

Proteolytic system parameters in the brain of rats with hyperhomocysteinemia

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Abstract

Hyperhomocysteinemia (HHcy) is now being actively studied as a potential risk factor and/or biomarker for numerous pathologi-

cal conditions, including brain diseases. This study aimed to analyze the proteolytic processes in the brains of rats with HHcy. Total proteolytic activity, metal-dependent, and serine proteases activities, the content of matrix metalloproteinases (MMPs), tissue inhibitor of metalloproteinases-1, cytokines, serine proteases, total protein and medium and low molecular-weight substances (MLMWS), were evaluated. HHcy was induced by DL-homocysteine thiolactone (HTL) daily intragastric administration (200 mg·kg⁻¹ of body weight) to young and adult albino non-linear male rats for 8 weeks following rat sacrifice and brain harvesting. It was established that HHcy causes an increase in total proteolytic activity and a rise in MLMWS levels in rat brains. Serine protease activity increased to a greater extent compared to metal-dependent one, and bigger changes were observed in young rats. Rise in MMP-9 and -10 levels (in young animals), a decline in MMP-3 and -8 levels, and a decrease in the content of interleukin-1 β , interferon- γ , interleukin-4 and tumor necrosis factor- α (the last two in young animals) was also detected. No significant changes were found in serine protease content. Therefore, proteolysis intensification in the brain of rats with HHcy is more likely caused by protease up-regulation through mechanisms stimulated by homocysteine, HTL, and oxidative stress, without involving pro-inflammatory signaling pathways.

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Ethics approval: all experiments on animals were performed in compliance with international principles of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). The study was approved by the Ethical Committee of Taras Shevchenko National University of Kyiv (protocol №3 approved 25.11.2020).

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Introduction

Proteolysis is important for normal body functioning, and under physiological conditions it is closely regulated. A complex network of proteases, their inhibitors, and substrates, interconnected through several links, is called a degradome.¹ Under pathology, the balance between degradome components is changed with corresponding alterations in proteolytic activity.

Human proteases are represented by at least 580 enzymes.² More than a third of them belong to serine proteases involved in a variety of physiological processes including digestion and blood coagulation.³ Matrix metalloproteinases (MMPs) participate in extracellular matrix remodeling and bioactive molecule activation.⁴ However, under numerous pathologies these enzymes are dysregulated, particularly due to cytokines action, that through specific signaling cascades changes the expression of genes encoding proteases and their inhibitors. In turn, increased proteolysis is associated with medium and low molecular weight substances (MLMWS) accumulation. These compounds with a molecular weight of up to 5000 Da have direct action on biomolecules and also modulate intracellular signaling cascades.⁵

Homocysteine (Hcy) is a thiol-containing non-proteinogenic amino acid, formed endogenously during methionine metabolism.⁶ Normally plasma total Hcy (tHcy) level is 5-15 $\mu\text{mol}\cdot\text{L}^{-1}$, but under

hyperhomocysteinemia (HHcy) its value rises.^{7,8} Factors that lead to HHcy include hereditary defects in Hcy metabolism, vitamins B6, B9, and B12 deficiency, chronic alcohol consumption, smoking, and taking certain medications.⁷ Plasma tHcy also increases with aging through age-related vitamin deficiency and/or decline in renal function.^{9,10} An average of 5-10% of individuals in the population have mild to moderate HHcy ($15\text{--}40\ \mu\text{mol}\cdot\text{L}^{-1}$).¹¹

More than 100 pathologies have been identified being associated with HHcy.^{10,12,13} Recently, the link between HHcy and neurodegenerative and cognitive disorders, epilepsy, and ischemic stroke has been actively investigated.¹⁴ And it is already proven that Hcy metabolite homocysteine thiolactone (HTL), along with Hcy itself, participates in tissue injury under these pathologies causing N-homocysteinylated protein formation with proinflammatory, prothrombotic, and proatherogenic properties.¹⁵

Thus, it is an urgent task today to study the peculiarities of proteolysis and its regulation under chronic HHcy. This study aimed to evaluate the proteolysis system parameters in the brains of rats with HHcy.

Materials and Methods

Reagents

The following reagents were used in our research: DL-HTL hydrochloride (Acros Organics, Italy); casein, trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), hydrogen peroxide and o-phenylenediamine (Sigma-Aldrich, St. Louis, MO, USA); anti-rat monoclonal antibodies to cytokines, tissue inhibitor of metalloproteinases-1 (TIMP-1) and MMPs (Santa Cruz Biotechnology, Inc., Dallas, Texas, USA); horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA). All other reagents and chemicals were of analytical grade quality and available commercially.

Animals and experimental model

In this study, 40 male non-linear albino rats of various ages were used. They were first divided into young (one-two-month-old) and adult (six-eight-month-old) rats, with further splitting into control and experimental groups. Therefore, the following animal groups were formed ($n=10$ in each group): Young_Control; Young_HHcy; Adult_Control; Adult_HHcy.

The experiments were started 1 week after animal acclimatization in the animal facility of Taras Shevchenko National University of Kyiv. The animals were kept in polypropylene boxes under constant conditions of temperature ($22\pm 3^\circ\text{C}$), light (12:12-h light: dark cycle), and humidity ($60\pm 5\%$) with free access to water and standard rodent food.

The model of chronic HHcy was created by intragastric administration of HTL at a dose of $200\ \text{mg}\cdot\text{kg}^{-1}$ of body weight in a 1% solution of starch once daily for eight weeks.¹⁶ HTL concentration we used was also chosen based on this publication. An equal volume of 1% starch solution was administered to control animals. Eight weeks after the experiment started, animals were sacrificed by cervical dislocation.

HHcy state was confirmed by the high content of Hcy in blood plasma ($>15\ \mu\text{mol}\cdot\text{L}^{-1}$) determined by enzyme-linked immunosorbent assay (ELISA) using the kit «Homocysteine EIA» (Axis-Shield Diagnostics Ltd., Dundee, Scotland, UK). Blood was collected by cardiac puncture via the left ventricle using a 21-gauge needle after animals were sacrificed. Whole blood was withdrawn slowly into

polyethylene tubes with 3.8% sodium citrate in a ratio of 9:1. Plasma was prepared by blood centrifugation at $1,000\ \text{g}$ for 10 min at room temperature.

All animal experiments were performed according to the international principles of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986). The study was approved by the Ethical Committee of Taras Shevchenko National University of Kyiv (protocol №3 approved 25.11.2020).

Brain sample preparation

To obtain a whole brain homogenate the brains were immediately collected after animals had been sacrificed. Then, 1 g of brain tissue was homogenized in 9 mL of ice-cold Tris-buffered saline (TBS; 50 mM Tris-HCl, 140 mM NaCl; pH 7.4) with following centrifugation ($1000\ \text{g}$, 15 min, 4°C). After the supernatant was separated, it was centrifuged again ($10000\ \text{g}$, 30 min, 4°C). The supernatant was collected and immediately used for the measurements. Bradford method was used to determine protein concentration.¹⁷

Proteolytic activity determination

Total proteolytic activity was determined according to the method described by Munilla-Moran and Stark.¹⁸ Casein was used as a substrate. To prepare the 4% casein solution, 4 g of casein was dissolved in a mixture of 50 mM phosphate buffer, pH 7.4 (80 mL), and 1 M NaOH (1.6 mL). The resulting mixture was first kept at room temperature, and in 40 min it was boiled in a hot water bath. The obtained casein solution was then cooled, its pH was adjusted to 7.4 with 1 M NaOH, and its volume was adjusted to 100 mL using phosphate buffer.

Next, 0.5 mL of brain homogenate was brought to a volume of 1 mL with 50 mM phosphate buffer solution (pH 7.4), mixed, and then 1 mL of 4% casein solution was added followed by incubation in a thermostat at $+37^\circ\text{C}$ for 30 min. The enzymatic reaction was stopped by adding 3 mL of TCA (15%) to the reaction mixture with subsequent centrifugation ($15000\ \text{g}$, 30 min). The supernatant absorbance was read by a spectrophotometer (SmartSpecPlus, Bio-Rad, Hercules, California, USA) at 280 nm against the blank, containing an equal volume of phosphate buffer instead of brain homogenate.

To estimate the activities of metal-dependent and serine proteases, metal-dependent enzyme inhibitor (EDTA) and serine proteases inhibitor (PMSF) were used at the final concentrations of 5 mM and 2 mM, respectively: the residual proteolytic activity was determined using casein as a substrate after pre-incubation of the studied samples with the corresponding inhibitor (30 min, 4°C). Results were expressed as rel. units $\cdot\text{g}^{-1}$ of brain tissue.

Matrix metalloproteinases, tissue inhibitor of metalloproteinases-1, and cytokines immunoassay

MMPs, TIMP-1, and cytokine levels were determined by ELISA according to the standard instructions for soluble proteins.¹⁹ Brain homogenate samples were diluted to $10\ \mu\text{g}\cdot\text{mL}^{-1}$ with TBS and incubated overnight at 4°C in sterile ELISA plate wells with following well washing with TBS to remove unbound antigen. After washing, a 5% nonfat dry milk solution was added with following incubation (1 h, 37°C) to block the plate wells. Then plates were washed again with TBS containing 0.05% Tween-20, with further incubation with the corresponding primary antibodies against MMP-1,-2,-3,-8,-9,-10, TIMP-1, TNF- α , IL-1 β , -6,-4,-8,-10, IFN- γ (1:3000) for 1 h at 37°C . After that, plates were washed with TBS

containing 0.05% Tween-20 with subsequent incubation (1 h, 37°C) with secondary antibodies conjugated with horseradish peroxidase (1:9000). Next, the wells were washed again with TBS containing Tween-20 (0.05%), followed by the addition of substrates o-phenylenediamine and peroxide hydrogen to visualize the reaction. After 10 min, 1M H₂SO₄ was added to stop the reaction. The optical density of the samples was read using a microplate reader (μQuant™, BioTek Instruments, Inc, Winooski, Vermont, USA) at 492 nm.^{20,21} Results were expressed in rel. units·g⁻¹ of brain tissue.

Serine protease level determination

Affinity chromatography on benzamidine sepharose (GE Healthcare Life Sciences, Chicago, IL, USA) was used to purify serine protease fractions from rat brain homogenates.²² A benzamidine sepharose column pre-equilibrated with 10 volumes of binding buffer containing 10 mM Tris-HCl and 0.5 M NaCl, pH 8.0, was loaded with samples in the binding buffer. The column was then washed with 15 volumes of 10 mM Tris-HCl buffer (pH 8.0) to remove unabsorbed material, followed by elution of adsorbed proteins with buffer solution (50 mM glycine-HCl, 1 M NaCl, pH 3.0). Both sample loading and serine protease fractions collection were performed at a flow rate of 2 mL·min⁻¹. Serine protease concentration was determined by the Bradford method and expressed in mg·g⁻¹ of brain tissue.

Determination of the level of medium and low molecular weight substances

MLMWS fraction was isolated according to the method.²³ First, proteins were precipitated from the brain homogenates by the addition of 1.2 M HClO₄ in a 1:1 (v/v) ratio. Obtained solutions were centrifuged (5000 g, 20 min, 4°C) followed by supernatant neutralization with 5M KOH to a pH of 7.0. Then the mixtures were centrifuged again (2500 g, 20 min), and supernatants were transferred into microtubes with ethyl alcohol addition to the final concentration of 80% and kept for 30 min at 4°C with subsequent centrifugation. The optical density of the obtained supernatants was measured by a spectrophotometer Smart Spec™ Plus

(BioRad, Hercules, California, USA) at 210 nm, 238 nm, and 254 nm. Results were expressed as rel. units·g⁻¹ of brain tissue.²⁴

Statistical analysis of results

Statistical analysis was carried out using software MS Excel (MS Office) and StatSoft Statistica ver. 8.0 for Windows. Verification of the normal distribution of results was performed by the Shapiro-Wilk test, and the significance of differences between the studied groups was evaluated by one-way analysis of variance (ANOVA). Data in figures and tables were presented as mean ± standard error of the mean (SEM) (n=10 for each group). The difference between the studied groups was considered to be statistically significant at a p-value of <0.05.

Results

First, plasma tHcy levels were evaluated to confirm the HHcy state. They were 3.2 times higher in rats with HHcy compared with age-matched controls (Table 1), indicating mild HHcy.⁷

The next step of our research was to evaluate the proteolytic activity in the brains of rats with HHcy. Total proteolytic activity was found to be 44% and 30% higher in the Young_HHcy and Adult_HHcy groups, respectively, compared to the control group of the corresponding age (p<0.05) (Figure 1). In adult rat brains this parameter value exceeded that in young animals: in the control by

Table 1. Total homocysteine levels in plasma of rats with hyperhomocysteinemia.

Groups		tHcy, μmol·L ⁻¹
Young	Control	5.41±0.44
	HHcy	17.5±1.78*
Adult	Control	5.65±0.91
	HHcy	18.1±0.76*

Values are expressed as mean ± SEM (n=10); *p<0.05 compared to the control group of corresponding age.

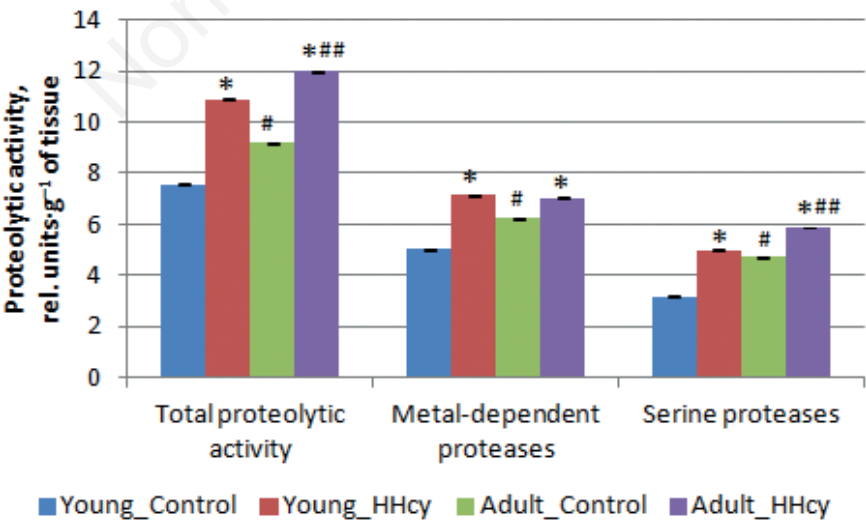


Figure 1. Peculiarities of proteolytic activity in the brain of rats with hyperhomocysteinemia. Values are expressed as mean ± SEM (n=10); *p<0.05 compared to the control group of corresponding age; #p<0.05 compared to the group “Young_Control”; ###p<0.05 compared to the group “Young_HHcy”.

21.5%, and in animals with HHcy to a lesser extent, by 10%. We also showed increased activities of serine proteases (1.6 times) and metal-dependent ones (1.4 times) in the brains of rats from the Young_HHcy group compared to the control of the same age. Similarly, in the Adult_HHcy group, serine protease activity exceeded control values by 25%, and metal-dependent protease activity by 13%. Activities of studied enzymes were also higher in the brain of adult control rats compared to those in the Young_Control group by 1.5 times (serine proteases) and 24% (metal-dependent proteases), whereas in adult rats with HHcy, serine proteases activity exceeded that in Young_HHcy group by 18%, and metal-dependent proteases activity did not significantly change.

Further, the content of MMPs and their regulators, such as TIMP-1 and cytokines, has been analyzed. No significant changes were revealed in MMP-1, -2, and TIMP-1 levels in the brains of rats with HHcy compared to those in age-matched controls (Figure 2).

Simultaneously, an increase in MMP-2 (by 11%) and TIMP-1 (by 15%) content with age was shown in control animals, wherein a rise in TIMP-1 can be considered as the activation of the compensatory mechanisms. A decline in MMP-3 and -8 levels in Young_HHcy (by 6% and 8.5%, respectively) and Adult_HHcy (by 9% and 10%, respectively) groups compared to the corresponding control groups was also found. MMP-3 content had no age-related changes, while MMP-8 levels increased by 7% with age in control rats only. MMP-9 and -10 levels in the brain of rats with HHcy, on the contrary, were higher by 33% and 10%, respectively, compared to the control animals, but in young animals only. MMP-10 content in the brain of rats from the Adult_Control group exceeded that in the Young_Control group by 18%.

Cytokine profile analysis revealed no significant differences in the levels of pro-inflammatory IL-6 and -8, and anti-inflammatory IL-10 in the brain of rats with HHcy compared to the age-matched controls (Figure 3).

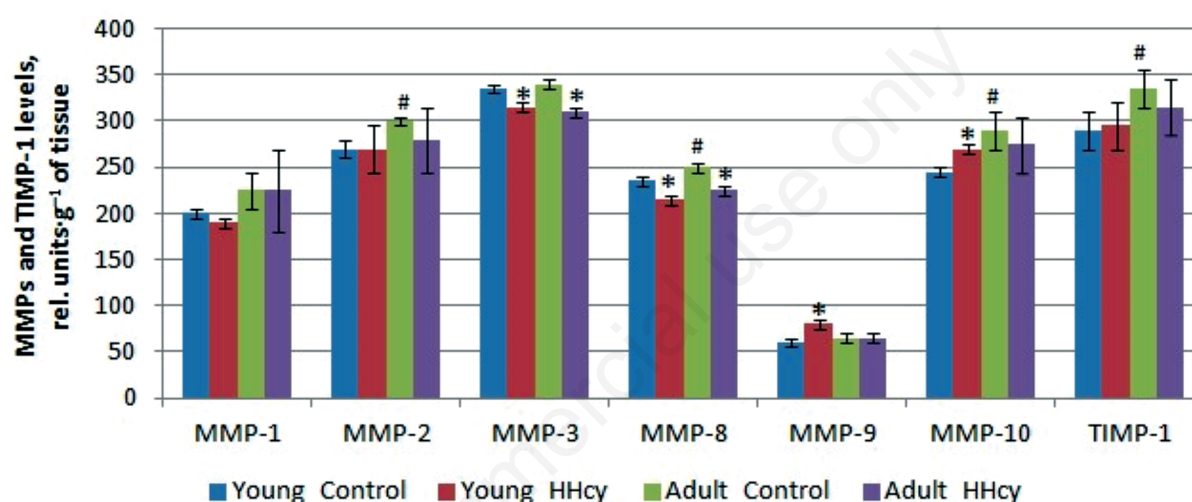


Figure 2. Levels of matrix metalloproteinases and tissue inhibitor of metalloproteinases in the brain of rats with hyperhomocysteinemia. Values are expressed as mean $\pm$ SEM (n=10); *p<0.05 compared to the control group of corresponding age; #p<0.05 compared to the group "Young_Control".

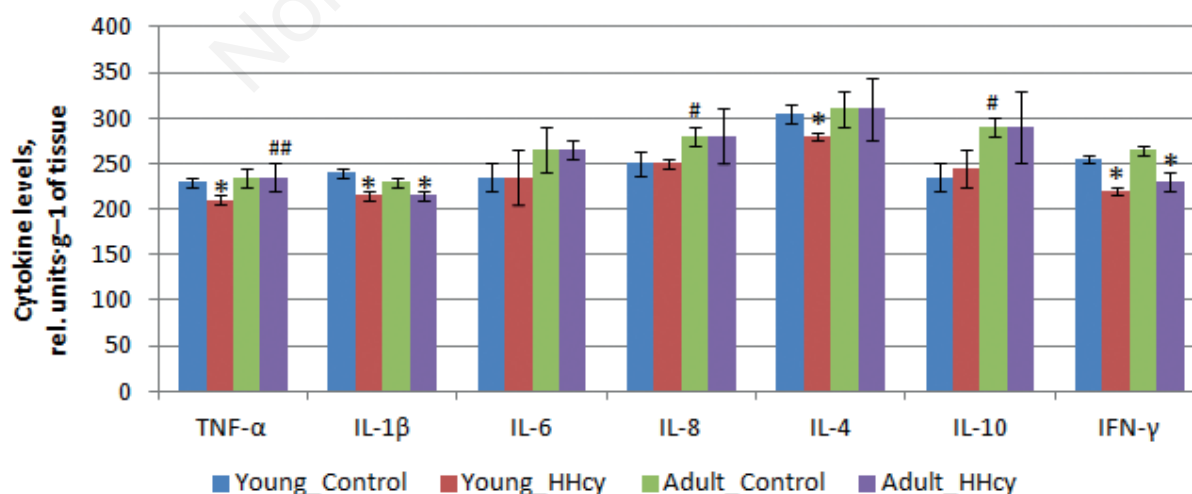


Figure 3. Cytokine profile of the brain of rats with hyperhomocysteinemia. Values are expressed as mean $\pm$ SEM (n=10); *p<0.05 compared to the control group of corresponding age; #p<0.05 compared to the group "Young_Control"; ##p<0.05 compared to the group "Young_HHcy".

Pro-inflammatory TNF- α and anti-inflammatory IL-4 content were changed in the Young_HHcy group only, declining by 8.5% compared to the corresponding control. The levels of pro-inflammatory IL-1 β and pleiotropic IFN- γ were reduced in the brains of rats from both Young_HHcy (by 10.5% and 14%, respectively) and Adult_HHcy (by 6.5% and 13%, respectively) groups. We also found that IL-1 β , -4, -6 and IFN- γ levels in brain homogenates did not change with rat age; IL-10 and -8 content in adult rats exceeded that in young animals only in the control (by 23% and 12%, respectively), and TNF- α content, only in animals with HHcy (by 12%).

Next, total protein, serine protease levels, and MLMWS content in the brains of rats were evaluated. Total protein content in the brains of rats from the Young_HHcy group was found to be 30% higher compared to the Young_Control group, and no changes were shown in group Adult_HHcys (Table 2). Simultaneously, total protein content decreased with age by 35% in the control, and by half in rats with HHcy.

No significant changes were found in serine protease content in the brains of rats with HHcy. However, it declined by 27% with age in the control animals.

A rise in MLMWS levels in the brains of rats with HHcy was also revealed. MLMWS210 (peptide fraction) content was 3.2 times (Young_HHcy group) and 2 times (Adult_HHcy group) higher compared to its value in the control group of corresponding age; the level of MLMWS238 fraction represented by non-aromatic peptides was 1.7 times and 2.2 times, and MLMWS254 content (non-aromatic sulfur-containing molecules, purine bases and nucleotides) 2 times and 3.2 times greater in Young_HHcy and Adult_HHcy groups, respectively, in comparison with age-matched control. Age-related changes were mainly observed in rats with HHcy (a 2-fold increase in MLMWS210, and a 1.4-fold rise in MLMWS254 and MLMWS238 levels), whereas in control rats only MLMWS210 level increased 3.2 times with age.

Discussion

HTL-induced HHcy is well known and one of the generally accepted methods of creating animal models of this pathology. The benefit of this model is attributed to the fact that, unlike a methionine-rich diet or folate deficiency (other common methods of inducing experimental HHcy), it avoids the direct effect of methionine excess or folic acid deficiency.¹⁶ At once after administration of HTL, it is metabolized by HTL hydrolases (found in the liver and other organs, as well as in the blood) to Hcy (increased levels of which are associated with HHcy).^{15,25} Another part of HTL is removed from the body with urine.^{15,26} Tissue damage under HHcy is considered to be complex and related both to the effect of Hcy itself and to the action of its metabolites formed directly in cells,

such as HTL, homocysteinic acid, homocysteinylated proteins, as well as may also involve other processes, including oxidative stress and inflammation.

Our results revealed an increase in plasma levels of Hcy, confirming the state of moderate HHcy. Plasma tHcy includes reduced (1%) and oxidized disulfide (99%) forms of Hcy and is regulated by Hcy use in cysteine and methionine biosynthesis and its reabsorption rate in the kidneys.¹² However, tHcy does not account for HTL that can be formed from Hcy in all cells during error-editing reactions by aminoacyl-tRNA synthetases, and also Hcy being a part of N-homocysteinylated proteins.^{6,15}

In HHcy, Hcy easily crosses the blood-brain barrier and enters the brain, where it can directly exert certain effects, or be converted back into HTL, which can either remain in the nervous tissue, causing its damage, or be transported into the blood and further be subject to neutralization or elimination.²⁷ HHcy effect on the brain results from both direct neurotoxic action and vascular changes induced by Hcy.⁸ Being the glutamate receptor agonist, Hcy can act as an excitotoxicity mediator. Its autooxidation generates reactive oxygen species (ROS), which damage brain cells through oxidative stress and nuclear factor-kappaB (NF- κ B)-dependent proinflammatory signaling pathways.¹⁴ HTL-induced neurotoxicity includes N-homocysteinylated proteins, and such modification changes their functions and predetermines the signs of many diseases.^{25,26} HHcy is also known to cause endothelial dysfunction and promote atherogenesis and thrombosis.⁸ With age, plasma tHcy increases, affecting the brain; but age-related changes also occur in the brain itself, particularly, due to antioxidant protection weakening and low-grade inflammation development.²⁸

The analysis of the proteolytic activity in the brain homogenates showed that total proteolytic activity in the brain of rats with HHcy increased (in young animals to a greater extent), and the rise in serine protease activity was more pronounced than that of metal-dependent ones. In animals with HHcy, age-related changes were smaller than in control, which can be explained by both higher values of studied parameters at a young age, and compensatory mechanisms involvement under HHcy.

Our previous studies detected an increase in proteolytic activity in the thyroid gland, lungs, spleen, heart, and liver of rats with HHcy.^{5,29,30} Under these conditions proteolysis intensification may result from the activation of already existing proteases and/or increase in their gene expression in oxidative stress and inflammation.^{6,10,31,32} HTL can also affect gene expression by epigenetic mechanisms.¹³ The increase in proteolysis during aging we found may be related to the protease's involvement in aging regulation and mechanisms preventing accumulation of misfolded and predisposed to aggregation proteins in the cells.³³

The present study results also revealed that the rise in MMP

Table 2. Levels of total protein, serine proteases, and medium and low molecular-weight substances in the brain of rats with hyperhomocysteinemia.

		Young		Adult	
		Control	HHcy	Control	HHcy
Total protein, mg·g ⁻¹ of tissue		58.8±0.6	76.6±1.7*	38.4±0.4#	39.2±1.5##
Serine proteases, mg·g ⁻¹ of tissue		4.01±0.01	3.7±1.38	2.91±0.81#	3.67±1.25
MLMWS, rel. units·g ⁻¹ of tissue	MLMWS210	0.12±0.04	0.39±0.01*	0.39±0.08#	0.77±0.03*##
	MLMWS238	0.14±0.02	0.24±0.01*	0.15±0.06	0.33±0.07*##
	MLMWS254	0.12±0.01	0.25±0.03*	0.11±0.07	0.35±0.04*##

Values are expressed as mean ± SEM (n=10); *p<0.05 compared to the control group of corresponding age; #p<0.05 compared to the group "Young_Control"; ##p<0.05 compared to the group "Young_HHcy".

activity in the brain of rats with HHcy we found was accompanied by an increase in MMP-9 and -10 content shown in the Young_HHcy group only, while MMP-3 and -8 levels, on the contrary, decreased in both Young_HHcy and Adult_HHcy groups compared with age-matched controls. The greatest changes in MMP content were related to MMP-9 and bigger shifts were observed in young rats. Age-related changes concerned only MMP-2,-8,-10, and TIMP-1 in control animals. Our data are consistent with results that highlight the MMP-9 role in blood-brain barrier dysfunction and explain increased expression of MMP-9 in the brain under HHcy by alterations in intracellular signaling caused by cytokines and ROS and HTL-triggered epigenetic changes.^{8,13}

Studying of brain cytokine profile of rats with HHcy established a considerable reduction in IFN- γ content (in animals of both age groups), and a less pronounced decline in the levels of TNF- α (young rats), IL-1 β (young, and adult animals) and IL-4 (young rats). So, the detected changes were also more expressed in young animals.

Therefore, while our previous investigations revealed mild inflammation in the heart and thyroid gland of rats with HHcy, we observed no signs of inflammation in the brains of experimental rats in this study.^{5,30} Similarly, earlier we showed no inflammation in the spleen of rats with HHcy.²⁹ Our data are also consistent with results that found MMP-9 activation and blood-brain barrier disintegration without neuro-inflammation in the brains of mice with mild HHcy.³⁴ Therefore, brain damage in rats with HHcy probably involves mechanisms unrelated to pro-inflammatory signaling pathway activation. Accordingly, the decrease in MMP-3 and -8 levels we observed may be caused by a decline in the content of their regulators (IL-1 β , TNF α , IFN- γ) as well as by epigenetic alterations or their molecules proteolysis. The rise in MMP-9 and -10 levels is rather related to the epigenetic mechanisms and/or signaling pathways modulation by oxidative stress under HHcy.

The observed age-related changes in MMPs and cytokines levels may be the result of a gradual rise in the number of senescent brain cells which are known to acquire a pro-inflammatory senescence-associated secretory phenotype (SASP).³⁵ SASP formation can be induced by oxidative stress, and protein misfolding/aggregation and is characterized by the production of a specific set of growth factors, chemokines, serine proteases, MMPs, and pro-inflammatory cytokines, which lead to low-grade inflammation.^{36,37} Thus, an increase in IL-8 and IL-10 levels along with MMP-2,-8,-10 and TIMP-1 content we revealed in the brain homogenates of animals from Adult_Control group in comparison with Young_Control may indicate the initial stages of age-associated inflammation. These changes will likely be more pronounced in old animals.

Our study also involved the evaluation of total protein, serine protease levels, and MLMWS content in the brains of rats with HHcy. The increase in total protein content we found in the brain of young rats with HHcy compared to the age-matched control may be an outcome of certain gene expression stimulation under HHcy caused by epigenetic mechanisms and (or) ROS-dependent modulation of cell signaling.^{6,13,32} However, no similar changes were shown in adult rats with HHcy. We also revealed a decrease in this parameter value with age, more pronounced in rats with HHcy, which may be a sign of proteolysis intensification in adult animals compared to young ones. Thus, no changes in total protein content in the brain of rats from the Adult_HHcy group compared to Adult_Control may be a consequence of an increase in protein biosynthesis under HHcy against age-related decrease in its level.

Our results showed no significant changes in serine protease content in the brains of rats with HHcy. Therefore, the rise in serine protease activity in all studied groups we found is probably not relat-

ed to their biosynthesis increase but may be the result of their up-regulation by post-translational mechanisms under oxidative stress.

Analysis of MLMWS levels revealed a significant increase in the content of all their fractions in the brains of animals with HHcy compared to the age-matched control, and age-related changes were more pronounced in rats with HHcy. Being the normal cell metabolites, MLMWS are usually found in tissues and blood at low levels. However, oxidative stress, inflammation, and/or increased proteolysis cause their accumulation that influences the biochemical processes, contributing to pathology progression. Therefore, these results confirm proteolysis intensification in brain homogenates of rats with HHcy and are consistent with our previous results that revealed a rise in MLMWS content in the plasma and thyroid gland of rats with HHcy.⁵

Conclusions

In conclusion, HHcy causes an increase in total proteolytic activity and a rise in MLMWS levels in rat brains. Serine protease activity increases to a greater extent compared to metal-dependent enzymes, and bigger changes are observed in young rats. HHcy leads to a rise in MMP-9 and -10 levels (in young animals) and a decline in MMP-3 and -8 levels; the levels of IL-1 β , IFN- γ , IL-4, and TNF- α are also diminished (the last two in young animals). No significant changes were found in serine protease content. Therefore, proteolysis intensification in the brain of rats with HHcy is more likely caused by protease up-regulation through mechanisms stimulated by Hcy, HTL, and oxidative stress, without pro-inflammatory signaling pathways involved. Obtained data could facilitate, at least partially, uncovering the mechanisms involved in the pathogenesis of brain damage under HHcy.

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