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Original Article

Effect of Hyperhomocysteinemia on Proteolytic Activity in the Spleen

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Abstract

Background: The aim of the study was to investigate the effect of hyperhomocysteinemia (HC) on proteolytic activity in the spleen of rats. **Methods:** Albino nonlinear male rats of different ages (young, adults, and old) were involved in the study. HC was induced by intragastric administration of DL-homocysteine thiolactone. The total proteolytic activity was evaluated using casein as a substrate. The activity of metal-dependent and serine proteases was determined using proteases inhibitors ethylenediaminetetraacetic acid and phenylmethylsulfonyl. The levels of matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs), and cytokines were measured by enzyme-linked immunosorbent assay. The fraction of serine proteases was isolated by affinity chromatography. **Results:** HC causes an increase in the total proteolytic activity, which is due to an increase in the activity of proteases of various catalytic types (serine, cysteine, aspartic proteases, and metal-dependent enzymes). The levels of MMP-2, MMP-3, MMP-8, and MMP-10 were significantly reduced in the spleen of adult and old rats and were at the control level in young rats. A disorder of the balance between MMP-1 and TIMP-1 in the spleen of rats with HC in favor of the active form of MMP-1 was revealed. At the same time, the level of serine proteases was increased in the spleen of rats of all groups. A decrease in the level of some pro-inflammatory cytokines was also revealed. **Conclusions:** HC causes disturbances in the proteolytic balance, manifested by an increase in proteolytic activity. The pathogenesis of this disease is not associated with the development of inflammation in the spleen.

Keywords: Cytokines, hyperhomocysteinemia, proteolytic enzymes, spleen

Introduction

Hyperhomocysteinemia (HC) is a condition characterized by an elevated level of homocysteine in the blood.[1] This condition is recognized as a separate disease, mainly due to the dysfunction of enzymes and cofactors of homocysteine metabolism. In addition to genetically determined defects, HC can be triggered by dietary factors, lifestyle factors, nutritional deficiencies in vitamin cofactors, hazardous habits, or certain medications. Accumulated data indicate a positive correlation between elevated homocysteine levels and the development of cardiovascular, neurodegenerative diseases, and malignant diseases.[2,3] HC is also associated with aging. Independent research groups have found that the blood level of homocysteine increases statistically every 10 years after age 60.[4,5]

Taking into account, the results of studies by a number of authors,[6,7] as well as our own works,[8,9] it can be concluded that the effect of elevated homocysteine levels is complex and extends to many organs. Literature reviews show that, in many cases, pathologies associated with the accumulation of homocysteine are accompanied by impaired functioning of the immune system. On the other hand, an intensive proliferation of immunocompetent cells can lead to the accumulation of homocysteine. For example, Schroecksnadel *et al*.[10] reported that the Th1-type immune response contributes to the development of HC and may be a major determinant of disease progression. Zhang *et al*.[11] demonstrate that reactive oxygen species formed as a result of homocysteine autoxidation are

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involved in the induction of B-lymphocyte proliferation. Since the spleen is the main organ of the immune system involved in the activation and control of immune responses, it seems reasonable to investigate whether HC affects this organ.

The aim of the study was to investigate the activity of proteases of various catalytic types (serine proteases, metal-dependent enzymes, cysteine, and aspartic proteases) in the spleen of rats with experimental HC. The levels of matrix metalloproteinases (MMPs), serine proteases, and cytokines were also investigated.

Methods

Reagents

Thiolactone D, L-homocysteine, ethylenediaminetetraacetic acid (EDTA), and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-rat monoclonal antibodies to MMPs, tissue inhibitors of metalloproteinases (TIMPs)-1, and cytokines were purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals and reagents used in this study were of analytical grade quality and available commercially.

Animals and experimental design

Sixty albino nonlinear male rats of different ages (1-month-old rats, 6-month-old rats, and 24-month-old rats) were used in the experiment. All animal manipulations 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. The animals were kept in polypropylene cages (595 mm × 380 mm × 200 mm, floor area 1820 cm^2; 5 rats in a cage) under constant conditions of temperature (22°C ± 3°C), humidity (60% ± 5%), and illumination (12 h light/12 h dark cycle). Standard food for rodents and water were provided *ad libitum*. In 1 week after the acclimatization, the animals were divided into the control and experimental groups by simple randomization. Thus, there were three experimental groups: Group #1 (1-month-old rats, $n = 10$), Group #2 (6-month-old rats, $n = 10$), and Group #3 (24-month-old rats, $n = 10$). The control groups ($n = 10$) consisted of animals of the same age as the rats of the corresponding experimental groups. HC was induced by intragastric administration of thiolactone D, L-homocysteine diluted in 1% starch solution (100 mg/kg of body weight), once per day for 28 days.[12] The rats of control groups were received an equal volume of 1% starch. The rats with plasma homocysteine levels above 15 mmol/L were considered HC rats. The levels of homocysteine were determined by enzyme-linked immunosorbent assay (ELISA) using the kit «Homocysteine EIA» (Axis-Shield, UK). On the 29th day since the start of the experiment, animals were sacrificed. The animals of Group #1 were killed by cervical dislocation; the animals of Groups #2, and 3 were killed by decapitation.

Preparation of spleen homogenate

Briefly, to obtain a spleen homogenate, 1 g of spleen tissue was homogenized in 9 ml of cold 50 mm Tris-buffered saline (pH 7.4). The sample was centrifuged at 1000 g (15 min, 4°C). The supernatant was collected and subjected to the second step of centrifugation at 10,000 g (30 min, 4°C). Protein concentration was determined by the Bradford method.[13]

Determination of the activity of various types of proteases

Total proteolytic activity was determined according to the method[14] using casein as a substrate. The aliquots of spleen homogenate (50 μg of total protein) were incubated with casein (2%) in 50 mm Tris-buffered saline (pH 7.4) at 37°C for 30 min. At the end of the incubation, trichloroacetic acid (7%) was added to the samples to stop the enzymatic reaction. After a 15-min incubation at 4°C, the samples were centrifugated for 30 min at 15,000 g. The absorbance of the supernatants was measured on a spectrophotometer (SmartSpecPlus, Bio-Rad, USA) using a wavelength of 280 nm. The blank sample was a sample in which the spleen homogenate has been replaced with an equal volume of Tris-buffered saline. The total proteolytic activity was expressed as rel. Units/g of spleen tissue. To assess the activity of metal-dependent enzymes and serine proteases, EDTA and PMSF were used, respectively. The inhibitors were used at a final concentration of 5 mm. The activity of enzymes of other catalytic types (cysteine and aspartic proteases) was calculated by subtracting the activity of metal-dependent enzymes and serine proteases from the total proteolytic activity.

Determination of the levels of matrix metalloproteinases, tissue inhibitors of metalloproteinases, and cytokines

The levels of MMP-1,-2,-3,-8,-10, TIMP-1, pro-inflammatory cytokines tumor necrosis factor-alpha, interleukin (IL)-1b, IL-8, IL-6, and anti-inflammatory cytokines interferon□gamma (IFN-γ), IL-4, and IL-10 were determined by ELISA according to the instructions of the manufacturers.[15] Plates were coated overnight at 4°C with samples of spleen homogenate diluted with 50 mm Tris-buffered saline (pH 7.4) to the concentration of proteins of 10 μg/mL. After washing, the plates were blocked with a solution of 5% nonfat dry milk in Tris-buffered saline for 1 h at 37°C and washed again. The samples were incubated first with specific primary antibodies to MMPs, TIMP-1, and cytokines, and then with secondary antibodies conjugated with horseradish peroxidase for 1 h at 37°C. After washing the plate, substrates o-phenylenediamine and hydrogen peroxide were added to the samples. The reaction was stopped with 2.5 N H_2SO_4. The absorbance was read at 492 nm.

Determination of the level of serine proteases

The fraction of serine proteases was isolated from the spleen homogenate by affinity chromatography on a Benzamidine Sepharose®4 Fast Flow (GE Healthcare, USA).[16] The samples were loaded to the column in binding buffer 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4. The absorbed material was

eluted with 50 mm glycine-HCl buffer, pH 3.0, containing 1 M NaCl. The samples were loaded, and the serine protease fractions were collected at a flow rate of 1 ml/min. The level of serine proteases was determined by the method of Bradford as described above, and the results were expressed as mg of serine proteases per g of spleen tissue.

Statistical analysis

Statistical analysis was performed with Statistica 8.0 software (StatSoft, GmbH, Hamburg, Germany, Europe). The data of biochemical estimations were reported as mean ± standard error of mean for each group (n = 10). The Kolmogorov–Smirnov test was used to verify the normal distribution of results. Statistical analyses were performed using a one-way analysis of variance. Differences were considered to be statistically significant when $P < 0.05$.

Results

Total proteolytic activity, and activity of proteases of various catalytic types

To test whether elevated homocysteine levels affect proteolytic homeostasis in the spleen of rats, the total proteolytic activity was examined. The data obtained [Table 1] indicate a significant increase in the total proteolytic activity in the spleen of rats with HC – the activity was higher by 1.54, 1.37, and 1.76 times in rats of groups #1, #2, and #3 compared with the values in the control groups. To elucidate the involvement of proteases of various catalytic types in the disorders of proteolytic balance, the total proteolytic activity was detected in the presence of EDTA and PMSF which are inhibitors of metal-dependent and serine proteases, respectively. The activity of metal-dependent enzymes increased 1.28 times in the spleen of rats of groups #1 and #2, and 1.58 times in the spleen of rats of group #3. The increase in the activity of serine proteases, more pronounced than in the case of metal-dependent enzymes, was found in all experimental groups. Thus, the activity of serine proteases increased 2.1 times in the rats of group #1; 1.4 times in the rats of group #2, and 2.4 times in the rats of group #3. Evaluation of the activity mediated by cysteine and/or aspartic proteases revealed a significant increase in this parameter in animals of group #1-proteolytic activity exceeded the control value by 9.2 times. At the same time, the activity mediated by cysteine and/or aspartic proteases were at the level of the corresponding control in the spleen of rats of group #3.

Total protein level and level of metal-dependent and serine proteases

To elucidate the possible reasons for the increase in protease activity in HC rats, the level of metal-dependent enzymes, namely, MMP, as well as the level of serine proteases, was determined. Our results [Table 2] demonstrated HC caused a significant increase in the level of serine proteases in the spleen of rats.

The most pronounced changes were found in the rats of group #2-the level of serine proteases was 0.37 ± 0.12 mg/g of tissue versus 0.11 ± 0.04 mg/g of tissue in the control group. The increase in the level of serine proteases occurred against the background of an increase in the level of total proteins in the rats of all experimental groups. As can be seen from Table 2, total protein levels increased 1.9, 2.1, and 1.7 times in the spleen of rats of groups #1, 2, and 3, respectively. In contrast, the levels of MMP-2, -3, -8, and -10 as well as TIMP-1 were significantly decreased in the spleen of rats of groups #2 and #3. At the same time, the levels of MMP-2, -3, -10, and TIPM-1 were within the corresponding controls in the rats of group #1. It should be noted, only the level of MMP-1 was found to be increased in the spleen of rats of all experimental groups-1.2 times for the rats of groups #1 and #2 and 1.3 times for the rats of group #3.

Level of pro-and anti-inflammatory cytokines

To determine whether inflammation in the spleen is involved in the pathogenesis of HC, a cytokine profile was examined. In our experiment, no increase in the level of pro-and anti-inflammatory cytokines in the spleen of rats with HC was found. Moreover, HC caused a significant decrease in the levels of IL-1b, IL-8, IL-6, IFN-γ, IL-4, and IL-10 in the rats of groups # 2 and # 3 [Table 3]. At the same time, the level of all tested cytokines (except IFN-γ) in rats of group #1 did not differ from the corresponding controls.

Discussion

To date, there exists a considerable body of literature on hyperhomocysteine pathogenesis. However, the exact mechanisms by which homocysteine exerts harmful effects

Table 1: Distribution of proteolytic activity mediated by various types of proteases in the spleen of rats with hyperhomocysteinemia

	rel. units/g of tissue					
	Group #1		Group #2		Group #3	
	Control	HC	Control	HC	Control	HC
Total proteolytic activity	6.13±0.30	9.45±0.47*	8.14±0.40	11.17±0.55†	4.01±0.20	7.07±0.34‡
Metal-dependent proteases	4.91±0.23	6.30±0.30*	5.48±0.27	7.06±0.35†	2.99±0.13	4.73±0.21‡
Serine proteases	1.14±0.05	2.41±0.12*	2.25±0.11	3.18±0.15†	0.88±0.04	2.19±0.11‡
Cysteine and aspartic proteases	0.08±0.004	0.74±0.03*	0.41±0.02	0.93±0.04†	0.14±0.007	0.15±0.007

*P<0.05 significantly different from the control of group #1, †P<0.05 significantly different from the control of group #2, ‡P<0.05 significantly different from the control of group #3. Values are expressed as mean±SEM (n=10). HC: Hyperhomocysteinemia, SEM: Standard error of mean

Table 2: Total protein level and levels of matrix metalloproteinases, tissue inhibitor of metalloproteinases, and serine proteases in the spleen of rats with hyperhomocysteinemia

	Group #1		Group #2		Group #3	
	Control	HC	Control	HC	Control	HC
Total proteins (mg/g of tissue)	23.13±3.57	44.72±11.37*	18.83±2.41	38.60±11.85†	19.75±6.93	33.65±7.29‡
Serine proteases (mg/g of tissue)	0.28±0.02	0.47±0.12*	0.11±0.04	0.37±0.12†	0.34±0.01	0.68±0.09‡
Matrix metalloproteinases (rel. units/g of tissue)						
MMP-1	0.20±0.01	0.24±0.02	0.24±0.01	0.29±0.01	0.26±0.01	0.34±0.01‡
MMP-2	0.35±0.03	0.35±0.02	0.43±0.03	0.33±0.01†	0.39±0.01	0.31±0.02‡
MMP-3	0.20±0.03	0.22±0.04	0.30±0.06	0.23±0.02†	0.29±0.01	0.21±0.01‡
MMP-10	0.28±0.04	0.27±0.03	0.41±0.04	0.27±0.01†	0.42±0.03	0.19±0.02‡
MMP-8	0.25±0.01	0.20±0.02	0.28±0.01	0.22±0.01†	0.31±0.01	0.21±0.02‡
TIMP-1	0.33±0.02	0.34±0.04	0.49±0.04	0.37±0.01†	0.51±0.02	0.38±0.01‡

*P<0.05 significantly different from the control of group #1, †P<0.05 significantly different from the control of group #2, ‡P<0.05 significantly different from the control of group #3. Values are expressed as mean±SEM (n=10). SEM: Standard error of the mean, MMP: Matrix metalloproteinases, TIMP-1: Tissue inhibitor of metalloproteinases-1, HC: Hyperhomocysteinemia

Table 3: Level of cytokines in the spleen of rats with hyperhomocysteinemia

	rel. units/g of tissue					
	Group #1		Group #2		Group #3	
	Control	HC	Control	HC	Control	HC
TNF-α	0.26±0.02	0.26±0.02	0.27±0.02	0.26±0.01	0.28±0.02	0.29±0.02
IL-1b	0.19±0.02	0.19±0.01	0.28±0.01	0.20±0.01†	0.23±0.02	0.16±0.01‡
IL-8	0.28±0.02	0.27±0.03	0.37±0.01	0.31±0.01†	0.33±0.01	0.24±0.02‡
IL-6	0.24±0.03	0.24±0.02	0.33±0.01	0.25±0.01†	0.35±0.02	0.21±0.02‡
IFN-γ	0.19±0.02	0.13±0.01*	0.21±0.01	0.15±0.02†	0.24±0.01	0.17±0.01‡
IL-4	0.28±0.02	0.28±0.03	0.36±0.05	0.31±0.02†	0.32±0.02	0.25±0.01‡
IL-10	0.30±0.01	0.29±0.03	0.47±0.01	0.37±0.03†	0.41±0.01	0.32±0.01‡

*P<0.05 significantly different from the control of group #1, †P<0.05 significantly different from the control of group #2, ‡P<0.05 significantly different from the control of group #3. Values are expressed as mean±SEM (n=10). HC: Hyperhomocysteinemia, SEM: Standard error of mean, TNF-α: Tumor necrosis factor-α, IL: Interleukin, IFN: Interferon

are still unknown, and more research is needed to elucidate the pathophysiology of HC. Undoubtedly, proteolysis is one of the fundamental processes underlying many vital functions.[17] Aberrant proteolysis, expressed in excessive activation or, conversely, in the suppression of the activity of proteolytic enzymes, is considered one of the triggers for the development of various pathologies.[18] In our previous studies, it was found that HC is characterized by an increase in proteolytic activity in a number of organs,[8,9] which may contribute to the progression of HC. To find out whether HC affects proteolysis in the spleen, the total proteolytic activity as well as the activity of the main types of proteolytic enzymes in the spleen of rats was investigated.

In the present study, it was revealed that HC causes a violation of the proteolytic balance in the spleen, as evidenced by a pronounced increase in the total proteolytic activity. The most pronounced change in the total proteolytic activity was found in the spleen of rats of group #3 (old animals). It should be noted that this parameter in the spleen of the rats of the control group for group #3 was lower than in the controls for groups #1 and #2. This may be due to age-related changes in cellular metabolism, namely, the physiological decline of proteolytic systems during aging.[18] Our data indicate an increase in the total proteolytic activity in the spleen of rats of all experimental groups is a result of an increase in the activity of metal-dependent, as well as serine proteases. The involvement of cysteine and aspartic proteases in enhancing proteolysis in the spleen has also been shown.

The exact mechanism by which elevated levels of homocysteine cause an increase in proteolysis is not fully understood. However, given the wide range of proteases that are activated in HC, several mutually reinforcing mechanisms may be involved in this process. First, this may be due to increased protease biosynthesis, which is partly confirmed by a significant increase in the protein content in the spleen of rats of all experimental groups. This explanation is not unreasonable: a review of the literature[19] shows that the expression of some genes is altered in subjects with elevated homocysteine levels. The influence of homocysteine on gene expression can be realized through disturbances in the cellular redox balance in response to the development of oxidative stress. The autoxidation of homocysteine, leading to the accumulation of reactive oxygen species,[20] is considered a possible trigger for the induction of oxidative stress during HC. For example, in their experiments,

Alge-Priglinger *et al*.[21] showed that oxidative stress causes an increase in mRNA for MMP-1 and MMP-3 in cultured human retinal pigment epithelium cells. Based on this fact, it can be assumed that the increase in the level of MMP-1 in the spleen of rats with HC may be a consequence of the induction of the biosynthesis of this enzyme. On the other hand, oxidative modification of cellular proteins by reactive oxygen species as well as their N-homocysteinylation by homocysteine is a signal for the proteolytic degradation of modified proteins to prevent the accumulation of molecules with potential cytotoxic, proinflammatory, prothrombotic, and proatherogenic properties.[22,23] In addition, N-homocysteinylation, affecting the physicochemical properties and biological activity of proteins, can provoke the activation of the immune response in HC. Autoantibodies against N-homocysteinylated protein are found in the plasma of patients with elevated levels of homocysteine.[24]

Analyzing our results, it seems that a significant increase in the activity of serine proteases in the spleen of animals with HC is due to an increase in the synthesis of new molecules. Since the spleen contains subpopulations of macrophages and neutrophils expressing a wide range of serine proteases,[25,26] an increase in both the content and activity of serine proteases displays the changes in the functional activity of the splenic immune cells. Despite the fact that a significant part of cellular metal-dependent proteases is represented by MMP, our results do not suggest that the increase in the activity of metal-dependent enzymes in the spleen of rats with HC is associated with an increase in the biosynthesis of MMP-2, MMP-3, MMP-8, and MMP-10 since the levels of these enzymes were even lower than in the corresponding control groups. To some extent, the increase in the activity of metal-dependent enzymes in the spleen of HC rats may be the result of MMP-1 activation, as evidenced by the increase in the level of this enzyme. At the same time, a decrease in the level of TIMP-1 may be an additional mechanism leading to the activation of MMP-1 in the spleen of rats of groups #2 and #3. It should be emphasized that MMP-1 activation is often observed during angiogenesis and cancer development,[27,28] therefore, more detailed studies of the functional state of the spleen in HC are needed. Taking into account the multilevel control of the activity of MMP, other mechanisms of regulation of enzyme activity, in particular, posttranscriptional ones, may also be involved in the activation of proteases in HC. Accumulating data from various studies indicates that serine proteases can act directly on some pro-MMPs,[29] generating active enzymes, which, in turn, activate other pro-MMPs, provoking a cascade mechanism for increasing proteolysis. Moreover, several works in the literature demonstrate that homocysteine can directly,[30] as well as through the extracellular signal-regulated kinase/mitogen-activated protein kinase signaling pathway,[31] activate pro-MMP.

Our results on the absence of changes in the level of pro-inflammatory cytokines in the spleen of rats of group #1 and the decrease in the level of these cytokines in rats of groups #2 and #3 are not consistent with the general concept that systemic inflammation is a necessary part of the pathogenesis of HC. A number of studies have established a close correlation between a high concentration of circulating homocysteine and the development of systemic inflammation.[32,33] Moreover, our previous studies showed a state of mild inflammation in the heart[9] and thyroid glands[34] of HC rats. According to the literature, cytokines are the key molecules by which HC leads to the development of such concomitant complications as atherosclerosis and associated thrombotic events in patients with HC. In contrast to the above, the results of determining the level of cytokines in the spleen of rats with HC indicate the absence of inflammatory processes in the spleen of rats with HC and suggest the involvement of other mechanisms for the implementation of negative effects of elevated levels of homocysteine.

Conclusion

The present study showed that HC affects the proteolytic balance in the spleen of rats of different ages. This is manifested in an increase in the total proteolytic activity, the activity of serine proteases, metal-dependent enzymes, as well as enzymes related to cysteine and aspartic proteases. The disorder of the proteolytic balance is not associated with an increase in the level of MMP; however, this may be due to an increase in the biosynthesis of serine proteases and the imbalance between MMP-1 and TIMP-1 in the spleen of rats with HC. This disease is not accompanied by the development of inflammation in the spleen.

Limitation of the study

The limitation was not applicable in this study.

Ethical statement

The experimental work with animals followed the international recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 1986) and was confirmed by the Ethical Committee of ESC "Institute of Biology and Medicine" of Taras Shevchenko National University of Kyiv (protocol No. 2 approved August 19, 2021).

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Conflicts of interest

There are no conflicts of interest.

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