhibition of complex I. A recent study showed that spare respiratory capacity rather then oxidative stress is involved in excitotoxic cell death (Yadava and Nicholls, 2007).

As shown by experimental and clinical studies, IRI –induced mitochondrial pathway of apoptosis is an important event leading to neuronal cell death after blood flow arrest. Impact of IRI and ischemic preconditioning on the level of apoptotic and anti-apoptotic proteins was assessed in both cortical and hippocampal mitochondria by Western blot analysis of p53, bax, and bcl-x (Racay et al., 2007, 2009b). Remarkably, IRI led to increase of p53 level in hippocampal mitochondria, with significant differences after 3 h (217.1 ± 42.2% of control), 24 h (286.8 ± 65% of control), and 72 h (232.9 ± 37.3% of control) of reperfusion. Interestingly, translocation of p53 to mitochondria was observed in hippocampus but not in cerebral cortex. However, levels of both the apoptotic proteins bax and the anti-apoptotic bcl-xl were unchanged in both hippocampal and cortical mitochondria. Ischemia-induced translocation of p53 to mitochondria was completely abolished by IPC since no significant changes in mitochondrial p53 level were observed after preconditioned ischemia. Similar to naive ischemia, the levels of both bax and bcl-xl were not affected by IPC. In addition, IPC had significant protective effect on ischemia-induced DNA fragmentation, as well as on number of positive Fluoro-Jade C staining cells. Thus, it indicates that IPC abolished almost completely both initiation and execution of mitochondrial apoptosis induced by global brain ischemia in vulnerable CA1 layer of rat hippocampus (Racay et al., 2007, 2009b).

The studies showed that ischemia induced inhibition of mitochondrial complexes I and IV, however inhibition is not accompanied by a decrease of mitochondrial Ca^{2+} uptake rate apparently due to the excess capacity of the complex I and complex IV. On the other hand, depressed activities of complex I and IV are conditions favourable of initiation of cell degenerative pathways, e.g. opening of mitochondrial permeability transition pore, ROS generation and apoptosis initiation, and might represent important mechanism of ischemic damage to neurons.

Accordingly, ischemic preconditioning acts at the level of both initiation and execution of ischemia-induced mitochondrial apoptosis affording protection from ischemia associated changes in integrity of mitochondrial membranes. IPC also activates inhibition of p53 translocation to mitochondria. Inhibition of the mitochondrial p53 pathway thus might provide a potentially important mechanism of neuronal survival in the face of ischemic brain damage (Otani, 2008).

5. Stress reaction of neuronal endoplasmic reticulum after IRI and IPC

Ischemic tolerance can be developed by prior ischemic non-injurious stimulus or preconditioning. The molecular mechanisms underlying ischemic tolerance are not yet fully understood yet. Therefore a series of papers (Urban et al., 2009; Lehotsky et al., 2009; Pavlikova et al.,2009) have focused attention at the mRNA and protein levels of **the ER stress** genes after **ischemic/reperfusion damage (IRI)** in naive and preconditioned groups of rats.

In the UPR response, an activated IRE1 specifically cuts out the coding region of X-box protein 1 (XBP1) mRNA (Calfon et al., 2002) which after translation functions as a transcription factor specific for ER stress genes including GRP78 and GRP94. In these experiments, the hippocampal mRNA for XBP1 showed elevated levels in the naive IRI group of animals during the ischemic phase (about 43%) as well as persistent non-significant changes in all other analyzed periods (Urban et al., 2009; Lehotsky et al., 2009).

Preischemic treatment (IPC) induces the level of hippocampal mRNA in ischemic phase only slight but not significant differences compared to controls, followed by significant decreases at 24 hours of reperfusion (by about 12.8 ± 1.4% compared to controls). When analyzed the translational product, the hippocampal **XBP1 protein** level in naive IRI animal group showed significant differences in ischemic phase (39.2 ± 1.6% compared to controls) and the levels were significantly elevated at later reperfusion periods (3 and 24 h) (82 ± 2.4% and 24.1± 1.6% respectively compared to controls). The influence of preischemia (IPC) on protein levels was significant mainly in later ischemic times. The protein level reached a maximum at 3 h of reperfusion (about 230% of controls) and stayed elevated in the later reperfusion (40.3 ± 4.9% compared to controls) (Urban et al., 2009; Lehotsky et al., 2009).

Endoplasmic reticular chaperone, the Ca^{2+} binding, **glucose regulated protein 78 (GRP78)** was shown to prevent neuronal damage (Morimoto et al., 2007). Under ER dysfunction and GRP78 dissociation it subsequently induced expression of ER stress genes. At the level of mRNA for GRP78 in hippocampus from naive IRI group of animals, the authors observed that maximal differences appeared in later reperfusion phases. Preischemic pretreatment (IPC) led to elevated mRNA hippocampal levels in the reperfusion period by about 11.7 ± 3.6 during the first hour and by about 8.7± 1.8% the next 24 hours of reperfusion in comparison to mRNA levels in corresponding ischemic/reperfusion times. Remarkably, the level of GRP78 protein in naive IRI showed rapid increases in ischemic time (by about 217% of controls) and remained elevated throughout 3 to 24 hours of reperfusion (about 213% and 43%, respectively, compared to controls). Increased mRNA values in preconditioned animals also corresponded with the significant increase of the levels of GRP78 protein. The changes are documented in the ischemic phase and also in all reperfusion times (by about 250% of controls and about 50% of corresponding ischemic/reperfusion times) (Urban et al., 2009; Lehotsky et al., 2009).

ATF6 works as a key transcription factor in the resolution of the mammalian UPR (Yoshida et al. 2001). As shown in this experiment, the mRNA level for ATF6 in naive IRI animals showed gradual significant increases up to 24 hours of reperfusion (9.2 ± 4 % higher than control) and preconditioning (IPC) did not significantly alter mRNA levels in all analyzed periods. Similarl to mRNA levels, the hippocampal ATF6 protein level in naive IRI animals followed the same patterns. IPC on the other hand, induced remarkable changes in the protein levels at ischemic phase achieving significant increased levels (about 170%) in comparison to controls and stayed elevated in earlier reperfusion times (about 37 and 62 % higher than in controls) and later reperfusion time (about 15% of controls).

In general, IRI initiates suppression of global proteosynthesis, which is practically recovered in the reperfusion period with the exception of the most vulnerable neurons, such as pyramidal cells of CA1 hippocampal region (de la Vega et al., 2001). 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 fact, IRI induces transient inhibition of translation, which prevents the expression of UPR

proteins and hinders recovery from ischemia-induced ER dysfunction (Kumar et al., 2001; Paschen et al., 2003a) which possibly leads to a pro-apoptotic phenotype (DeGracia and Montie, 2004). Similarly, Thuerauf et al. (2006) found that myocardial ischemia activates UPR with the increased expression of XBP1 protein and XBP1-inducible protein. They contribute to protection of the myocardium during hypoxia. Also the results of Paschen et al. (2003a) using semi-quantitative RT-PCR showed a marked increase in XBP1 mRNA levels after focal ischemia in the cerebral cortex.

Preischemia induced elevation of mRNA and protein GRP78 levels in reperfusion periods. GRP78 is a member of the 70kDa heat shock protein family that acts as a molecular chaperone in the folding and assembly of newly synthesized proteins within the ER. As shown by Yu et al. (1999) the suppression of GRP78 expression enhances apoptosis and disruption of cellular calcium homeostasis in hippocampal neurons that are exposed to excitotoxic and oxidative insults. This indicates that a raised level of GRP78 makes cells more resistant to the stressful conditions (Aoki et al. 2001). Similar results were obtained by Morimoto et al. (2007) in the focal ischemia model. Also Hayashi et al. (2003) and Garcia et al. (2004), who demonstrated an increase in GRP78 expression after 2 days of preconditioning proposed that the development of tolerance includes changes in PERK/GRP78 association, which were responsible for the decrease in eIF2a phosphorylation induced by preconditioning. On the other hand, Burda et al. (2003), failed to find any differences in the level of GRP78 protein in rats with or without acquired ischemic tolerance. This was probably due to exposure to very short reperfusion times. ATF6 is an ER-membrane-bound transcription factor activated by ER stress, which is specialized in the regulation of ER quality control proteins (Adachi et al., 2008). Haze et al. (1999) found that the overexpression of full-length ATF6 activates transcription of the GRP78 gene. Explanation of generally higher levels of protein p90ATF6 in preischemic group is probably connected to an increased promotor activity of GADD153 to UPR genes (Oyadomari et al., 2004).

The data from these experiments (Urban et al. 2009; Lehotsky et al. 2009) suggest that IRI initiates time dependent differences in **endoplasmic reticular gene expression** at both the mRNA and protein levels and that endoplasmic gene expression is affected by preischemic treatment. These data and recent experiments of Bickler et al. (2009) also suggest that preconditioning paradigm (preischemia) may exert a role in the attenuation of ER stress response and that InsP3 receptor mediated Ca^{2+} signaling is an important mediator in the neuroprotective phenomenon of acquired ischemic tolerance. Changes in gene expression of the key proteins provide an insight into ER stress pathways. It also might suggest possible targets of future therapeutic interventions to enhance recovery after stroke (Yenari et al., 2008; Pignataro et al., 2009).

6. Effect of ischemic preconditioning on secretory pathways Ca^{2+}-ATPase gene expression

The Golgi apparatus, as a part of **secretory pathways (SP)** in neural cells, represents a dynamic Ca^{2+} store. Ca^{2+} ions play an active role in processes such as secretion of neurotransmitters and secretory proteins for the growth/ reorganization of neuronal circuits, synaptic transmission, neural plasticity, and remodeling of dendrites (Michelangeli et al., 2005). In addition, SP are involved in the stress sensing, neuronal aging, and transduction of apoptotic signals (Maag et al., 2003; Sepulveda et al., 2008). On the other

hand, a high luminal Ca^{2+} concentration, and Mn^{2+}, is required in the Golgi apparatus for the optimal activity of many enzymes and for post-translational processing and trafficking of the newly formed proteins. For both cytosolic and Golgi Ca^{2+} and Mn^{2+}homeostasis, **the secretory pathway Ca^{2+}-ATPases (SPCAs)** play an important role.

The 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; Murin et al., 2006). Two isoforms sharing 64% of sequence identity, namely SPCA1 and SPCA2, are expressed in mammalian cells (Wootton et al., 2004; Xiang et al., 2005). While SPCA2 expression seems to be more restricted to specific cell types, the SPCA1 is considered as a house-keeping isoform with pronounced expression in neural cells (Wootton et al., 2004; Murin et al., 2006; Sepulveda et al., 2008). The higher expression levels of SPCA1 in the brain coincide with a relatively high ratio of SPCA activity (thapsigargin insensitive) to the total activity of Ca^{2+}-dependent ATPases. Therefore, implying a significant role of SPCA-facilitated transport of Ca^{2+} for calcium storage within the brain (Wootton et al., 2004).

As shown by previous studies, the SPCA plays a pivotal role in normal neural development, neural migration, and morphogenesis (Sepulveda et al., 2007, 2008). In addition, as shown in SPCA1 knockout mice, SPCA1 deficiency caused alteration in neural tube development and Golgi stress. These animals presented structural changes in the Golgi such as dilatation and the reduction in the number of stacked leaflets (Okunade et al., 2007). In apoptosis, a morphological change in the Golgi complex, for example its fragmentation, represents an early causative step rather than a secondary event, and it is very commonly associated with several neurodegenerative diseases, such as amyotrophic lateral sclerosis, corticobasal degeneration, Alzheimer's and Creutzfeldt-Jacob diseases, and spinocerebelar ataxia type 2 (Gonatas et al., 2006).

6.1 Effect of oxidative damage on SPCA1

Collective studies confirm that reactive oxygen species contribute to neuronal cell injuries secondary to ischemia and reperfusion (Lehotsky et al., 2004; Burda et al., 2005; Danielisova et al., 2005; Shi and Liu, 2007). Oxidative burst lasting several minutes upon the onset of reperfusion is followed by dysregulation of antioxidant mechanism and moderate but persistently elevated production of oxygen radicals which might initiate cell death signaling pathways after cerebral ischemia and parallels with selective postischemic vulnerability of the brain (Valko et al., 2007; Shi and Liu, 2007).

One of the main aims of the study of Pavlikova et al. (2009) was to determine whether IRI and IPC would affect the physical and functional properties of hippocampal membrane vesicles including Golgi SP. Neuronal microsomes are vulnerable to physical and functional oxidative damage (Lehotsky et al. 1999, 2002a; Urikova et al. 2006). The nature of the effect of free radicals on SPCA1 protein is not yet known. Authors show here for the first time that SPCA activity is also selectively damaged by free radicals in vitro, the property which is similar to other P-type ATPase such as SERCA and PMCA (Lehotsky et al. 2002b). In the study, authors showed that transient ischemia for 15 min induces considerable LPO and protein oxidation in hippocampal membranes. Protein oxidation pursues disturbances in oxidant/antioxidant balance and depression of enzymatic activities of main antioxidant enzymes detected at later stages after the ischemic insult (Lehotsky et al., 2002a; Urıkova et al., 2006). Thus, oxidative alterations detected after IRI may at least partially explain

functional post-ischemic disturbances of neuronal ion transport mechanisms (Lipton, 1999; Lehotsky et al., 2002a; Obrenovitch, 2008) and inhibition of global proteosynthesis (Burda et al., 2003), which both are implicated in neuronal cell damage and/or recovery from ischemic insult.

IPC caused significant reductions of LPO products and it reduced protein oxidative changes induced by ischemia in the hippocampal membranes in both the ischemic time and in reperfusion period. One of the possible explanations comes from the studies describing upregulation of defense mechanisms (antioxidant enzymes) against oxidative stress due to the preconditioning challenge (Danielisova et al., 2005; Gidday, 2006; Obrenovitch, 2008). In addition, forebrain ischemia causes small but significant drops in **the SPCA-associated Ca^{2+}-ATPase activity** (by about 9%). The activity increases in early reperfusion times. However, it did not reach the control level and reached the highest depression after 24 h reperfusion to 88% of control. In the experiments, the IPC had a partial protective effect on the SPCA-associated Ca^{2+}-ATPase activity. Ischemic insult after IPC pretreatment initiate only non-significant inhibition of Ca^{2+}-ATPase activity compared to preconditioned control. After 1 and 3 h of reperfusion, the activity exceeded the control levels and reached it again after 24 h of reperfusion. However, the changes were not statistically significant at any reperfusion time. As shown in earlier studies, preconditioning upregulates defense mechanisms against oxidative stress (Danielisova et al., 2005; Gidday, 2006; Obrenovitch, 2008), which might partially restore the depression of enzyme activity. Additionally, as shown in the study by Western blot analysis, IPC induced an elevation of SPCA protein level in comparison to corresponding naive ischemic control.

In summary, the experiments conclusively showed that cerebral IRI-induced depression of SPCA activity and lipid and protein oxidation in rat hippocampal membranes. IRI also activates induction of SPCA1 gene expression in later reperfusion periods. IPC partially suppresses oxidative changes in hippocampal membranes and also partially restores the ischemic-induced depression of SPCA activity.

In addition, IPC initiates earlier cellular response to the injury by the significant elevation of mRNA expression (to 142% comparing to 1 h of corresponding reperfusion) and to 154 and 111% comparing to 3 and 24 h of corresponding reperfusion, respectively. Similar patterns were observed on the translational level by Western blot analysis. Results of Pavlikova et al. (2009) indicate the specific SPCA1 expression pattern in injured ischemic hippocampus and might serve to understand the molecular mechanisms involved in the structural integrity and function of the Golgi complex after ischemic challenge. They also suggest for the correlation of SPCA function with the role of SP in response to preischemic challenge.

Collective studies confirm, that reactive oxygen species (ROS) contribute to neuronal cell injuries secondary to ischemia and reperfusion (Lehotsky et al., 2004; Burda et al., 2005; Danielisova et al., 2005; Shi and Liu, 2007) and might initiate cell death signaling pathways after cerebral ischemia and parallels with selective post-ischemic vulnerability of the brain (Valko et al., 2007; Shi and Liu, 2007; Otani, 2008; Dirnagl et al., 2009). As shown by measurement of steady state fluorescence of ANS in hippocampal mitochondria (Racay et al., 2007, 2009a), naive IRI induced significant increase in ANS flurescence (it binds to hydrophobic part of membrane lipids and proteins) of the forebrain in both ischemic and reperfusion periods. These results support data from previous experiments (Lehotsky et al., 2004; Babusikova et al., 2008), which showed that IRI induced structural changes on hippocampal membrane lipids and both, the lipoperoxidation dependent and the direct

oxidative modifications of membrane proteins. Remarkably, preconditioning (IPC) induces significant decrease of ANS fluorescence, which indicates protective effect of IPC on mitochondrial membranes.

SP are involved in the stress sensing, neuronal aging and transduction of apoptotic signals (Maag et al., 2003; Sepulveda et al., 2008). In order to evaluate whether the severe metabolic stress induced by IRI and/or IPC affects transcription of SPCA1 gene, the mRNA and protein levels of SPCA1 was analyzed (Lehotsky, 1999, 2002a, 2004). As shown by Pavlikova et al. (2009), RT-PCR clearly detected, that hippocampal cells respond to the IRI by induction of mRNA level in reperfusion period with maximum at 3 h reperfusion (to 171% of control). Preconditioning (IPC) initiates earlier tissue response to the injury by the significant elevation of mRNA expression already at 1 h of reperfusion and the level of mRNA expression reached 142% comparing to 1 h ischemia, and to 164% comparing to control.

Neuronal microsomes are vulnerable to physical and functional oxidative damage (Lehotsky et al., 1999, 2002a, 2004; Urikova et al., 2006). The authors showed (Pavlikova et al. (2009) that SPCA activity, similar to other P-type ATPases, is also subject to ischemic damage most likely due to free radicals action (Lehotsky et al., 2002b). In addition, oxidative alterations detected in mitochondria and microsomes after IRI in our experiments, may at least partially explain functional postischemic disturbances of neuronal ion transport mechanisms (Lipton 1999; Lehotsky et al., 2002a; Obrenovitch, 2008) and inhibition of global proteosynthesis (Burda et al., 2003), which are both implicated in neuronal cell damage and/or recovery from ischemic insult, IPC-induced reduction of lipoperoxidation products and protein oxidative changes (Racay et al., 2009; Pavlikova et al., 2009). These may all be probably due to upregulation of defence mechanisms (antioxidant enzymes) against oxidative stress in the preconditioning challenge (Danielisova et al., 2005; Gidday, 2006; Obrenovitch, 2008).

One of the most pronounced morphological features following IRI is the mitochondrial and Golgi swelling and activation, which could be suppressed by neuroprotective treatment (Hicks and Machaner, 2005; Strosznajder et al., 2005; Gonatas et al., 2006). The secretory pathways are apparently involved in sensing stress and transducing signals during the execution phase of apoptosis (Maag et al., 2003; Hicks and Machamer, 2005). Data from Pavlikova (2009) showed a partial recovery of Ca^{2+}-ATPase activity and earlier hippocampal response to later ischemia by the induction of mRNA and protein expression.

Cross-talk between the function of intracellular organelles following ischemic insult and reperfusion (Fig.2) and response of the tissue to the preischemic challenge (Fig 3) is depicted bellow .

6.2 Effect of hyperhomocysteinemia on SPCA expression

Homocysteine (Hcy) is a sulfur-containing amino acid, which is derived from methionine metabolism. Hyperhomocysteinemia, condition in which Hcy concentration exceeds 16 μmol/l, is the result of perturbed Hcy metabolism and dietary deficiencies in folic acid, vitamin B6, and/or vitamin B12 (Obeid et al., 2007).

Hyperhomocysteinemia has been implicated as an independent risk factor for arteriosclerosis and coronary heart disease (Refsum et al., 1998; Thambyrajah et al., 2000). Severe forms of hyperhomocysteinemia results in convulsions and dementia (Watkins et al., 1989; van den Berg et al., 1995) corresponding multiple participation of homocysteine (Hcy) in diverse pathologies that affect the CNS. Likewise, homocysteine has also been associated

with several CNS disorders, such as stroke (Obeid et al., 2007), epilepsy (Sachdev, 2004; Herrmann et al., 2007), neurodegenerative (Clarke et al., 1998; Mattson et al., 2002) and neuropsychiatric diseases (Diaz-Arrastia, 2000; Bottiglieri, 2005), as well as inborn errors of metabolism (Mudd et al., 2001). In addition, even moderate hyperhomocysteinemia is a factor stimulating the development of dementia and Alzheimer's disease (Seshadri et al., 2002).

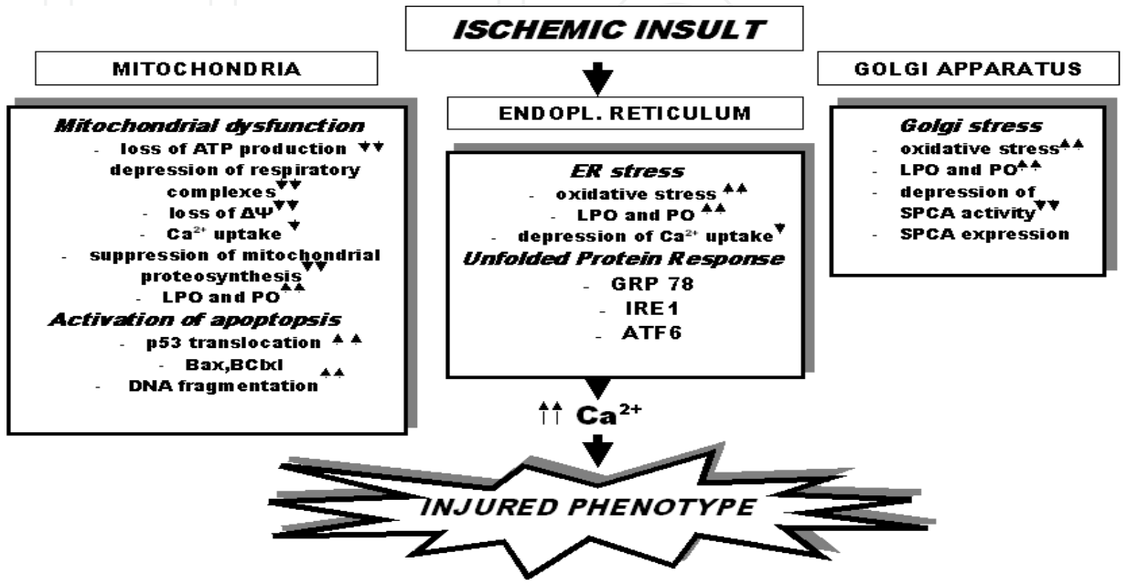

Fig. 2. Cross-talk between the fuction of intracellular organelles which follows ischemic insults and results in injured phenopyte in vulnerable neurons. Adapted from Lehotsky et al. (2009c).

Ischemic brain stroke in humans represents very complex cerebrovascular disease. A number of conventional risk factors for ischemic stroke are known, such as a previous occurrence of stroke, previous transient ischemic attack (TIA), arterial disease, atrial fibrillation, poor diet and/or obesity and physical inactivity (Prasad, 1999). It has been reported that hyperhomocysteinemia may also be associated with the incidence of ischemic brain stroke (Refsum et al., 1998), mainly due to pleiotropic activity of homocysteine and acceleration of atherosclerotic changes (Refsum et al., 1998; Thambyrajah et al., 2000). In fact, Hcy suppresses NO production by endothelial cells (Upchurch et al., 1997) and platelets (Mutus et al., 2001) and increases 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 (Jeremy et al., 1999; Domagala et al., 1998).

In addition, Hcy has been shown to inhibit methyltransferases, to suppress DNA reparation and to facilitate apoptosis when accumulated inside the cells (Duan et al., 2002; Kruman et al., 2002). Autooxidation of Hcy metabolites results in H_20_2 accumulation (Gortz et al., 2004; Boldyrev, 2005) and long term incubation of neurons with Hcy metabolites induces necrotic cell death (Zieminska et al., 2003; Boldyrev et al., 2004). Homocysteine has also been shown to be elevated in other disorders of the CNS, e.g. Alzheimer disease or Parkinson disease (Toohey, 2007).

A series of papers (Urban et al., 2009; Lehotsky et al., 2009b; Pavlikova et al.,2009) found that ischemia/reperfusion injury (IRI) initiates time dependent differences in endoplasmic reticular gene expression at both the mRNA and protein levels in rat hippocampus and that endoplasmic gene expression is affected by pre-ischemic treatment. More recently, Pavlikova et al. (2011) conducted an investigation into the differences between naive control and hyperhomocysteinemic control animals in each group independently. They showed for the first time that experimental 2 weeks hyperhomocysteinemia significantly decreased the level of SPCA1 mRNA gene expression in cerebral cortex which also led to the non-significantly decreased expression levels in hippocampal area. In cortex, ischemic challenge for 15 min. did not change significantly the level of mRNA SPCA1 expression in comparison to controls. Conversely, the gene response to pre-ischemic challenge was clearly shown within the homocysteine group by abrupt stimulation of the mRNA expression level to 249 % of hyperhomocysteinemic ischemic group and to 321% of hyperhomocysteinemic control. Notably, values far exceed those observed in the naive control. However, the effect of IPC challenge was not observed in the naive groups.

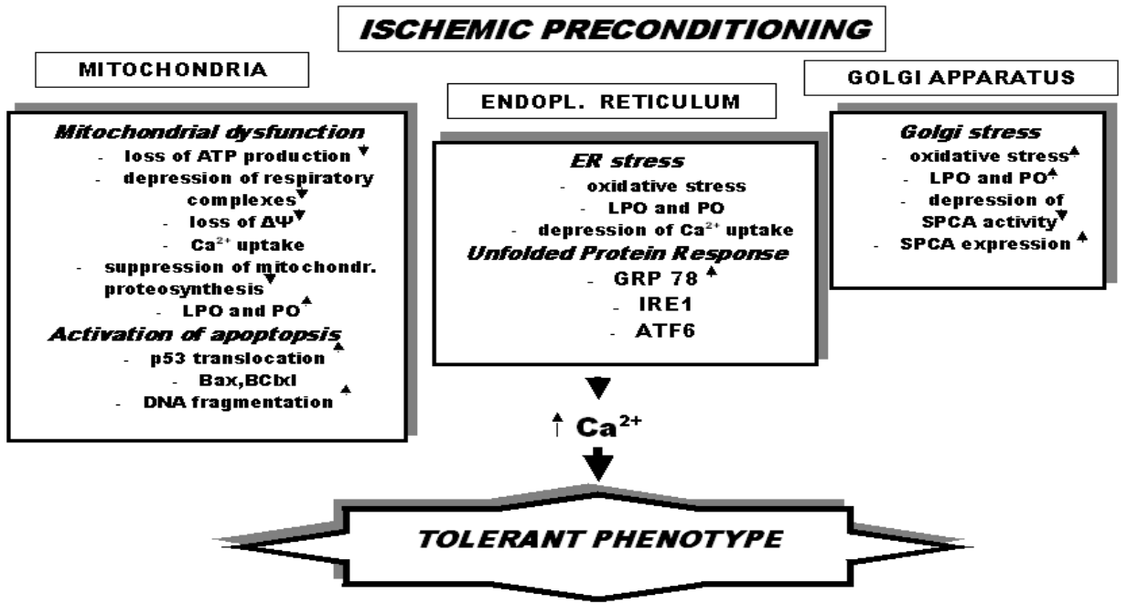

Fig. 3. Cross-talk between the fuction of intracellular organelles which follow preischemic maneuver and results in tolerant phenopyte in vulnerable neurons. Adapted from Lehotsky et al. (2009c).

The expression level decreased in the hyperhomocysteinemic control to 259% ($p<0.05$) of naive control and to 277% of control with IPC. When changes were compared between all ischemic groups, the following were observed: low level of mRNA expression in hyperhomocysteinemic ischemic group (to 201% of naive ischemia and to 185% of ischemic preconditioning. However, there were no significant differences between Hcy-control group and Hcy- ischemic group. Preischemic challenge initiated stimulation of the mRNA expression to 249% of hyperhomocysteinemic ischemic group. This response may be

attributed to a part of the protective tolerant phenomenon induced by preconditioning treatment.

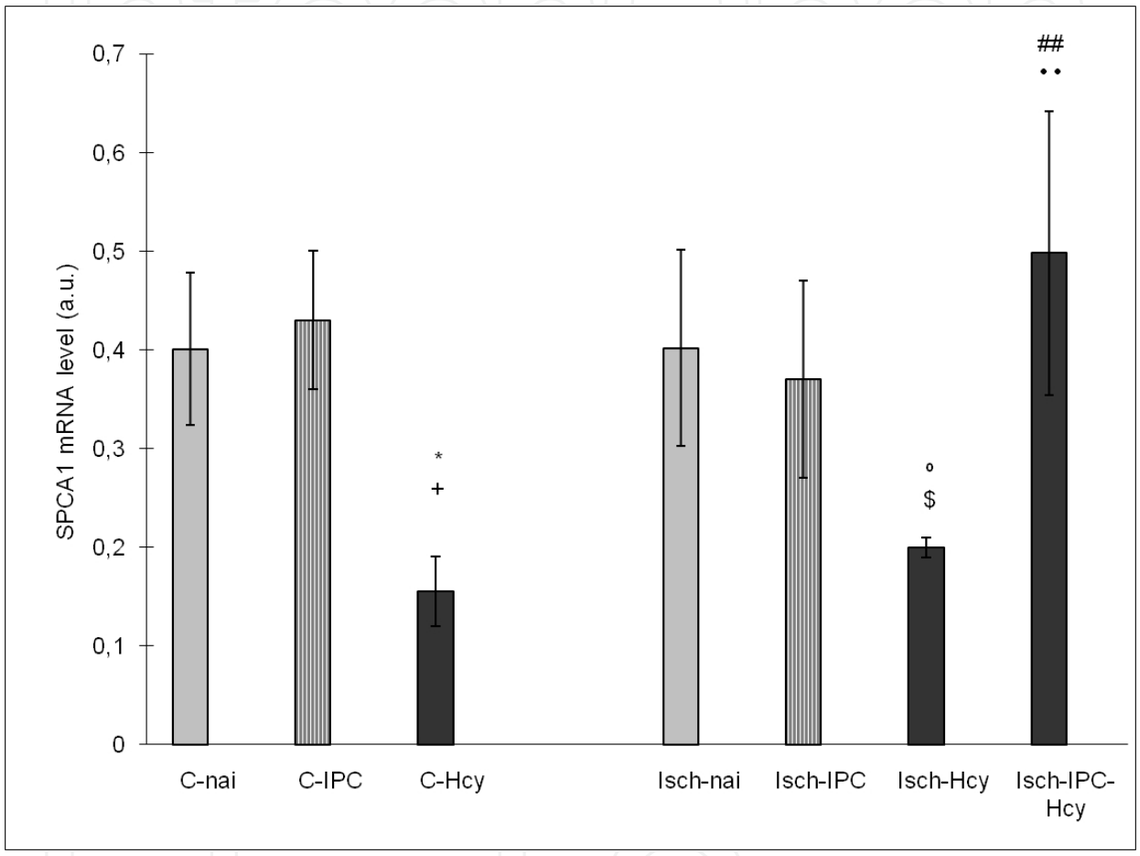

Fig. 4. Comparison of mRNA levels of SPCA1 between naive group (C-nai, Isch-nai), IPC group (C-IPC, Isch-IPC) and hyperhomocysteinemic group (C-Hcy, Isch-Hcy, Isch-IPC-Hcy) in rat cortex. Results are presented as mean ± SEM for $n = 6$. * $p < 0.05$ compared to C-nai group, + $p < 0.05$ compared to C-IPC groups, † $p < 0.05$ compared to Isch-Hcy group, ‡ $p < 0.05$ compared to C-IPC group, ## $p < 0.01$ compared to Isch-IPC-Hcy group, && $p < 0.01$ compared to Isch-IPC-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. Adapted from Pavlikova et al. (2011).

In results of mRNA SPCA1 expression in hippocampal area no statistically significant changes were found between naive control and IPC control groups. Hyperhomocysteinemia for 14 days supressed mRNA expression, however the changes were not statistically significant. Similarly, as shown in the cortex, the preischemic challenge in hippocampal region initiated stimulation of the mRNA expression by 159% of hyperhomocysteinemic control and by to 131% hyperhomocysteinemic ischemic group. The suggestion was, that this response might also be part of the protective tolerant phenomenon induced by preconditioning treatment.

The previous results showed that IRI insult alters time expression profile of SPCA1 on mRNA and protein level (Pavlikova et al. 2009), and that preischemic challenge (induction of tolerance), not only preserved majority of surviving neuron but also activates partial recovery of the secretory pathways SPCA Ca^{2+}-ATPase activity and earlier hippocampal response to later ischemia by the induction of SPCA1 mRNA and protein expression. We shown here for the first time that chemically induced experimental 2 weeks hyperhomocysteinemia significantly decreased the level of SPCA1 mRNA gene expression in cerebral cortex and also led to the non-significant decreased expression level in hippocampal area. There are no literature data on how the Hcy might affect the expression profile of the Ca^{2+}-transport proteins in neuronal cells. 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 the gene regulation by the cis-enhancing elements in 5´-untranslated regions (Kawada et al., 2005). Another possibility is the expression of the putative endogenous activator of SPCA or the changes in local membrane environment are suggested as a cause for the increase in SPCA activity (Sepulveda et al. 2008). In fact, hyperhomocysteinemia often results in intracellular Ca^{2+} mobilization, endoplasmic reticulum (ER) stress, with the subsequent development of apoptotic events, chronic inflammation leading to endothelial dysfunction and remodeling of the extracellular matrix. Homocysteine has also been reported to induce modulation of gene expression through alteration of the methylation status (Dionisio, 2010).

In conclusion, our results indicate that chemically induced hyperhomocysteinemia initiates supression of the SPCA1 gene expression in both brain regions cerebral cortex and hippocampus. Documented response of SPCA gene to preischemic challenge in hyperhomocysteinemic group of animals might suggest for the correlation of SPCA expression with the role of secretory pathways in the proposed phenomenon of ischemic tolerance (Dirnagl et al., 2009; Pignataro et al., 2009). This might also serve to understand the molecular mechanisms involved in the structural integrity and function of the Golgi complex after ischemic challenge.

7. Conclusion

Ischemic induced alterations of mitochondria, endoplasmic reticulum and Golgi apparatus shed more light on understanding the cross-talk between intracellular Ca^{2+} stores in cerebral ischemia/reperfusion injury. Documented neuroprotective response of intracellular organelles in the phenomenon of ischemic tolerance may also form a basis for future therapeutic interventions to enhance recovery from stroke. Finally, exploration of the protective mechanisms could lead to the recognition of newer strategies and suggestions for development of novel prophylactic/therapeutics for neuronal apoptosis-related diseases.

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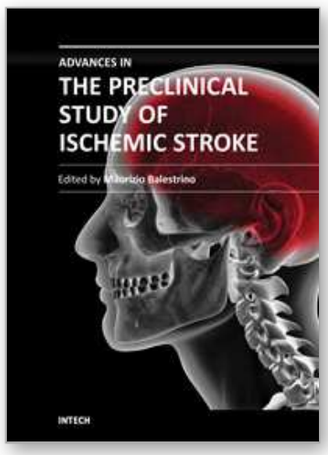

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This book reports innovations in the preclinical study of stroke, including - novel tools and findings in animal models of stroke, - novel biochemical mechanisms through which ischemic damage may be both generated and limited, - novel pathways to neuroprotection. Although hypothermia has been so far the sole "neuroprotection" treatment that has survived the translation from preclinical to clinical studies, progress in both preclinical studies and in the design of clinical trials will hopefully provide more and better treatments for ischemic stroke. This book aims at providing the preclinical scientist with innovative knowledge and tools to investigate novel mechanisms of, and treatments for, ischemic brain damage.

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