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Hyperhomocysteinemia-induced oxidative stress differentially alters proteasome composition and activities in heart and aorta ☆

Faouzia Derouiche [a,b], Christine Bôle-Feysot [c,d], Dalila Naïmi [a], Moïse Coëffier [c,d,e,*]

[a] Laboratory of Microbiological Engineering and Application (team of cellular physiology and molecular biology), Constantine University 1, Constantine, Algeria
[b] Khenchela University, Khenchela, Algeria
[c] INSERM Unit 1073, Rouen, France
[d] Institute for Research and Innovation in Biomedicine, Rouen University, Rouen, France
[e] Rouen University Hospital, Nutrition Unit, Rouen, France

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ABSTRACT

Background and aims: Hyperhomocysteinemia (HHcy) is associated with cardiovascular diseases and is thought to induce endogenous oxidative stress and causes many cellular damages. Proteasome that degrades oxidized and ubiquitinated proteins can regulate the cellular response to oxidative stress. We aimed to investigate whether hyperhomocysteinemia induces oxidative stress and alters proteasome function and composition in heart and aorta tissues of rat.

Methods and results: To create hyperhomocysteinemia, male Wistar rats (Pasteur Institute-Algiers) were received daily intraperitoneal injections of DL-homocysteine (0.6–1.2 μM/g body weight) for 3 weeks. Biomarkers of oxidative stress (malondialdehyde (MDA), protein carbonyl (PC), superoxide dismutase (SOD) and catalase (CAT)) were first measured by biochemical methods and tissue damages by histological sections. Proteasome activities were quantitated using fluorogenic synthetic peptides; ubiquitinated proteins and proteasome subunits expression were then evaluated by SDS PAGE and Western blot analysis. We showed increased MDA and PC but decreased SOD and CAT levels both in plasma, heart and aorta accompanied by histological changes. A significant decrease of proteasome activities was observed in heart, whereas proteasome activity was not affected in aorta. However proteasome composition was altered in both tissues, as the accumulation of ubiquitinated proteins.

Conclusion: Data demonstrated an alteration of the ubiquitin–proteasome system in hyperhomocysteinemia as a result of accumulating oxidized and ubiquitinated proteins in response to oxidative stress. Further studies must be conducted to better understanding mechanisms responsible of proteasome alterations in hyperhomocysteinemia.

1. Introduction

Hyperhomocysteinemia (HHcy), defined as an elevated plasma total homocysteine (Hcy) is a consequence of genetic defects of some enzymes, nutritional deficiencies, methionine rich diet, related of some diseases and some drugs [1,2]. Hcy is an amino acid derived from methionine and containing sulphur. Oxidative stress plays a key role in the pathophysiology of hyperhomocysteinemia and its complications [3]. Previous studies showed that increased level of Hcy altered morphological tissue structure and reduced antioxidant defences in heart and aorta tissues of rats [3,4].

HHcy remains a major risk factor leading to endothelial cell dysfunction and induces apoptotic cell death through reactive oxygen species (ROS) production in endothelial and smooth muscle cells. HHcy also causes accumulation of damaged proteins resulting in modification and alterations of their function [2]. ROS are physiologically generated by metabolic processes or as consequences of injury [5,6]. It has been suggested that HHcy is associated with ROS formation, such as superoxide anion, hydrogen peroxide and hydroxyl radicals which are normally eliminated by antioxidants enzymes [7]. It has been postulated that free radicals and lipid peroxidation products such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), a major end products of lipid peroxidation caused damage to proteins [8].

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* Corresponding author at: INSERM unit 1073, Rouen University, 22 Boulevard Gambetta, 76183 Rouen Cedex 1, France. Fax: +33 2 35 14 82 26.
E-mail address: moise.coeffier@chu-rouen.fr (M. Coëffier).

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Numerous studies have demonstrated that the ubiquitin proteasome system (UPS) plays a pivotal role in the removal of oxidized proteins [9–11]. 26S proteasome is a large, multicatalytic protein complex, composed of a 20S core particle (CP) that carries the catalytic activity and by two 19S regulatory particle (RP). The 20S CP is a barrel-like structure composed of four rings, two identical outer α-rings and two identical inner β-rings. α and β rings are composed each of seven distinct subunits. The catalytic sites are localized to some of the β-subunits. The 19S (RP) is implicated in the recognition of polyubiquitinated substrates [12]. However, the regulation of UPS remains unknown during HHcy.

Thus, the present study aimed to evaluate the regulation of ubiquitin–proteasome system during hyperhomocysteinemia in rats.

2. Material and methods

2.1. Animal model of hyperhomocysteinemia

Male Wistar rats (3 months, 240 ± 10 g), obtained from Pasteur Institute in Algiers, were maintained under controlled conditions. Treated animals were injected intraperitoneally, once a day with DL-homocysteine (Sigma Aldrich) for 3 weeks as previously described [13].

2.2. Plasma and tissue homogenates

Blood was collected 24 h after the last DL-homocysteine injection from the sinus venipuncture of control and treated rats with EDTA treated tubes and centrifuged at 3000 rpm for 20 min at 4 °C. Plasma was immediately aspirated for malondialdehyde (MDA) assay and stored at −80 °C for homocysteine and protein carbonyl groups (PC) analyses. Heart and aorta were homogenized in ice-cold PBS pH 7.4. Homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatants were taken for total protein determination by the method of Bradford using bovine serum albumin (BSA) as standard [14]. Supernatants were rapidly frozen and stored at −80 °C for antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) and PC content measurement, and directly used for MDA assay.

For proteasome assays, tissues were homogenized for 2 min with homogenizer (Janke – Kunkel, Inka-Labotecknik) in ice-cold lysis buffer containing 30 m M Tris, p H 7.2, 1 m M dithiothreitol (DTT), 1% Triton X and then centrifuged at 12,000 rpm for 15 min at 4 °C. Supernatants were immediately frozen at −80 °C after total protein determination by Bradford's method for later proteasome activities assays and supplemented with both 1% protease inhibitor cocktail and phosphatases inhibitors for western blot analysis as previously described [15,16].

2.3. Homocysteine assay

Plasma total homocysteine concentration was assayed by Axis® Homocysteine EIA Kit (IBL International, Germany), an enzyme immunoassay detection, by following manufacturer's instructions.

2.4. MDA assay

The level of malondialdehyde MDA was determined by a previously spectrophotometric described method [17]. MDA levels were expressed as nmol/mg protein.

2.5. Protein carbonyl (PC) assay

PC content was measured using an OxiSelect™ Protein Carbonyl Spectrophotometric Assay Kit (Cell Biolabs, Inc). Results were expressed as nmol PC/mg protein.

2.6. SOD assay

SOD activity was carried out as previously described [18] by using the inhibition of autoxidation of pyrogallol by SOD enzyme. SOD activity was expressed as units per milligram of protein (U/mg protein).

2.7. Catalase assay

CAT activity was measured as previously reported [19] and expressed as units of CAT by mg of protein of enzymatic preparation (U/mg protein).

2.8. Histological analysis

Specimens of abdominal aorta and heart were fixed in 10% formalin solution, dehydrated through a graded series of ethanol solutions and embedded in paraffin. Paraffin longitudinal sections of 5 μm thick were then stained with hematoxylin–eosine (H–E) for histological examination under light microscope.

2.9. Proteasome activities assays

Peptidase activities of the proteasome were assayed using fluorogenic peptides, Suc-LLVY-MAC (Calbiochem) for chymotrypsin-like activity, Boc-LSTR-AMC (Sigma Aldrich) for trypsin-like activity and Z-LLE-MCA (Sigma Aldrich) for caspase-like activity and Z-LLL-MCA (Sigma) for peptidases activity as previously described [15]. Proteasome activities were monitored in a microtiter plate fluorometer Mithras LB940 (Berthold Technologies, Bad Wilbad, Germany) in absence or in presence of 10 μM of proteasome inhibitor MG132 (Sigma Aldrich), the difference between the two values was attributed to proteasome activity expressed in relative fluorescent units (RFU)/60 min/mg protein.

2.10. Western blot analysis of proteasome subunits expression and ubiquitinated proteins

As previously described [16], 25 μg of proteins were added to lauryl dodecyl sulfate buffer (Nu-PAGE LDS 4×) plus 500 mM DTT, boiled at 100 °C for 5 min and then separated on 4–12% sodium dodecyl sulfate polyacrylamide gel (Invitrogen, Cergy-Pontoise, France). After separation, they were transferred to a nitrocellulose membrane (GE Healthcare, Orsay, France) for 1 h at 100 V. The membrane was blocked for 1 h at room temperature with 5% (w:v) non-fat dry milk in Tris-buffered saline (TBS, 10 m mol/l, Tris, pH 8; 150 m mol/l NaCl) plus 0.1% Tween 20 followed by overnight incubation at 4 °C with specific primary antibodies, mouse anti 20S subunit β1, anti 20S subunit β2 (1: 1000, Biomol) or anti β-actin (1:1000, Sigma Aldrich); rabbit anti 20S α1, anti β5, anti 20S core (1:1000, Biomol) or anti ubiquitin (1:1000, Calbiochem). After three washes in TBS/0.1% Tween 20, a 1 h incubation with secondary antibodies, horseradish peroxidase-conjugated goat anti rabbit or anti mouse IgG (1:5000, Santa Cruz Biotechnology) was performed, followed by three additional washes, immunoreactive proteins were revealed by using enhanced chemiluminescence (ECL) detection system (GE Healthcare). Protein bands were quantified by densimetry using Image scanner III and Image Quant TL software (GE Healthcare).

2.11. Statistical analysis

All results were expressed as mean ± SD. Results were compared by student *t*-test, Mann–Whitney test or two way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison

Table 1
Oxidative stress biomarkers.

Parameters	Controls	Treated
Plasma MDA (nmol/ml)	1.56 ± 0.18	2.96 ± 0.51*
Heart MDA (nmol/mg protein)	5.25 ± 1.36	18.09 ± 0.90*
Aortic MDA(nmol/mg protein)	053 ± 026	1.71 ± 0.03*
Plasma PC (nmol/ml)	1.32 ± 0.14	2.69 ± 0.13*
Heart PC (nmol/mg protein)	1.56 ± 0.21	9.50 ± 1.24*
Aorta PC (nmol/mg protein)	0.45 ± 1.10	1.87 ± 0.09*
Heart SOD (U/mg protein)	18.81 ± 2.3	9.22 ± 0.08*
Aorta SOD (U/mg protein)	13.26 ± 1.71	6.78 ± 0.54*
Heart CAT (U/mg protein)	12.64 ± 0.81	5.34 ± 0.38*
Aorta CAT (U/mg protein)	19.88 ± 0.76	12.79 ± 1.36*

Malondialdehyde (MDA) and protein carbonyl (PC) levels were measured in plasma, heart and aorta tissues of rats treated or not with DL-homocysteine during 3 weeks. Superoxide dismutase (SOD) and catalase (CAT) activities were measured in heart and aorta. Values are means ± SE ($n = 5$ in each group).

* $p < 0.05$ vs. controls.

test, as appropriate. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Total homocysteine levels

Plasma homocysteine levels were significantly higher (7.53 fold change) after DL-homocysteine administration as compared to controls (42.2 ± 3.4 vs. 5.6 ± 1.8 μM, $p < 0.05$).

3.2. Oxidative stress biomarkers levels

Biomarkers of oxidative stress, MDA, PC, SOD, CAT in plasma, heart and aorta tissues of control and treated rats are displayed in Table 1. Levels of plasma, cardiac and aortic MDA were

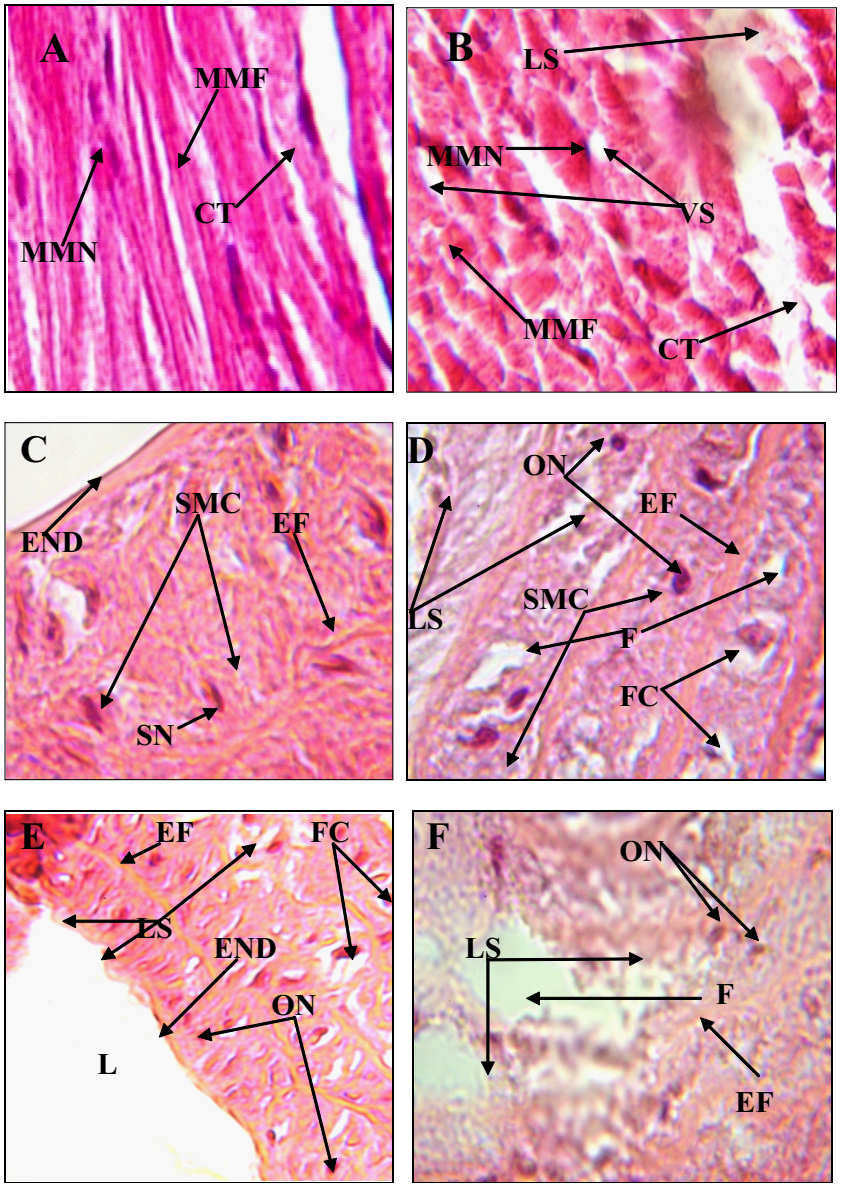

Fig. 1. (A) Longitudinal section of heart control rat, H–E (×400). (B) Longitudinal section of heart rat 3 weeks DL-homocysteine application, H–E (×400). (C) Longitudinal section of abdominal aorta control rat, H–E (×1000). (D), (E), (F) Longitudinal sections of abdominal aorta rat 3 weeks DL-homocysteine application, H–E (×1000), H–E (×400), H–E (×1000) respectively. MMF: muscular myocardial fiber, MMN: muscular myocardial nuclei, LS: lysis, VS: Vacuoles, CT: connective tissue, END: endothelium, SMC: smooth muscle cells, EF: elastic fiber, SN: spindle nuclei, ON: oval nuclei, F: fenestration, FC: foam cell, L: lumen.

significantly increased (1.89, 3.43 and 3.22 fold change, respectively) in DL-homocysteine treated rats as compared to controls. PC content in plasma, heart and aorta in treated rats were significantly higher than in controls (2.03, 6.08 and 4.15 fold change, respectively). In contrast, SOD and CAT activities were significantly reduced both in heart (2.04 and 1.95 fold change, respectively) and aorta (1.86 and 1.55 fold change, respectively) in DL-homocysteine-treated rats.

3.3. Histological investigations

Light microscopy of the heart in control rats showed normal tissue structure (Fig. 1A). In contrast, intracytoplasmic vacuolizations of muscular fiber were observed in the heart of DL-homocysteine treated rats (Fig. 1B). The aorta of control rats had intact endothelium and spindle shape mediocyte nuclei (Fig. 1C). In contrast, formation of foam cells and thick elastic fiber were observed in the aorta of DL-homocysteine treated rats. In addition, smooth muscle cell nuclei changed from spindle to oval nuclei (Fig. 1D–F) and desquamation processes of endothelium was observed (Fig. 1E) as well as elastolysis (Fig. 1F).

3.4. Proteasome activities

Chymotrypsin-like, trypsin-like and caspase-like activities in heart were significantly decreased in treated rats compared with controls (Fig. 2A, D, E). Chymotrypsin-like activity was significantly decreased from 35 min to 90 min incubation with substrate (Fig. 2B). Peptidase activity in heart was unaltered (Fig. 2C). All proteasome activities in aorta were unchanged (not shown).

3.5. Proteasome subunits and total ubiquitinated protein expression

Western blot analysis of subunits proteasome composition demonstrated significant decreases of the expression of 20S α1 subunit, 20S β1 subunit, 20Score and slightly 20S β2 subunit, whereas 20S β5 subunit remained unchanged in heart of DL-homocysteine treated rats as compared to controls (Fig. 3). Aortic 20S proteasome subunits 20S α1, 20S β2, 20S β5 were unchanged (Fig. 4) between both controls. In contrast, 20S core was significantly reduced in DL homocysteine-treated rats compared with controls (Fig. 4). Ubiquitinated protein expression was significantly increased both in heart and aorta (Figs. 3 and 4) of DL-homocysteine treated rats.

4. Discussion

In the present work, we showed that the plasma level of homocysteine was elevated after DL-homocysteine treatment to [42 ± 3 μM]. This value was in the range of pathophysiological concentrations [20–60 μM] found in humans [1] and also in some experimental models [3,13,20]. We thus created a rat model of intermediate hyperhomocysteinemia (HHcy).

Increased MDA and PC levels and decreased SOD and CAT activities observed in Hcy-treated treated rats were in accordance with a previous study [4], even if another study reported increased SOD and CAT levels [13]. Hcy induced oxidative damages both in heart and aorta by increasing lipid peroxidation and oxidized proteins since MDA, a biomarker of lipid peroxidation and PC, a parameter of protein damage were higher. This condition could be related to increasing ROS generated in part by Hcy autoxidation and also may be mainly related to mitochondria that generated more ROS in response to oxidative stress as previously reported [7]. SOD and CAT compose the first antioxidant enzymes responsible for removal of ROS by $O_2^{\cdot-}$ dismutation and H_2O_2 conversion respectively. Thus, inhibition of SOD and CAT activities altered ROS elimination [21]. We supposed that SOD and CAT were targeted for oxidation by free radicals that reduced their activities in hyperhomocysteinemia rats. Excessive production of ROS may induce protein damages resulting in a significant accumulation of oxidized and ubiquitinated proteins observed both in heart and aorta tissues. DL-homocysteine administration leads to cardiac and aortic injury as alteration of the histological structure in these tissues of treated rats were observed, in accordance with previous results [3].

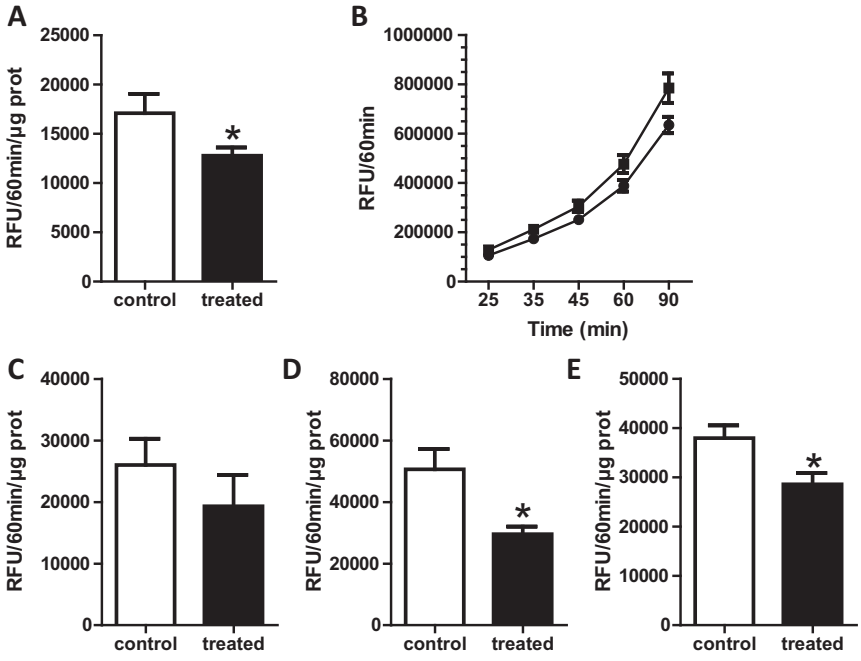

Fig. 2. Effect of DL-homocysteine on proteasome activities in rat heart. Chymotrypsin-like activity (A and B), peptidase (C), trypsin-like (D) and caspase-like (E) activities were evaluated in heart of DL-homocysteine-treated and controls rats (n = 8/group). Values are means ± SE. *, $p < 0.05$ vs. controls.

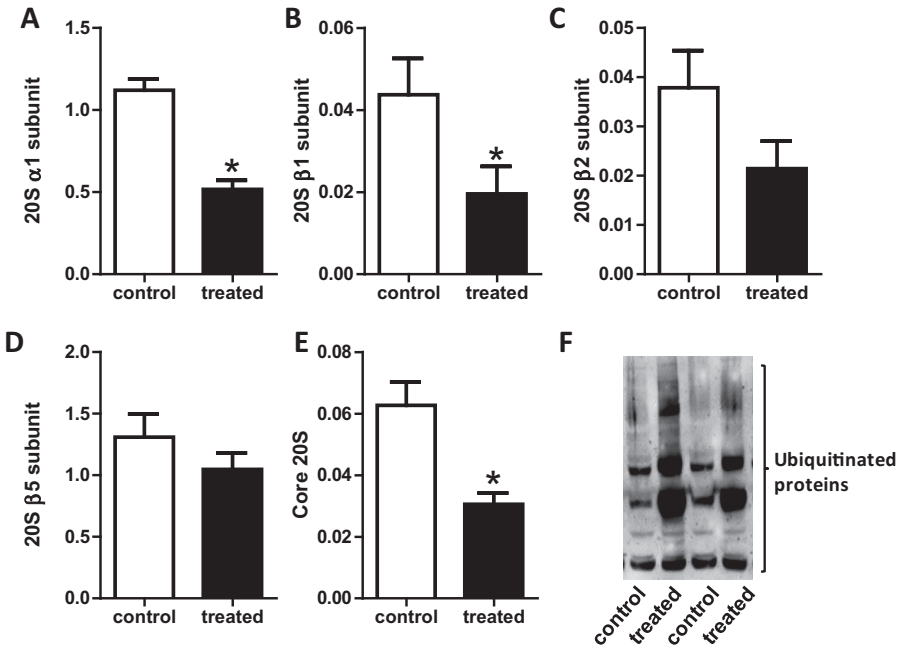

Fig. 3. Effect of DL-homocysteine on proteasome subunit expression in heart. Proteasome subunits, 20S α1 (A), 20S β1 (B), 20S β2 (C), 20S β5 (D) and 20S core (E) and ubiquitinated proteins (F) were evaluated in heart of DL-homocysteine-treated and controls rats ($n = 8$/group). Values are means ± SE. *, $p < 0.05$ vs. controls.

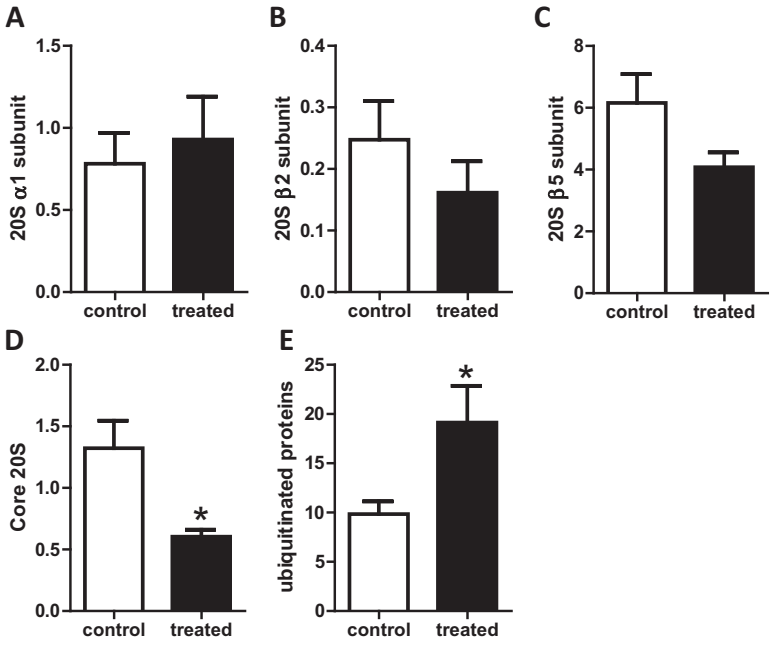

Fig. 4. Effect of DL-homocysteine on proteasome subunit expression in aorta. Proteasome subunits, 20S α1 (A), 20S β2 (B), 20S β5 (C) and 20S core (D) and ubiquitinated proteins (E) were evaluated in aorta of DL-homocysteine-treated and controls rats ($n = 8$/group). Values are means ± SE. *, $p < 0.05$ vs. controls.

Proteasome normally mediated degradation of oxidized and ubiquitinated proteins to prevent their toxicity [9,10,12]. The regulation of proteasome during HHcy has been poorly documented [20] and to our knowledge has not yet been studied in heart and aorta. In the present study, we observed reduction of chymotrypsin-like, trypsin-like and caspase-like proteasome activities in heart of Hcy treated rats. Interestingly, these modifications were associated with decrease of 20S α1, 20S β1, 20S β2 and 20S core subunits expression, while 20S β5 expression remained unchanged. Decreased β1 and β2 catalytic subunits may explain respectively the decrease of caspase-like and trypsin-like activities. In addition decreased of all three activities could also be related to decrease of the regulatory 20S α1 and 20S core subunits expression. It has been reported that reduction of proteasome activity may occur in the appearance of oxidized and ubiquitinated proteins in the cytosol [22]. Thus, inhibition of proteasome could be due to the accumulation of oxidized and ubiquitinated proteins. In contrast, in aorta, we found no significant changes in proteasome activities. In previous paper [20] that reported no activation of proteasome in liver of cystathionine beta synthase deficient

mice developing HHcy. In addition, in aorta of hyperglycemic mice, chymotrypsin-like activity was reduced, whereas the trypsin-like and caspase-like activities remained unchanged [23]. In the present study, 20S core expression was decreased while 20S β5, 20S β2 and 20S α1 subunits expressions were not changed.

Proteasome inhibition in cardiac cells may contribute to accumulation of damaged proteins and in turn would lead to cellular consequences [24]. Further investigations should be conducted to identify oxidized and ubiquitinated substrates of proteasome. In addition, oxidative inactivation of the proteasome has been reported in many cell types and tissues [25,26], but the mechanisms remain poorly documented and should be elucidate.

In conclusion, we reported alteration of 26S proteasome during hyperhomocysteinemia in heart and aorta resulting to an increase of oxidized and ubiquitinated proteins. In these pathophysiological conditions, the role of proteasome inhibition on cardiovascular disorders remains to be elucidated.

Conflict of interest

None to declare.

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