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Forebrain Ischemic Stroke and the Phenomenon of Ischemic Tolerance: Is Homocysteine Foe or Friend?

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Additional information is available at the end of the chapter

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Abstract

Hyperhomocysteinemia (hHCy) is a recognized comorbid risk factor of human brain stroke. We overview here the recent data on the homocysteine (Hcy) metabolism and on the genetic and metabolic causes of hHCy-related neuropathologies. In context of our results which detected an increased oxidative stress in hyperhomocysteinemic rats, we discuss here the role of free radicals in this disorder. Brain ischemia-reperfusion causes delayed neuronal death. Ischemic tolerance evoked by preconditioning (IPC) represents a phenomenon of central nervous system (CNS) adaptation to any subsequent ischemia. The paper describes changes in the mitogen-activated protein kinases (MAPKs) protein pathways, and apoptotic markers were used to follow the degeneration process. Our studies provide evidence for the interplay and tight integration between extracellular signal-regulated kinase (ERK) and p38 MAPKs signaling mechanisms in response to the hHCy and also in association with brain ischemia/IPC challenge. Recognition of the effects of risk factors in the ischemic tolerance would lead to improved therapeutics, especially the brain tissue.

Keywords: hyperhomocysteinemia, ischemic tolerance, oxidative stress, brain, preconditioning

1. Introduction

Comorbidities are widely recognized as possible risk factors for cardiovascular and cerebrovascular diseases [1–4]. Clinically described comorbidities such as earlier incidence of any type of stroke, prior transient ischemic attack (TIA), arterial disease, atrial fibrillation, unpro-

diet and/or obesity, and physical inactivity are known to elevate risk for ischemic stroke [3, 4]. As it has been proved by several studies, even mild hyperhomocysteinemia (hHcy) increases the incidence of ischemic brain damage, probably due to pleiotropic effect of homocysteine (Hcy) and the impact of venous and arterial atherosclerotic changes [2, 5–7]. In fact, Hcy inhibits NO synthesis by endothelial cells and platelets and stimulates the production of reactive oxygen species (ROS) by the release of arachidonic acid from platelets. In parallel, it also inhibits glutathione peroxidase and therefore stimulates the proliferation of endothelial cells (see [6] for review). In addition, Hcy has been shown to inhibit methyltransferases, to suppress DNA repair, and to facilitate apoptosis when accumulated inside the cells. Auto-oxidation of Hcy metabolites results in H_2O_2 accumulation [8], and long-term incubation of neurons with Hcy metabolites induces necrotic cell death [9]. Consequently, homocysteine level has been shown to be comorbidly elevated in neurodegenerative and acute disorders of the central nervous system (CNS), for example, Alzheimer's disease or Parkinson's disease [10]. Thus, the incorporation of animal models more consistent with the clinical population afflicted by stroke is urgently needed for proper exploration of the disease's etiology such as stroke and other cerebrovascular diseases. In fact, only limited number of literature data can be found to describe the mutual influence of comorbid hyperhomocysteinemia to ischemic damage on animal models of ischemic stroke.

2. Phenomenon of ischemic tolerance evolution

The brain is highly susceptible to hypoxia or ischemia, and numerous endogenous mechanisms exist to protect neural tissue from its effects and to produce a protective state known as ischemic tolerance [3, 4, 11]. It represents an evolutionally conserved endogenous neuroprotection/plasticity, which can be induced by various paradigms/stressors. Preconditioning is one of the recognized neuroprotective strategies, which is induced prior to stroke as a preventative measure in a high-risk individuals. It also can be used as a precaution against secondary stroke following medical procedures such as aneurysm repair or cardiac surgery [1, 3, 4, 12]. In the clinical settings of human stroke, which is hardly predicted, a novel algorithm of postconditioning may have a very high therapeutic value. Remarkably, it could be used afterward of ictus to stimulate protective and regenerative pathways or as a precaution against stroke recurrence [1, 12, 13]. Clinical studies are needed to test the safety and efficacy of these novel strategies in humans [3, 4]. Although the cascade of molecular processes determining ischemic preconditioning is not fully understood, it has been shown to influence receptor activities, mitogen-activating protein (MAP) and other kinases and apoptotic mechanisms. Additionally, ischemic tolerance in the brain can also be stimulated remotely, for example, by the application of a tourniquet to one of the limbs also in human patients with subarachnoid hemorrhage. More detailed studies are still needed to clinically validate this phenomenon [1, 3, 12, 14–16].

In spite of the high clinical relevance, only limited number of experiments can be found in the literature to describe the mutual influence of comorbid hyperhomocysteinemia to ischemic damage on animal models of human stroke. This paper summarizes current knowledge of the homocysteine metabolism and the genetic and the metabolic causes of hyperhomocysteine-

mia-related neurotoxicity. Based on results from our laboratory, in this context, we also found that the combination of experimental hyperhomocysteinemia (hHCy) with ischemic insult and/or with the pre-ischemic challenge affects the extent of neuronal degeneration as well as the MAP kinase pathways involved in the preconditioning phenomenon.

3. Toxicity of homocysteine as recognized from its metabolism

Homocysteine (Hcy) is an intermediate sulfhydryl-containing amino acid derived from methionine with recognized toxicity to neural cells and vascular endothelial cells. It originates from a dietary protein through S-adenosyl methionine conversion [17]. Human patients with severe hyperhomocysteinemia (hHCy) are characterized by a perplex of typical clinical cardiovascular manifestations, which include neurological abnormalities, such as cerebral atrophy, dementia, and seizures [18]. A large number of epidemiological investigations have revealed the association of folate deficiency and hyperhomocysteinemia with an increased risk of vascular diseases and brain ischemic stroke [6, 19].

Homocysteine metabolism includes three independent alternative pathways:

- i. re-methylation,
- ii. transmethylation to methionine, or
- iii. transsulfuration to cysteine.

In addition to the already recognized causative role of mutations or polymorphisms in the key genes encoding enzymes of Hcy metabolism to cardiovascular disorders and also stroke, a novel observation already proved that epigenetic mechanisms, such as DNA methylation, chromatin remodeling, RNA editing, noncoding RNAs (ncRNAs), and microRNAs (miRNAs), might be equally relevant in the stroke etiopathogenesis [3, 20]. Earlier genetic studies suggest that polymorphisms of the genes, which encode the metabolic pathways, such as methylenetetrahydrofolate reductase (MTHFR), cystathionine β -synthase (CBS), DNA methyltransferase (DNMT), and nicotinamide N-methyltransferase (NNMT), might play an important role in stroke development during elevated level of Hcy. Additionally, nutritional supplements, for example, folic acid (a cofactor in one-carbon metabolism), can regulate the epigenetic alterations of neuronal cells and may play an important role in the maintenance of neuronal integrity [18, 20].

General Hcy metabolism in liver represents mainly methyl group transfer and re-methylation and requires vitamin B₁₂ and folic acid for N-5-methyltetrahydrofolate-homocysteine methyltransferase. Additionally, the transsulfuration reaction of Hcy depends on the presence of vitamin B₆. Remarkably in the CNS, the Hcy metabolism differs from other organs. The transsulfuration pathway is practically absent and the re-methylation pathway using betaine is not present [6, 21]. Thus, the effectivity to metabolize or convert homocysteine mostly relies on externally delivered folate and cobalamine in the form of vitamins. The glial cells contain very low deposits of vitamin B₁₂, which is exhausted rather quickly during its negative balance.

The harmful effect of the homocysteine to CNS neurons is quite well known. It has an influence to both the neuronal survival rate and the functionality of neurons to the signal transmission. As a consequence, it also affects the formation of functional neural networks with the effect surpassing simple neuronal survival.

In our laboratory, we have analyzed the neurotoxic properties of Hcy on glial cells, using a glioblastoma cell line as a study model. The viability of cells was assayed both biochemically and cytologically. As proved, Hcy concentration around 50 $\mu\text{mol/l}$ induced cell death. It is worth noting that Hcy induces cell death of human glial cells at concentrations which corresponds to the mild clinically occurred hyperhomocysteinemia. We propose that Hcy-induced impairment of neuronal functions along with the damage of glial cells may contribute to the etiopathogenesis of neurological disorders associated with hyperhomocysteinemia [22]. As shown in clinical studies, an elevated Hcy level associates with CNS disorders, such as stroke, Alzheimer's disease, dementia, as well as with classical homocystinuria [2, 6].

In humans, plasma Hcy concentration varies from 5 to 10 $\mu\text{mol/l}$ and its elevated level is classified as hyperhomocysteinemia (hHcy) with mild, moderate, intermediate, and severe (for concentrations higher than 100 $\mu\text{mol/l}$), manifested also with homocystinuria [19]. As generally believed, homocysteine is produced merely in all tissues. On the other hand, its metabolic inactivation proceeds only in the liver/kidney, mainly through the transsulfuration pathway. As the outcome, in tissues such as the blood vessels and the brain, where re-methylation enzymes are merely absent, the reduction of MTHFR activity leads to homocysteine accumulation. As analyzed in various genetic cohorts, the MTHFR C677T polymorphism has been established as a risk factor for ischemic stroke in different laboratories [23].

Selective neurotoxic effect of Hcy includes various pathomechanisms such as glutamate receptor-mediated neurotoxicity [2, 24]. Notably, glutamatergic excitotoxicity is also associated with the brain damage caused by ischemic insult. Hcy was shown as an inductor of caspase-dependent neuronal apoptosis, by a mechanism involving several detrimental steps, such as the DNA damage, poly-ADP-ribose polymerase (PARP) dysregulation, and mitochondrial dysfunction by caspase-3 activation. Hcy appears to also be critically involved in the glial-vascular interface communications as part of the blood-brain barrier. The important role of astrocytes in the regulation of overall brain metabolism and in particular in the brain energy metabolism has already been proved [4, 22]. As generally believed, the elevation of the Hcy activates an excitatory glutamatergic neurotransmission in different brain areas, which stimulates neuronal damage derived from an excessive Ca^{2+} influx and reactive oxygen species generation.

Principally, dysbalance in redox state and oxidative stress has been proposed as one of the primary etiologies for hHcy-related pathogenesis [6, 19]. Previous observations proved that dysequilibrium in redox balance may be a key factor in the pathogenesis of vascular hypertrophy, thrombosis, and atherosclerosis in hyperhomocysteinemic animals [25]. In fact, reactive oxygen species (ROS) are side products of the Hcy-free thiol group oxidation, mainly when Hcy binds via a disulfide bridge with plasma proteins—mainly albumin—or with other low-molecular plasma thiols, or secondarily with other Hcy molecule.

Already proposed reactions responsible for Hcy-induced oxidative stress include:

- i. inhibition of the activity of cellular antioxidant enzymes,
- ii. Hcy auto-oxidation,
- iii. nitric oxide synthase (NOS)-dependent generation of superoxide anion via uncoupling of endothelial NOS (eNOS),
- iv. disruption of extracellular superoxide dismutase from endothelial surfaces, and
- v. activation of NADPH oxidases.
- vi. Interestingly, mutual tissue production of strong oxidant peroxynitrite stimulates tyrosine nitration, which leads to protein functional alterations and cellular dysfunction [26]. Auto-oxidation of Hcy metabolites results in H_2O_2 accumulation and as shown by [9] and later by [8] prolonged incubation of neurons with Hcy metabolites leads to necrotic cell death.

Homocysteine is also converted to the thioester forming the Hcy-thiolactone in an error-editing reaction in translation mechanism. Accidentally in proteosynthetic process, Hcy is erroneously selected in place of methionine by methionyl-tRNA-synthetase. As shown by animal and human experiments, the Hcy-thiolactone remarkably contributes to Hcy pathobiology. Its toxicity is based on reaction which leads to protein N-homocysteinylation through the formation of amine bonds with protein lysine residues [27], which impairs or alters the structural and functional properties of particular protein. Several clinical studies have reported that increased plasma homocysteine levels may provoke neurological seizures. Systemic administration of homocysteine at high doses is able to induce convulsions in mice and it can be suggested that similar detrimental effects might occur in patients affected by temporal lobe epilepsy [28].

A number of papers from our and also other laboratories [13, 29–31] documented that global ischemia/reperfusion injury (IRI) in rats follows with the time-dependent dysbalance of redox balance in cortex and hippocampus. The insult also activates different gene expression at both the mRNA and protein levels. In addition, experiments of [32] proved that the redox status and gene expression are influenced by preconditioned pre-ischemic treatment. In spite of high clinical relevance, the data from literature which deals with the mutual effect of IRI and endogenously produced homocysteine as verified risk factor to ischemic injury are very limited [2]. Observations of [6] which described the effect of chronic dietary supplementation of Hcy for 2 weeks (to initiate hyperhomocysteinemia-hHCy) documented remarkable elevation of lipoperoxidative and protein oxidative products in rat cortex and hippocampus. Experimental hyperhomocysteinemia was induced by the subcutaneous administration of homocysteine in saline solution ($0.45 \mu\text{mol/g}$ body weight) twice a day at 8-h interval for 14 days [6, 30]. It has been proved that Hcy crosses the blood/brain barrier and exhibits a maximum level in the cerebrum and parietal cortex between 15 and 60 min after subcutaneous injection. Remarkably, plasma Hcy concentration in rats treated this way achieved levels similar to those found in homocystinuric patients (moderate hyperhomocysteinemia). Groups of rats were subdivided as described earlier [6, 30]:

1. sham-operated control (naive) animals,
2. sham-operated control (preconditioning) animals,
3. the animals that underwent 15-min ischemia (naive),
4. the animals with induced 5-min IPC following 15-min ischemia,
5. sham-operated hyperhomocysteinemic control animals,
6. the hyperhomocysteinemic animals that underwent 15-min ischemia,
7. the hyperhomocysteinemic animals with induced preconditioning animals following 15-min ischemia.

Experiments evidently document that hyperhomocysteinemia induces remarkable elevation of lipoperoxidative and protein-oxidative products, the results which are in a good correlation with previously published experiments [24]. Moreover, as documented by the number of Fluoro-Jade B-positive- and TUNEL-positive cells (as indicator of neuronal degeneration) [30, 33–37], the number and proportion of degenerated neurons over intact cells evidently exceeds in the hippocampus of hHCy animals and reached the level which almost competes with the levels documented after ischemic insult in nontreated, naive group. As was shown in earlier studies, an auto-oxidation of Hcy metabolites results in higher H_2O_2 production, and prolonged incubation of neurons with Hcy metabolites elevates the number of necrotic cell death [8, 9]. Interestingly, as was documented by [38], single intracerebroventricular injection of homocysteine was followed by the elevation of typical apoptotic features of cells of *substantia nigra*, which results to the typical Parkinson's disease-like behavior in rats. As was detected earlier, an increased Hcy level is potent to induce and accumulate hydroxyl radicals as the most potent, powerful-free oxygen species with the high efficiency to remove electrons from other cellular biomolecules such as lipids, proteins, carbohydrates, and DNA [39]. Remarkably, results of experiments from our laboratory and also previous results have documented that in hyperhomocysteinemic model in rats [6, 30, 36, 37, 40], the elevation of homocysteine was followed by significant neurotoxic effect. As was proposed earlier, the effect is probably caused by the hHCy-induced oxidative dysbalance and cellular stress. Interestingly, as was detected in a human study on Alzheimer's patients and patients with the mild cognitive impairment, an increased level of plasma homocysteine positively correlated with alterations of hippocampal volume. This effect is not mediated by cerebral beta-amyloid deposition and vascular burden but likely due to the oxidative dysregulation induced by homocysteine as part of direct homocysteine adverse effect [2, 41].

Results from another study from this laboratory [42] have shown that hyperhomocysteinemic differently express mRNA and protein of calcium pump in secretory pathways (SPCA1). SPCA has a remarkable role in the development of neural cells and their migration [43] and its loss can finalize to the stress of Golgi apparatus expressed by alterations of membrane structure and redox dysregulation in neuronal cells. The effect of Hcy on the expression profile of the Ca^{2+} -transport proteins in neuronal cells is not yet fully described. The transcription factors Sp1 and YY1 were identified in the gene expression regulation by the cis-enhancing elements in 5'-untranslated regions. Hyperhomocysteinemia is followed in intracellular Ca^{2+} elevation,

release of calcium and endoplasmic reticulum (ER) stress [6, 44], leading to apoptotic events, endothelial dysfunction, and remodeling of the extracellular matrix also in the brain parenchyma. Moreover, homocysteine itself by metabolic interfering with the level of S-adenosylmethionine (donor of methyl group) has also been reported to induce the modulation of gene expression through the alteration of the gene methylation status [10].

Remarkably, the processes, such as modifications of protein structure, have been proved in the etiology eventually leading to homocysteine-induced neurotoxicity. Homocysteinylation can be subdivided into the two main types:

- i. S-homocysteinylation and
- ii. N-homocysteinylation,

both of which are typical examples of posttranslational protein modifications. The extent of the homocysteinylation correlates with increased plasma Hcy level [39], and the chemical conversion of Hcy to Hcy-thiolactone followed by protein N-homocysteinylation is an etiological factor, which contributes to the expressed Hcy neuronal and tissue toxicity. As shown by [45], homocysteinylation follows functional protein modification and enhances protein degradation processes which can finalize to cell damage.

In this context, Petras et al. [6] in a recent study observed remarkable decrease of Mn^{2+} -activated superoxide dismutase (Mn-SOD) activity in cortical mitochondria in the 14-day hHCy model in rats. This catalytic activity is included in the first line of cellular defense against oxidative injury, and has been shown to be suppressed in the hHCy group compared to the control group. These results might be explained by putative increased posttranslational modifications of Mn-SOD due to the higher level of Hcy leading to the enzyme homocysteinylation and thiolation. As was shown recently by Li et al. [46], the imbalance among antioxidant enzymes caused by an increased Hcy level might alter ROS elimination, and thus lead to the increasing amount of free radicals and likely to homocysteine-induced stress of endoplasmic reticulum, which can result in the dysfunctional consequences in rat hippocampus.

4. Impact of hyperhomocysteinemia on ischemic/reperfusion injury and ischemic tolerance induced by ischemic preconditioning

Now, the clinical comorbid effect of hHCy to the stroke incidence and severity is clearly recognized. However, a bit surprisingly, the experimental results, which are focused on the mutual influence of the comorbid hyperhomocysteinemia to ischemic damage on the animal models of human stroke, are only limited [12, 32, 47]. Ischemic insult/reperfusion insult in the animal models is followed by the degeneration of majority (more than 64%) of hippocampal neurons [3, 33]. Experiments from this laboratory [33, 34, 42], which combine 14 days of hyperhomocysteinemia with 15-min forebrain ischemia/reperfusion, show that insult initiates manifestations of morphologically changed neurons and disturbances of glial cells of the hippocampal area. On the other hand, the combination of both approaches elevated the percentage of morphologically intact and probably nondegenerated cells in comparison to the

naive ischemic/reperfusion insult. In this context, Sato et al. [47] in experiments on hippocampal CA1 neurons after ischemic insult in rats have proved concentration-dependent cell protection by S-adenosyl-L-methionine (SAME). The effect was concealed by the concomitant administration of S-adenosyl-L-homocysteine, a potent inhibitor in transmethylation. From these results, the authors considered that the enhancement of intraparenchymal SAME level followed by the activation of transmethylation using SAME as a methyl donor in postischemic brain is inevitable for the tissue protection and prevention of delayed neuronal death.

The pre-ischemic treatment has been recognized to save the majority of hippocampal neurons which exceeds more than 75% of all neurons [1, 33]. If we combined 14 days of hyperhomocysteinemia with preconditioning [6, 33, 34, 36, 37, 40, 42], this particular treatment has finalized by the larger suppression of cell degeneration not crossing 5% of the total number of neurons. It is remarkable that in this particular hyperhomocysteinic model in rats, the protective effect of IPC maneuver is influenced by not-yet fully clarified mechanism. One appealing candidate that has been described is a novel regulatory epigenetic mechanism, which encompasses both the gene environment interactions and also tissue functionality. The recent evidence proves that several epigenetic mechanisms are also involved in the stroke pathogenesis and the tolerance etiology. Participation of several important enzymes regulating DNA methylation including DNA-N-methyltransferase has been proved, and the high level of Hcy may eventually finalize to the increase in the level of S-adenosyl methionine. As a result, its higher enzymatic activity might stimulate hypermethylation of the genomic DNAs and silencing of functional genes [44].

In the recent study [35], the authors have analyzed whether hyperhomocysteinemia (risk factor of brain ischemia) alone or in combination with the ischemic preconditioning (IPC) affects the ischemia-induced neurodegenerative changes and imbalance in the intracellular-signaling pathway including the MAPK/p-ERK1/2 and MAPK/p-p38 gene and protein expression in the rat brains. We have used the model of hyperhomocysteinemia induced by the subcutaneous administration of homocysteine as well as the preconditioning maneuvers followed by global ischemia as was described above. Here, we suggest that hHCy alone (as an example of metabolic stress) and/or in combination with IR injury affects mechanisms of the ischemic tolerance induced by IPC maneuver. The study reveals that hHCy alone significantly increased neurodegeneration by Fluoro-Jade C and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) positive cells in hippocampus as well as in the cortical brain area. Prominent features of those changes were detected by using markers of cell damage/degeneration and changes in MAPKs expression in the CA1 hippocampal region and M1 cortical sector. We have also found in these experiments an elevated level of MAPK/p-ERK and the decreased level of MAPK/p-p38 after pre-ischemic challenge by Western blot and fluorescent immunohistochemistry.

Experiments from this laboratory previously showed that IR insult leads to the neurodegeneration of neurons in the CA1 region of hippocampus as detected by Fluoro-Jade C and TUNEL analysis. As expected, IPC leads to the suppression of the number of positive cells and conferred neuroprotection [33, 34]. Interestingly, the effect of homocysteine on cellular degeneration and following morphological changes was observed in the rat hippocampal and

cortical regions. The increased number of Fluoro-Jade C+ and TUNEL+ neuronal and glial elements supports this effect of hHCy. The thickened and collapsed processes that poorly extend to the area of pyramidal neurons in CA1 region and M1 cortex are presumably due to the morphological alterations of astrocytes and cytoskeletal remodeling. This might suggest for the ensuing development of hHCy-associated neuronal cell damage [23, 48, 49]. Astrocytes are highly plastic cells and their dynamic morphological changes could affect the intercellular communication with surrounding synapses that are important in the development of brain lesions [40]. Maler et al. [50] reported that Hcy doses of 2 mmol/l and above induced a dose-dependent cytotoxic effect on cortical astrocytes. Astrocytes regulate the expression of the N-methyl-D-aspartate (NMDA) receptor subtypes, which increase neuronal sensitivity to glutamate toxicity and thus accelerate the initial step in a program of reactive astrogliosis and dynamics of the astrocyte response to damage [51]. On the other hand, in response to the injury, astrocytes synthesize a number of factors that may play either neuroprotective or neurotoxic roles.

Recently, we have shown that IPC prior to lethal ischemia affects MAPK/ERK and MAPK/p38 pathways in the cerebral cortex [33] as well as in hippocampus [34]. There is only sparse literature data focusing on the effect of Hcy on the MAPKs protein expression in neuronal cells [48, 52]. Our results suggest that the combination of both stressors (ischemia + Hcy) affects considerably the MAPKs pathways expression. hHCy-IR induces the MAPK/p38 expression as detected by Western blot and immunoanalysis. Poddar and Paul [52] showed a biphasic response of MAPK/p38 activation in the Hcy-NMDA-induced neuronal damage *in vitro*, characterized by the initial rapid elevation followed by a delayed and more prolonged secondary increase, where the later peak was primarily involved in mediating the Hcy-induced cell death. They also showed that this secondary activation of MAPK/p38 correlates with the upstream MAPK/ERK activation, which plays a role in facilitating of the Hcy with the conclusions of this study. Interestingly, we found that the MAPK/p38 pathway was activated also in hHCy control group. We previously reported that IR injury induces only slight increase of MAPK/p38 expression [33, 34]. However, the combination of both stressors (ischemia + hHCy) leads to the massive activation of MAPK/p-p38 with maximum at 24 h after reperfusion. This dynamic MAPK/p38 activation could contribute to more extensive progression of tissue injury [52, 53]. Remarkably, the effect of the IPC maneuver on the decreasing of MAPK/p-p38 expression in ongoing reperfusion times indicates for proposed neuroprotective mechanism. The MAPK/p-p38 pathway was suppressed also by the combination of two stressors.

MAPK/ERKs are versatile protein kinases that are ubiquitously expressed in the CNS. We have already shown that in the hippocampus and cerebral cortex activated MAPK/ERK parallels neuroprotection induced by IPC [33, 34]. The robust expression changes in hippocampus and modest posttranslational changes in MAPK/ERK pathway in less sensitive vulnerable neurons of the cortical layers III and V corresponds with results of similar experimental models [53, 54]. Extensive studies have shown an interplay and tight integration of MAPK/ERK signaling in promoting neuronal cell death both in development and in neurodegenerative disorders [49, 52]. It has been proposed that transient activation of MAPK/ERK has different consequences as compared with sustained activation [49, 52]. Transient activation of MAPK/ERK plays

a pivotal role in neuronal maturation, survival, and long-term potentiation. On the other hand, the sustained activation of MAPK/ERK may play a critical role in triggering proapoptotic signals and neuronal cell death. As documented in our study [35], the immunoreactivity of MAPK/p-ERK after IPC with induced hHCy was found in the early stages of reperfusion, with maximum level at 24 h, and its activation is probably associated with neuronal protection induced by IPC [54]. The slight activation of MAPK/ERK was detected also in the hHCy-control group. It is well known that hHCy mediates glutamate-mediated NMDA receptor stimulation, which eventually leads to the activation of both stimulatory and inhibitory pathways involved in the modulation of MAPK/ERK signaling [49]. In fact, the dual role of MAPK/ERK kinases in cell survival and death suggests that a unique profile of gene expression may be elicited depending on the duration and/or magnitude of MAPK/ERK kinase activation [52]. Thus, the duration of MAPK/ERK kinase activation following MAPK/p38 stimulation depends on the nature of the extracellular stimuli and may have different consequences on intracellular signaling pathways eventually leading to different cellular responses.

To summarize, results from our and other experiments show that hHCy is associated with a selective degeneration of cortical and limbic brain structure including hippocampal area. The degeneration involves the loss of neurons, glial growth, hypertrophy of astrocytes, and probably sprouting of new connections. The morphological findings indicate that the astrocytes are the first neural cells participating in the deleterious actions of Hcy on the CNS. Apparently, astrocytes are able to respond to mild hHCy by reorganizing their cytoskeleton, surviving and protecting neurons from the damage [55].

Moreover, our studies also suggest that there are at least two different ways in which the neuronal tissue responds to hHCy. The induction of hHCy alone leads to neuronal cell death and morphological changes in the hippocampus and cortex that corresponds with findings of previous reported studies [48, 55, 56]. Combination of hHCy with more intense stimuli (ischemia) causes more prominent changes in cortex than in hippocampus. Importantly, IPC maneuver, even if combined with hHCy, still preserves the neuronal tissue from lethal ischemic effect. The other important finding that arose from our studies is that MAPK/p38 promotes neuronal cell death, whereas MAPK/ERK activation opposes apoptosis. Finally, all the above-mentioned studies provide evidence for the interplay and tight integration between ERK and p38 MAPK-signaling mechanism in response to mild hHCy and also in association with IR insult/IPC challenge in rat brain. Conclusively, preconditioning even if combined with hHCy could still preserve the neuronal tissue from lethal ischemic effect.

The one carbon unit metabolic pathway, which is involved in the regulation of homocysteine metabolism, is also part of the process which methylates amino acids in functional proteins and histones as well as the bases within the RNA and DNA. As a result, demethylation of S-adenosyl methionine, which is converted to S-adenosyl-homocysteine, exclusively provides methyl groups for the cells. Derangement of this phase might cause wide implications on plenty of cellular processes including the modulation of the expression of functional genes as well as the epigenetic regulation [10, 44, 21].

Elimination of Hcy-thiolactone, a metabolite of Hcy, is performed by the high-density lipoprotein (HDL)-associated enzyme, Hcy-thiolactonase/paraoxonase 1 (PON1). This enzyme

hydrolyzes Hcy-thiolactone in human serum [57] and similarly in the brain, it has been proposed that PON1 protects mice against Hcy-thiolactone neurotoxicity by enzymatic hydrolysis [58].

In summary, the experiments proved that pre-ischemic challenge in 14 days of hyperhomocysteinemic model in rats initiates responses of neuronal cells, which probably coordinate several etiological/protective mechanisms such as antioxidant defense [58], Ca^{2+} transport, and epigenetic mechanisms, such as DNA methylation and chromatin remodeling in the manifestation of the phenomenon of ischemic damage and ischemic tolerance [1, 12, 20, 32, 33].

5. Conclusion, challenges, and future directions

Hyperhomocysteinemia manifested as an elevated plasma level of homocysteine is now a widely recognized risk factor of human ischemic stroke. Elevated plasma homocysteine level leads to an increase in cerebrovascular permeability and causes several biochemical alterations such as thiolation and homocysteinylation to plasma proteins and enzymes and remarkably also in the brain parenchyma. These hHCy induced posttranslational protein modifications can have a causative role to the altered function and activity of the functional enzymes involved in the free radical protection also in brain parenchyma. Homocysteine metabolism itself leads also to the redox imbalance and to increased oxidative stress and the production of free radicals with the consequence in the damaging neuronal lipoperoxidation and cellular protein oxidation. The paper also highlights protective effect of pre-ischemic challenge to the subsequent lethal ischemia in rats. The pre-ischemic maneuver itself and also combined with hyperhomocysteinemia diminished the extent of neuronal degeneration as well as the intracellular signaling. Recent studies also underscore an opposing effect of MAPK/ERK and MAPK/p38 on cell survival and cell death in hyperhomocysteinemic conditions in brain parenchyma also if combined with IPC challenge in rat model of global ischemic stroke. Interestingly, a novel protective paradigm, such as postconditioning and/or remote conditioning, has already been observed as an effective in reducing ischemic reperfusion injury. As was proved in the experimental studies and also in clinical trials, those paradigms affect all the cells of the neurovascular network (consisting of neurons, glial cells, vascular endothelial cells, pericytes, smooth muscle cells, and venule/veins) [59, 60]. In the context of clinical applications, it is strongly suggested that the identification of the effects of comorbid factors in the mechanisms of ischemic damage and if combined with the ischemic tolerance evolution can potentially lead to improved therapeutics, especially the brain tissue. Additional studies of these molecular pathways are strongly needed to validate the role of hHCy in the etiology of neurological disorders.

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