ORIGINAL ARTICLE

PROTEOLYTIC ACTIVITY IN THE HEART OF RATS WITH HYPERHOMOCYSTEINEMIA

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ABSTRACT

The aim: To investigate the distribution of proteolytic activity and cytokine profile in the heart of rats with hyperhomocysteinemia.

Materials and methods: A total of 60 albino non-linear male rats was used in the study. Hyperhomocysteinemia was induced by intragastric administration of DL-homocysteine thiolactone. Total proteolytic activity was measured using casein as a substrate. To determine the activity of metal-dependent and serine proteases, ethylenediaminetetraacetic acid, and phenylmethylsulfonyl fluoride were used. The level of matrix metalloproteinases, tissue inhibitor of metalloproteinases-1, and cytokines was studied by enzyme-linked immunosorbent assay.

Results: It was found an increase in the total proteolytic activity in the heart of young, adult, and old animals. In addition, the redistribution of proteolytic activity was revealed – the portion of metal-dependent enzymes increased in all groups while the percentage of serine proteases decreased in the old animals with hyperhomocysteinemia. The state of mild inflammation, evidenced by the increased level of some pro-inflammatory cytokines, was found in the heart of young and old animals with hyperhomocysteinemia.

Conclusions: The pathogenesis of hyperhomocysteinemia is accompanied by a change in the proteolytic activity in the heart as well as a change in the cytokine profile.

KEY WORDS: hyperhomocysteinemia, proteolytic activity, cytokines

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INTRODUCTION

A number of studies have demonstrated that the elevated level of the amino acid homocysteine is an independent risk factor for cardiovascular diseases [1,2]. Moreover, a high serum homocysteine level is strongly associated with myocardial infarction, atherosclerosis, and thrombosis. Despite numerous scientific efforts, the exact mechanisms of pathological effects of increased homocysteine levels are not yet fully established. The fact that excess homocysteine is harmful to most tissues suggests that the harmful effects of homocysteine are mediated through common fundamental mechanisms. Homocysteine has been shown to induce both oxidative stress and inflammation [3,4]. Among the direct consequences of the accumulation of reactive oxygen species, as well as changes in the cytokine profile, are increased proteolysis and redistribution of proteolytic enzymes. Proteolytic homeostasis undoubtedly plays an important role in maintaining overall cell health. Therefore, the rate of proteolysis must be tightly controlled in order to prevent pathological consequences and the development of the disease. The activation of proteolytic enzymes in the heart is considered an important factor in the progression of heart disease. Given the crucial function of the extracellular matrix (ECM) in maintaining the appropriate structural integrity of the heart, abnormalities in EMC metabolism can trigger changes in myocardial structure that ultimately affect function. Among the enzymes, that involve in the tissue remodeling processes are matrix metalloproteinases (MMPs) – a family of enzymes responsible for the breakdown of extracellular matrix, as well as basal membrane of the vessels [5]. Uncontrolled MMP activation can manifest as a variety of pathological conditions, including rheumatoid arthritis, tumor cell metastasis, periodontal disease, atherosclerosis, and heart disease.

THE AIM

The current study was conducted to assess the distribution of proteolytic activity and cytokine profile in the heart of rats with hyperhomocysteinemia (HM).

MATERIALS AND METHODS

REAGENTS

Thiolactone D,L-homocysteine, tris(hydroxymethyl) aminomethane, o-phenylenediamine, hydrogen peroxide, ethylenediaminetetraacetic acid, phenylmethylsulfonyl fluoride, trichloroacetic acid, and casein were purchased from

Sigma-Aldrich (St. Louis, MO, USA). Anti-rat monoclonal antibodies to TNFα, INFγ, IL-1b, IL-4, IL-6, IL-8, IL-10, MMP-1, -2, -3, -8, -3/10, and TIMP-1 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

A total of 60 albino non-linear male rats was used in the study. All experiments on animals were performed in the 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 experiments were started after 7 days of animal acclimation in the animal facility of Taras Shevchenko National University of Kyiv, maintained under constant conditions of temperature (22 ± 3°C), humidity (60 ± 5 %), and light (12 h light/12 h dark cycle). Standard rodent food and water were provided *ad libitum*. The animals of different ages were used in the current study – one-month-old rats that are corresponded to young animals; six-month-old rats that are corresponded to adult animals, and twenty-month-old rats that are corresponded to old animals. Hyperhomocysteinemia was induced by intragastric administration of DL-homocysteine thiolactone diluted in 1 % starch solution (100 mg·kg^{-1} of body weight), ones per day for 28 days [6]. The control rats were received an equal volume of 1 % starch. HM development was confirmed by the high blood level of homocysteine (more than 15 μmol·L^{-1}). The level of homocysteine in the blood plasma was determined by enzyme-linked immunosorbent assay using the kit «Homocysteine EIA» (Axis-Shield, UK). On the 29th day since the start of the experiment, animals were killed. Thus, there were 3 experimental groups each of them consists of control rats (ten animals) and rats with HM (ten animals): 1) Group #1 (young animals); 2) Group #2 (adult animals); and 3) Group #3 (old animals).

HEART SAMPLE PREPARATION

The heart was immediately collected after the animals have been killed. The tissue (1 g) was homogenized in 9 mL ice-cold 50 mM Tris-HCl buffer (pH 7.4) and further centrifuged at 12 000 g for 30 min at 4°C. The supernatant was collected and stored at 80°C for further biochemical analysis. The protein concentration was determined by the Bradford method [7].

DETERMINATION OF TOTAL PROTEOLYTIC ACTIVITY, ACTIVITY OF METAL-DEPENDENT AND SERINE PROTEASES

Total proteolytic activity was measured using casein as a substrate according to the method [8]. The activity of metal-dependent and serine proteases was assessed using corresponding inhibitors – ethylenediaminetetraacetic acid for the estimation of metal-dependent enzymes and phenylmethylsulfonyl fluoride for the estimation of serine proteases. Both inhibitors were used at the final concentration of 5 mM. The tested samples were preincubated with inhibitor for 30 min at 4°C and then the remaining enzyme activity was estimated using casein as a substrate.

MMPS, TIMP-1, AND CYTOKINES IMMUNOASSAY

MMPs, tissue inhibitor of metalloproteinases (TIMP-1), and cytokines measurements were done by enzyme-linked immunosorbent assay (ELISA) according to the standard instructions [9]. ELISA plates were coated overnight at 4°C with samples of thyroid gland previously diluted with Tris–HCl buffer (pH 7.4) to obtained concentration of proteins 10 μg·mL^{-1}. After being washed, plates were blocked with 5 % nonfat dry milk for 1 h at 37°C and washed again. Next plates were incubated for 1 h at 37°C with specific primary antibodies against the cytokines (IFN-g, IL-1b, IL-12, IL-4, IL-10), MMPs (-1, -2, -3, -8, 3/10), and TIMP-1. Plates were washed and incubated for 1 h at 37°C with corresponding secondary antibodies conjugated to horseradish peroxidase. After washing, substrates (o-phenylenediamine and hydrogen peroxide) were added. The reaction was stopped by addition of 2.5 N H_2SO_4. Plates were read at 492 nm by a microplate reader (mQuant™, BioTek Instruments, Inc).

STATISTICAL ANALYSIS

The data of biochemical estimations were reported as mean±SEM for each group (n = 10). Statistical analyses were performed using one-way analysis of variance (ANOVA). Differences were considered to be statistically significant when $p < 0.05$.

RESULT

First, the total proteolytic activity in the heart of HM rats was assessed. The obtained results showed an increase in this parameter in the rats of all studied groups (Table I). The total proteolytic activity increased by 1.88-fold, 2-fold, and 2.23-fold in the heart of HM rats of Group #1, Group #2, and Group #3, respectively. The distribution of proteolytic activity in the heart of rats with HM was also assessed. It was found that HM is accompanied by an increase in the fraction of metal-dependent enzymes in the heart; the change in the level of metal-dependent enzymes was more pronounced in the group of old animals (22 % in control vs 58 % in HM rats) (Fig. 1).

The present findings showed an increase in the level of MMPs in the heart of HM rats of Group #1 – the level of MMP-1, -2, -3, and -3/10 was higher than the corresponding control values by 25 % ($p<0.05$) while the level of MMP-8 was increased by 15 % ($p<0.05$) (Table II). Analysis of MMP levels in the HM rats of Group #2 has revealed a less pronounced change in this parameter.

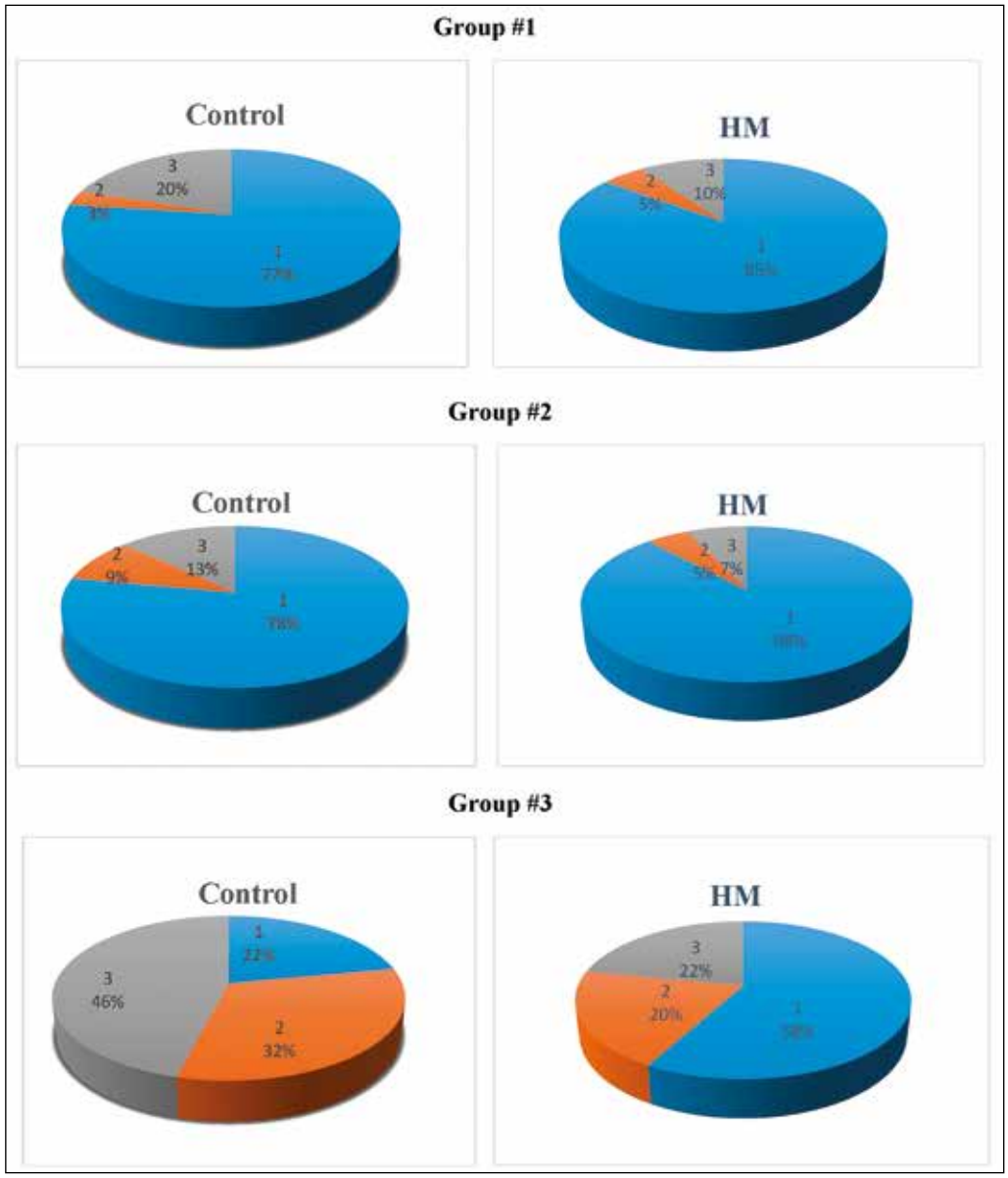

Fig. 1. The distribution of proteolytic activity in the heart of rats with hyperhomocysteinemia: 1 – metal-dependent proteases; 2 – serine proteases; 3 – other proteases

Table I. Total proteolytic activity in the heart of rats with hyperhomocysteinemia

	Group #1		Group #2		Group #3	
	Control	HM	Control	HM	Control	HM
rel.units·g-1 of tissue	3.17±0.21	5.99±0.03*	4.52±0.04	9.43±0.02**	2.13±0.01	4.77±0.03***

Values are expressed as mean ± SEM (n = 10); *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

Thus, the level of MMP-1, -2, and -3/10 increased by 21 % (p<0.05), 17 % (p<0.05), and 15 % (p<0.05), respectively. In contrast, the levels of MMP-3 and MMP-8 were within control values. The most significant changes in the levels of MMPs in Group #3 were detected for MMP-8 – the level of this enzyme was 27 % (p<0.05) higher than in the corresponding control group. According to the obtained data, the level of TIMP-1 in the rats of Group #1 increased by 29 %. The opposite situation was observed in the rats of Group #2 and #3 – the level of inhibitor decreased by 35 % (p<0.05) and 7 % (p<0.05), respectively.

At the same time, the level of anti-inflammatory cytokines in the heart of HM rats was also increased. Thus, the levels of IL-4 and IL-10 increased by 42 % (p<0.05) and 14 % (p<0.05), respectively. Analysis of the cytokine profile in the rats of Group #2 has revealed an increase in the concentration of TNFα by 12 % (p<0.05); other cytokine levels were at the control values. The levels of TNFα and IL-6 in the heart of rats of Group #3 increased by 20 % (p<0.05); the levels of IL-1b and IL-8 were also higher than those in the control group. No significant changes in the level of anti-inflammatory cytokines IL-4 and IL-10 were detected in the rats of Group #3.

Table II. Level of MMPs and TIMP-1 in the heart of rats with hyperhomocysteinemia

		MMP-1	MMP-8	MMP-2	MMP-3	MMP-3/10	TIMP-1
		rel. units·mg-1 of proteins					
Group #1	Control	80.0±4.0	95.0±4.7	180.0±9.0	140.0±7.0	180.0±8.5	170±8.0
	HM	100.0±5.0*	110.0±5.5*	225.0±9.5*	175.0±6.5*	230.0±9.5	220.0±8.0*
Group #2	Control	140.0±5.5	140.0±5.0	285.0±10.5	250.0±10.5	315.0±10.0	340.0±11.0
	HM	170.0±8.5**	135.0±5.5	335.0±15.0**	235.0±8.5	365.0±16.5**	305.0±15.0
Group #3	Control	160.0±6.0	165.0±6.0	315.0±10.5	265.0±10.0	350.0±15.5	360.0±16.0
	HM	190.0±8.5***	210.0±9.5***	345.0±12.5***	280.0±10.5	370.0±16.5	315.0±12.0***

Values are expressed as mean ± SEM (n = 10); *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

Table III. Level of cytokines in the heart of rats with hyperhomocysteinemia

		Pro-inflammatory cytokines					Anti-inflammatory cytokines	
		TNFα	INFγ	IL-1b	IL-6	IL-8	IL-4	IL-10
		rel.units·mg -1 of proteins						
Group #1	Control	100.0±5.0	100.0±5.0	85.0±3.5	105.0±5.0	150.0±7.5	130.0±6.5	175.0±8.7
	HM	135.0±6.7*	105.0±4.5	100.0±4.5*	115.0±4.5*	195.0±8.7*	185.0±6.5*	200.0±9.5*
Group #2	Control	165.0±5.5	140.0±6.5	140.0±6.5	155.0±6.5	300.0±10.0	265.0±8.5	290.0±14.5
	HM	185.0±8.0**	135.0±6.5	130.0±6.0	150.0±7.5	295.0±10.0	250.0±9.5	300.0±13.0
Group #3	Control	175.0±7.5	145.0±6.0	160.0±7.0	175.0±7.5	305.0±13.5	270.0±9.5	325.0±13.5
	HM	210.0±9.5***	155.0±6.0	185.0±8.5	210.0±7.5***	375.0±16.5***	265.0±8.0	345.0±15.0

Values are expressed as mean ± SEM (n = 10); *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

DISCUSSION

Uncontrolled and excessive proteolysis may cause cardiac dysfunction and even cell death due to necrosis and/or apoptosis. In the current study, we have demonstrated that hyperhomocysteinemia affects proteolytic balance in the heart. This is manifested, first of all, in a significant increase in the total proteolytic activity and the activity of metal-dependent proteases. Taking into account the results on the distribution of proteolytic activity in the heart of rats with HM, particularly, a significant part of metal-dependent enzymes, it seems reasonable to analyze the level of MMP as the dominant part of metal-dependent enzymes present in the heart. The family of MMPs includes a number of zinc-containing endoproteases with different substrate specificity. In order to obtain a comprehensive assessment of the involvement of MMPs in the pathogenesis of HM, the level of MMPs belonging to different groups of enzymes was investigated. Our attention was focused on the collagenases (MMP-1, -8) that degrade fibrillar collagen types I, II, and III; the gelatinases (MMP-2) due to their ability to degrade type IV collagen in basement membranes, and the stromelysins (MMP-3, -3/10) which are active against a broad spectrum of ECM components, including proteoglycans, laminins, fibronectin, vitronectin [5,10]. The synthesis and degradation of collagen is a strictly balanced process, therefore, an increase in the level or/and activity of MMPs in the rats with HM can adversely affect the metabolism of collagen. Given that most of the myocardial collagen fibers consist of collagen types I and III, an increase in the level of MMP-1 and MMP-8 may be one of the reasons for structural and functional changes in the heart under HM. The situation is exacerbated in the case of older animals, for which a decrease in the production of collagen is a physiological process. Taking into account the role of MMPs in the development of cardiovascular diseases, our results can be considered as a poor prognostic criterion for those with HM.

Due to the strong proteolytic potential of MMPs, their concentration is usually maintained at a low level in physiological conditions. MMP activity may be regulated by different mechanisms. The binding of MMPs by endogenous inhibitors is one of the most effective ways to prevent the over-activation of MMPs [11]. Considering TIMP-1 suppresses the activity of most MSMs, the level of this inhibitor in the heart of HM rats was assessed. According to obtained data, an increased level of TIMP-1 in the heart of young rats with HM (Group #1) may indicate the activation of compensatory mechanisms aimed at reducing the level of active enzymes and maintaining homeostasis. In contrast, a decreased level of TIMP-1 and increased levels

of MMPs in older rats (Group #2 and Group #3), are factors leading to the enhancement of proteolysis in the cardiac tissue. Loss of tight control over the activity of MMP can be a driving force for the destruction of the myocardial matrix. This may result in the weakening of cardiac activity in HM conditions.

It is known that a long-term increase in the concentration of blood homocysteine can provoke the development of systemic inflammation [4, 12]. Among the serious consequences of inflammation is an impairment of proteolysis, partly due to an increase in the levels of MMPs, an expression of which is controlled by some inflammatory cytokines. The induction of the synthesis of most MMPs is mediated by pro-inflammatory cytokines such as IL-1, IL-6, TNFα, while other cytokines such as IL-4 or IL-10 have been shown to inhibit the expression of MMP genes. Thus, the revealed increase in the level of cytokines, especially IL-6 and TNFα, in the heart of HM rats may be the cause of an increase in the levels of MMPs. It should be noted that the increase in the level of MMPs can be explained not only by the influence of cytokines but also by a disturbance of oxidative balance. It was found that the metabolism of homocysteine is associated with the formation of reactive oxygen species. These metabolites can directly activate pro-MMPs, as well as activate key transcription factors that affect the expression of MMPs. Obtained data revealed an increase in the level of cytokines in the control animals with age. This may indicate a predisposition of old animals to the development of inflammation in the heart.

CONCLUSION

1. An increase in the total proteolytic activity was observed, which was more pronounced in older animals with HM.
2. The pathogenesis of HM is associated with an increase in the level of MMP-1, -2, -3, -8 in the heart of young animals, MMP-1, -2 in the heart of adult animals, and MMP-1, -2, -8 in the heart of old animals. An increase in the percentage of metal-dependent enzymes was found, especially in the group of old rats with HM.
3. The state of mild inflammation, evidenced by the increased level of some pro-inflammatory cytokines, was revealed in the heart of young and old animals with HM.

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Conflict of interest:

The Authors declare no conflict of interest.

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