**Review**

# Cross-talk of intracellular calcium stores in the response to neuronal ischemia and ischemic tolerance

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**Abstract.** Ischemic/reperfusion brain injury (IRI) is a very severe event with the multiple etiopathogenesis. Ischemic preconditioning (IPC) is an important phenomenon of adaptation of CNS to subsequent ischemia. An altered cross-talk between intracellular calcium stores is presumed in the mechanisms of ischemic damage/protection. We show here that IRI leads to the inhibition of mitochondrial respiratory complexes I and IV, however due to the excess of their capacities, the mitochondrial $Ca^{2+}$ uptake rate is not significantly depressed. IPC acts at the level of both initiation and execution of IRI-induced mitochondrial apoptosis and protects from IRI-associated changes in integrity of mitochondrial membranes. IPC also activates inhibition of p53 translocation to mitochondria. Inhibition of the mitochondrial p53 pathway might thus provide a potentially important mechanism of neuronal survival after ischemic brain damage.

In addition, IRI initiates a time dependent differences in endoplasmic reticular (ER) gene expression of the key UPR proteins at both the mRNA and protein levels. Moreover, gene expression of the UPR proteins is affected by preischemic treatment 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 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 lipo- and protein oxidation of hippocampal membranes and leads to partiall recovery of the ischemic-induced depression of SPCA activity. The data suggests the correlation of SPCA function with the role of secretory pathways (Golgi apparatus) in response to preischemic challenge.

Documented functional alterations of mitochondria, ER and Golgi apparatus put light into the understanding of cross-talk between intracellular $Ca^{2+}$ stores in cerebral ischemia and ischemic tolerance and might suggest for possible targets of future therapeutic interventions to enhance recovery after stroke.

**Key words:** Cerebral ischemia — Ischemic tolerance — Intracellular calcium stores

## Introduction

Ischemic/reperfusion brain injury (IRI) is a very severe event with the multiple, parallel and sequentional pathogenesis (Endres 2008). Its etiology includes dysregulation of the energetic metabolism with intracellular derangement of ion homeostasis. Altered $Ca^{2+}$ dysregulation, triggering $Ca^{2+}$-dependent bio-polymer degradation and mitochondrial and bioenergetic failure, ultimately culminates in the activation of reactions leading to necrotic/apoptotic cell death. It is not yet clear which sources of $Ca^{2+}$ and which pathways are involved, however the defective cross-talk between intracellular stores is presumed in the etiopathogenesis of the injury (Bano and Nicotera 2007).

Ischemic preconditioning (IPC) represents an important phenomenon of adaptation of CNS to sub-lethal short-term

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ischemia, which results in increased tolerance of CNS to the lethal ischemia (Kirino 2002; Dirnagl et al. 2003, 2009; Gidday 2006; Obrenovitch 2008). The mechanisms underlying ischemic tolerance are rather complex and not yet fully understood. In all multiple paradigms for IPC, two windows have been identified. One window that represents very rapid and short-lasting post-translational changes and second, which develops slowly (over days) after initial insult as a robust and long lasting transcriptional changes which culminate to prolonged neuroprotection (Gidday 2006; Obrenovitch 2008; Yenari et al. 2008; Dirnagl et al. 2009).

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 event 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 $Ca^{2+}$ signalling, $Ca^{2+}$ -dependent exocytosis and stimulation of oxidative metabolism and ATP production (Rizzuto 2001; Gunter et al. 2004). On the other side, 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 the 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).

The endoplasmic reticulum (ER) of neural cells responds to the interruption of blood flow 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, such as 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, activities of these factors are suppressed by binding of the ER chaperone: $Ca^{2+}$ binding, glucose regulated protein 78 (GRP78). Remarkably, induction of GRP78 prevents neuronal damage and its increased expression may correlate with the degree of neuroprotection (Morimoto et al. 2007). Under ER dysfunction, GRP78 dissociates and subsequently induces expression of the ER stress genes. ATF6 is a key transcription factor in the resolution of the mammalian UPR, and unlike IRE1 and PERK, there is no evidence that ATF6 is involved in proapoptotic pathways (Yoshida et al. 2001). 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. Previous studies shown that changes of the UPR gene expression induced by IRI occur during the first 24 h (Paschen 2003) or the first few days after the insult (Qi et al. 2004).

The secretory pathways (SP) in neural cells represents a dynamic $Ca^{2+}$ store where high luminal $Ca^{2+}$ concentration, and also $Mn^{2+}$ are required for optimal activity of many enzymes and for processes such as secretion of neurotransmitters and secretory proteins (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). The SP derived $Ca^{2+}$-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; Murín et al. 2006). The SPCA1 isoform is considered as a house-keeping isoform with pronounced expression in neural cells (Wootton et al. 2004; Murín 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, implying a significant role of SPCA-facilitated transport of $Ca^{2+}$ for calcium storage within the brain (Wootton et al. 2004).

Remarkably, the SPCA plays a pivotal role in normal neural development, neural migration and morphogenesis (Sepulveda et al. 2007, 2008). Likewise, deficiency of SPCA in knock-out mice caused alteration in neural tube development and Golgi stress with its dilatation and reduction in the number of stacked leaflets (Okunade et al. 2007). Such morphological changes in the Golgi complex, like its fragmentation, represents an early causative step rather than a secondary event, and it is very commonly found 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).

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

Mitochondria are involved in the control of neuronal $Ca^{2+}$ homestasis and neuronal $Ca^{2+}$ signalling. In a series of recent

papers (Racay et al. 2007, 2009a,b,c), we have studied the effect of global cerebral ischemia/reperfusion injury (IRI) and ischemic preconditioning (IP) on mitochondrial $Ca^{2+}$ homeostasis and mitochondrial way of apoptosis.

At first, we have analyzed effect of global brain ischemia-reperfusion on mitochondrial $Ca^{2+}$ uptake in relation to ischemia-induced inhibition of complex I and IV. Although in previous experiments was detected decrease of $Ca^{2+}$ uptake capacity in isolated hippocampal mitochondria (Friberg et al. 2002), in our experimental setting we did not observe any significant changes on the rate of active $Ca^{2+}$ uptake in isolated mitochondria from hippocampi of rats submitted to ischemia-reperfusion. Neither ischemia nor reperfusion had significant impact on the rate of active $Ca^{2+}$ uptake, statistically significant reduction of mitochondrial capacity to accumulate $Ca^{2+}$ was observed only after 15 min of global ischemia (80.8% of control). The capacity of mitochondria to retain $Ca^{2+}$ was completely restored during reperfusion (Racay et al. 2009a,b).

It is generally accepted that the rate of mitochondrial $Ca^{2+}$ uptake is proportional to changes of membrane potential which is generated by respiratory chain complexes (Duchen, 2004). Our results have shown that rate of mitochondrial $Ca^{2+}$ uptake decreased proportionally with decrease of membrane potential (Racay et al. 2009a). Inhibition of key mitochondrial enzyme complexes is thought to be a cause of ischemia-induced mitochondrial dysfunction. It seems that mitochondrial complex I, the initial complex of respiratory chain, plays crucial role in mitochondrial functioning since complex I inhibition has been implicated in a number of brain pathologies (Duchen 2004). Indeed, ischemia induced progressive inhibition of complex I with the minimal activity expressed at 24 h after ischemia (63% of control). In addition, an inhibition of complex IV activity to 80.6% of control was observed 1 h after ischemia. This discrepancy between unaltered rate of $Ca^{2+}$ uptake and activities of both complexes was explained by titration experiments (Racay et al. 2009a). As shown from relationship between inhibition of respiratory complexes and generation of mitochondrial transmembrane potential, complex I and IV activities must be decreased by approximately 40, and 60%, respectively, before significant decline of the transmebrane potential. Thus, mitochondrial $Ca^{2+}$ uptake was not significantly affected by IRI, apparently due to excess capacity of the complexes I and IV. Inhibition of complex I is favourable of reactive oxygen species (ROS) generation. Maximal oxidative modification of membrane proteins was documented 1 h after ischemia. Although enhanced formation of ROS might contribute to neuronal injury, depressed activities of complex I and IV together with unaltered rate of $Ca^{2+}$ uptake are conditions favourable of initiation of other cell degenerative pathways like opening of mitochondrial permeability transition pore or apoptosis initiation, and might represent important mechanism of ischemic damage to neurones.

Ischemic preconditioning (IPC) represents an important phenomenon of adaptation of CNS to sub-lethal short-term ischemia, which results in increased tolerance of CNS to the lethal ischemia (Kirino 2002; Dirnagl et al. 2003, 2009; Gidday 2006; Obrenovitch 2008). 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 pretected by IPC and the net effect of IPC was the shift of its mininal actvity from 1 h to 3 h after reperfusion (Racay et al. 2009c).

As shown earlier by several studies, 10 min of global cerebral ischemia initiates decreased capacity for active $Ca^{2+}$ sequestration by isolated forebrain mitochondria, while a 5-h period of reperfusion after 30 min of forebrain ischemia in the rat also inhibited the ability of isolated mitochondria both to actively accumulate and retain $Ca^{2+}$ (Sciamanna et al. 1992). Decreased $Ca^{2+}$ uptake capacity was observed in isolated hippocampal mitochondria during reperfusion (Friberg et al. 2002). The discrepancy between our and previous studies might be attributable to different models of ischemia and different anaesthetics. As shown in our laboratory, the effect of ischemia on active $Ca^{2+}$ accumulation by endoplasmic reticulum depends on ischemic model and used anaesthetics (Racay et al. 2000). In addition, the ischemia/reperfusion-induced inhibition of mitochondrial $Ca^{2+}$ transport was parallel with decline of mitochondrial respiration (Sciamanna et al. 1992). In our study, the ischemia-induced inhibition of complex I and IV was not accompanied by significant changes in the rate of mitochondrial $Ca^{2+}$ uptake. It is generally accepted and was confirmed in our studies (Racay et al. 2007, 2009a,b,c) that the rate of mitochondrial $Ca^{2+}$ uptake is proportional to transmebrane potential which is generated by respiratory chain complexes. However, based on our titration experiments, we suppose that the initial rate of mitochondrial $Ca^{2+}$ uptake was not altered apparently due to excess capacity of the complex I and complex IV documented by energy thresholds (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*

(Panov et al. 2005) 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. 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, level 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).

Interestingly, Bcl-xl can prevent mitochondrial membrane permeabilization by competing with Bax (Billen et al. 2008), and it seems that Bax is already inserted in outer membrane of hippocampal mitochondria but the pore forming properties of bax are neutralized by high mitochondrial level of bcl-xl. Thus, our results are consistent with the recent view, that p53 protein can directly induce permeabilization of the outer mitochondrial membrane by forming a complex with protective Bcl-xl protein, resulting in oligomerization of Bax, cytochrome c release, and initiation of neuronal apoptosis after cerebral ischemia (Endo et al. 2006).

A considerable delay from the preconditioning stimulus until onset of ischemic tolerance is consistent with a role for transcriptional changes in adaptation (Kirino 2001; Dirnagl et al. 2003, 2009; Gidday 2006; Obrenovitch 2008; Otani 2008). Tanaka and co-workers (2004) have shown that IPC acts downstream of caspase-3 activation and upstream of its target caspase-activated DNase to prevent the onset of apoptotic cell death (Tanaka et al. 2004). The IPC-induced inhibition of caspase-activated DNase was consistent with observations that IPC induces over-expression of heat shock protein 70 kDa, in which protective effect from cerebral ischemia via inhibition of caspase death cascade and mitochondrial apoptosis is well documented. Recently, it has also been shown that hsp70 inhibits apoptosis upstream of mitochondria by preventing bax translocation (Gidday 2006; Obrenovitch 2008; Otani 2008). The molecular mechanisms driving translocation of p53 to mitochondria after brain ischemia are not yet known. Thus, we can only speculate about the possible mechanism involved in inhibition of mitochondrial p53 translocation observed after IPC. Since several different mechanisms, like IPC-induced over expression of heat shock protein 70 kDa (Tanaka et al. 2004) or activation of Akt pathway (Gidday 2006; Obrenovitch 2008; Pignataro et al. 2009), might be considered, the exact mechanism of IPC-induced prevention of p53 translocation to mitochondria has to be clarified by further experiments.

Collectively, our studies showed that ischemia induced inhibition of mitochondrial complexes I and IV, however inhibition is not accompanied by 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. In line of this, ischemic preconditioning acts at the level of both initiation and execution of ischemia-induced mitochondrial apoptosis and protects 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).

## Stress reaction of neuronal endoplasmic reticulum after IRI and IPC

Ischemic tolerance can be developed by prior ischemic non-injurious stimulus - preconditioning. The molecular mechanisms underlying ischemic tolerance are not yet fully understood, therefore in a series of papers (Urban et al. 2009; Lehotsky et al. 2009; Pavlikova et al.2009) we focused our attention on both the mRNA and the protein levels of ER stress genes after ischemic/reperfusion damage (I/R) 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. As we have observed in our experiments,

the hippocampal mRNA for XBP1 has shown elevated level in the naive IRI group of animals in ischemic phase (about 43% ) and persisted non-significantly changed in all analyzed periods (Lehotsky et al. 2009; Urban et al. 2009). Preischemic treatment (IPC) induces on the level of hippocampal mRNA in ischemic phase only slight, not significant differences compared to controls, followed by significant decrease at 24 hours of reperfusion (by about 12.8 ± 1.4% compared to controls). When we analyzed 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 level was significant mainly in later ischemic times. The protein level reached maximum at 3 h of reperfusion (about 230% of controls) and persisted elevated in the later reperfusion (40.3 ± 4.9% compared to controls) (Lehotsky et al. 2009; Urban 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 induces expression of ER stress genes. On the level of mRNA for *GRP78* in hippocampus from naive IRI group of animals we have observed that maximal differences are seen in later reperfusion phases. Preischemic pretreatment (IPC) led to the elevated mRNA hippocampal levels in reperfusion period by about 11.7 ± 3.6 at the first hour and by about 8.7± 1.8% at 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 increase in ischemic time (by about 217% of controls) and remained elevated also at 3 and 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) (Lehotsky et al. 2009; Urban et al. 2009).

ATF6 works as a key transcription factor in the resolution of the mammalian UPR (Yoshida et al. 2001). As shown in our experiments, the mRNA level for *ATF6* in naive IRI animals showed gradual elevation reached to significant increase at 24 hours of reperfusion (9.2 ± 4 % higher than control) and preconditioning (IPC) did not change significantly mRNA levels in all analyzed periods. Similarly to mRNA levels, the hippocamapal ATF6 protein level in naive IRI animals followed the mRNA levels. Interestingly, IPC induced remarkable changes in the protein levels at ischemic phase reaching significantly (about 170%) increased levels in comparison to controls and remained 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 in the most vulnerable neurons, such as pyramidal cells of CA1 hippocampal region (de la Vega et al. 2001). 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 fact, IRI induces transient inhibition of translation, which prevents the expression of UPR proteins and hindered recovery from ischemia-induced ER dysfunction (Kumar et al. 2001; Paschen et al. 2003) which possibly leads to a pro-apoptotic phenotype (DeGracia and Montie 2004). Similarly to our data, 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. (2003) by 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 70-kDa 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 ichemia model. Our results are similar to the findings of Hayashi et al. (2003) and Garcia et al. (2004), who documented an increase in GRP78 expression after 2 days of preconditioning. Authors 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). Interestingly, 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 with an increased promotor activity of GADD153 to UPR genes (Oyadomari et al. 2004).

The data from these experiments (Lehotsky et al. 2009; Urban 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 $InsP_3$ 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; Pignatato et al. 2009).

**Secretory pathways $Ca^{2+}$ ATPase (SPCA1) gene expression is altered following ischemic precondtioning**

The secretory pathways (SP) in neural cells represents a dynamic $Ca^{2+}$ store required for optimal activity of enzymes and for secretion of neurotransmitters and secretory proteins (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). The SPCA $Ca^{2+}$-ATPase has a significant role for calcium storage within the brain (Wootton et al. 2004) and was shown to play a pivotal role in normal neural development, neural migration and morphogenesis (Sepulveda et al. 2007, 2008).

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 in our laboratory 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) in both ischemic and reperfusion periods. These results support data from our 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.

In addition, as shown by recent paper of Pavlikova et al. (2009), microsomes prepared from injured hippocampus after IRI also manifested considerable lipoperoxidation and protein oxidation as analyzed from the level of TBARS and fluorescence intensities of tryptophan (Trp) and bityrosine (biTyr). The experiments also indicated an IRI-induced depression of $Ca^{2+}$ ATPase activity which is attributable to SPCA-associated thapsigargin insensitive activity. Interestingly, preconditionig (IPC) partially protects hippocampal membranes from oxidative damage, as shown by recovery of both the Trp and biTyr fluorescence intensities and lower levels of TBARS. However, the oxidation of membrane proteins still persists (to 94 and 105%, respectively). It is important from functional aspects, that IPC had also partial protective effect on the SPCA-associated $Ca^{2+}$-ATPase activity. Thus, results from these experiments showed that IPC incompletely suppresses lipo- and protein oxidation in hippocampal membranes and led to partiall recovery of the ischemia-induced depression of SPCA activity.

As shown by earlier studies, 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, we analyzed the mRNA and protein levels of SPCA1. RT-PCR clearly detected, that hippocampal cells responded 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.

Western blot analysis demonstrated that immunosignal for SPCA1 showed similar profile to that of the mRNA and it increased in the later reperfusion period. Although IPC did not induce any statistically significant changes at 3 and 24 h of corresponding ischemic levels, in the line of results of mRNA expression, the IPC induced a 149% rise in the level of SPCA1 protein corresponding to a 1 h reperfusion level. Thus, these results showed that the SPCA expression and the post-translational changes induced by ischemia are modulated by the IPC. These data might also serve to understand the molecular mechanisms involved in the structural integrity and function of the secretory pathways after ischemic challenge. Data also suggests that there is a correlation between SPCA function and the role of SP in the response to pre-ischemic challenge.

Neuronal microsomes are vulnerable to physical and functional oxidative damage (Lehotsky et al.1999, 2002a, 2004; Urikova et al. 2006). We have shown here that SPCA activity, similarly to other P-type ATPases, is also subject of ischemic damage 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; Lehotský 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. Ischemic preconditioning induced reductions of lipoperoxidation products and protein oxidative changes (Racay et al. 2009b; Pavlíková et al. 2009), probably due to upregulation of defence mechanisms (antioxidant enzymes) against oxidative stress in the preconditionig challenge (Danielisova et al. 2005; Gidday 2006; Obrenovitch 2008).

In addition, IRI causes significant drops in the SPCA associated $Ca^{2+}$-ATPase activity and ischemic preconditioning had a partial protective effect on this activity. As shown in earlier studies, preconditioning upregulates defence mechanisms against oxidative stress (Danielisova et al. 2005; Pignataro et al. 2009) which might partialy restore the depression of enzyme activity. Additionally, as shown in this study by Western blot analysis, ICP induced an elevation of SPCA protein level in comparison to corresponding naive ischemic control.

Golgi apparatus is strategically located which predict its common $Ca^{2+}$ signaling communication and contribution to the spatial and temporal complexity of $Ca^{2+}$ signals (Michelangeli et al. 2005). However, the Golgi $Ca^{2+}$ stores also reflect a number of different functions. The cis-Golgi appears to express SERCA and $InsP_3$ receptors, while the trans-Golgi contains SPCA1 and lacks $InsP_3$ receptors. In fact, we (Murin et al. 2006) and others (Sepulveda et al. 2008), have demonstrated the presence of SPCA1 protein in hippocampal neurons either in neuronal cell cultures or in rat hippocampus. Interestingly, particularly these cells are highly vulnerable to ischemic challenge.

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). Our results showed a partial recovery of $Ca^{2+}$-ATPase activity and earlier hippocampal response to later ischemia by the induction of mRNA and protein expression. In fact, the 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), or 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 conclusion, results of these series of experiments indicate for 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 the correlation of SPCA function with the role of secretory pathways in response to preischemic challenge.

## Conclusions

Collectively, our studies showed that ischemia induced inhibition of mitochondrial complexes I and IV without depression of mitochondrial $Ca^{2+}$ uptake rate, apparently due to the excess capacity of the complex I and complex IV. Depressed activities of complex I and IV are conditions favourable for initiation of cell degenerative pathways, e.g., opening of mitochondrial permeability transition pore, ROS generation and apoptosis initiation. Ischemic preconditioning acts at the level of both initiation and execution of ischemia-induced mitochondrial apoptosis and protects from ischemia associated changes in the integrity of mitochondrial membranes. IPC also activates inhibition of p53 translocation to mitochondria and thus IPC affects downstream processes connnecting mitochondrial dysfunction. Inhibition of the mitochondrial p53 pathway might provide a potentially important mechanism of neuronal survival in the face of ischemic brain damage (Fig. 1 and Fig 2).

Our data also suggests, 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. Preconditioning paradigm (preischemia) exerts a role in the attenuation of ER stress response in the neuroprotective phenomenon of acquired ischemic tolerance.

Results of experiments also indicate a 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 a correlation of SPCA function with the role of secretory pathways in response to preischemic challenge.

Ischemic induced alterations of mitochondria, endoplasmic reticulum and Golgi apparatus (Fig.1 and Fig. 2) shed light on the understanding of cross-talk between intracellular $Ca^{2+}$ stores in cerebral ischemia/reperfusion and in the phenomenon of ischemic tolerance. Documented neuroprotective response of intracellular organelles in the phenomenon of ischemic tolerance might suggest possible targets for future therapeutic interventions to enhance recovery after stroke (Dirnagl et al. 2009; Pignataro et al. 2009).

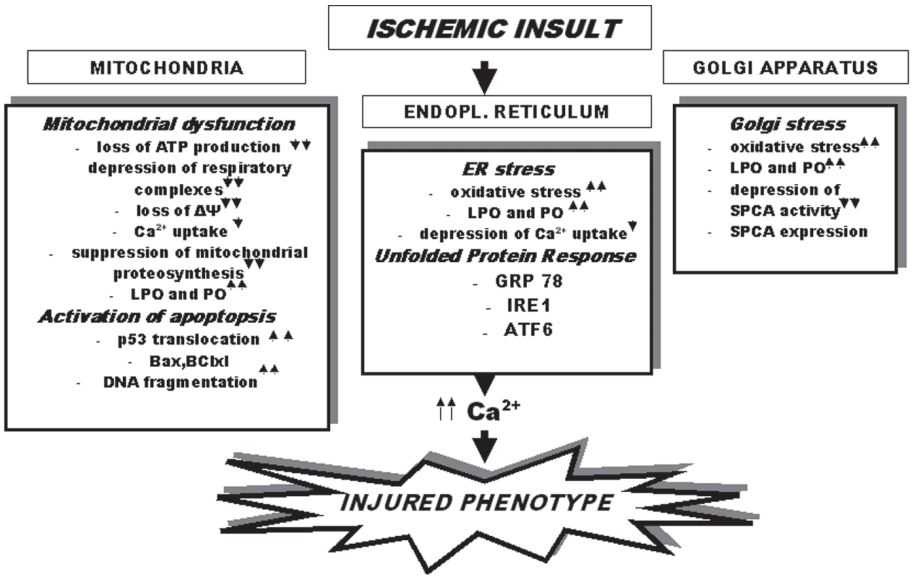


**Figure 1.** Schematic drawing of the documented mechanism for intracellular organelles dysregulation leading to ischemic injurious phenotype (Lehotsky et al. 2009; Pavlikova et al. 2009; Racay et al. 2007, 2009a,b,c; Urban et al. 2009). ↑↑ = significant alterations, ΔΨ = membrane potential, LPO = lipoperoxidation, PO = protein oxidation.

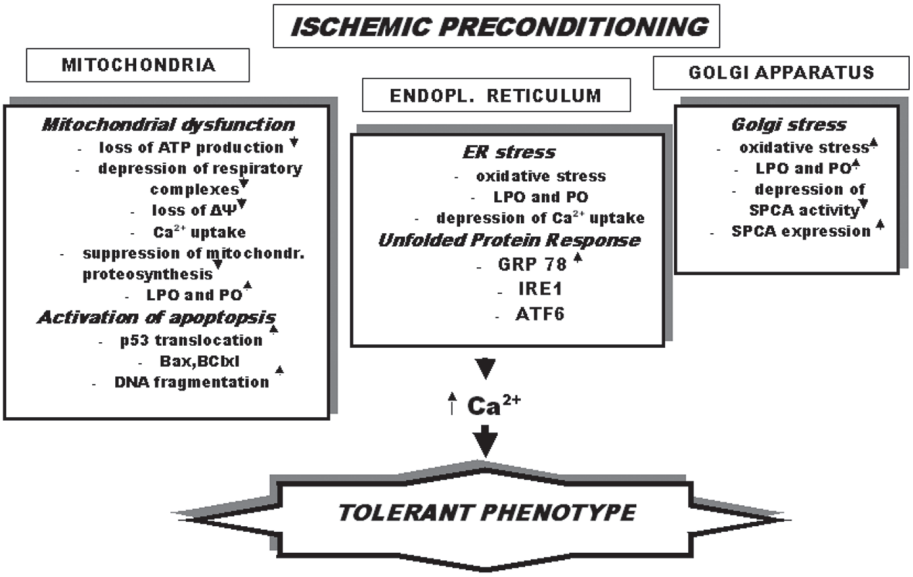


**Figure 2.** Schematic drawing of the mechanism for documented intracellular events activating after preconditioning which eventualy lead to ischemic tolerant phenotype (Lehotsky et al. 2009; Pavlikova et al. 2009; Racay et al. 2007, 2009a,b,c; Urban et al. 2009). ↑ = medium alterations, ΔΨ = membrane potential, LPO = lipoperoxidation, PO = protein oxidation.

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