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Homocysteine modulates the proteolytic potential of human vascular endothelial cells

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Abstract

Pathological levels of homocysteine induce a metalloproteinase-dependent degradation of the elastic structures in arterial wall. This elastolytic process is preferentially localized toward the internal elastic laminae and in the first layers of the media, suggesting endothelium could participate in extracellular matrix degradation induced by homocysteine. Therefore, we studied the effects of homocysteine on proteolytic potential of endothelial cells. Human umbilical vein endothelial cells were cultured with concentrations of homocysteine matching human physiological (10 μM) and pathological (50, 100, and 250 μM) plasma homocysteine levels. Pathological levels of homocysteine increased the secretion of elastolytic metalloproteinase-2 and -9, but not of metalloproteinase-3 and -7. Homocysteine also increased the expression of human tissue kallikrein, a potential activator of matrix metalloproteinase-2 and -9, while the expression of urokinase plasminogen activator was not altered. These results suggest vascular endothelial cells could participate in the subendothelial degradation of the arterial elastic structures occurring in hyperhomocysteinemia.

Keywords: Homocysteine; Matrix metalloproteinase; Urokinase; Endothelial cell; Tissue kallikrein; Extracellular matrix

Homocysteine is a physiological non-constitutive amino acid, well known as a risk factor for vascular occlusive diseases on the basis of a large set of epidemiological studies [1,2]. Homocystinuria, the most dramatic form of hyperhomocysteinemia in human, as well as moderate hyperhomocysteinemia lead to early onset atherosclerosis and arterial and venous thrombosis. The mechanisms by which homocysteine exert its atherothrombotic effect are still unclear. Accumulated evidences suggest that endothelium plays a major role in the homocysteine-induced pathological process in arteries through an alteration of its antithrombotic phenotype [3], an impairment of its regulative role in arterial vasorelaxation [4,5], and by acquiring a proinflammatory phenotype [6].

Among the mechanisms described in the progression of hyperhomocysteinemia-associated atherosclerosis, the arterial wall remodeling appears to be a major and early event [7,8]. One of the main features of this process is the degradation of the arterial elastic structures associated with an increase of the metalloproteinase-dependent elastolytic activity [8]. Interestingly, this elastolysis appears to be more intense towards the internal elastic lamina and the first elastic laminae of the media, in severe homocystinuria in human [7,9,10] as well as in experimentally induced mild hyperhomocysteinemia in animal [8,11]. We hypothesized that endothelial cells could participate in the subendothelial proteolytic matrix degradation induced by homocysteine. To address this point, we investigated the alteration of the proteolytic potential of endothelial cells cultured with homocysteine at concentrations matching the physiopathological levels encountered in human.

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We focused on the arterial elastolytic matrix metalloproteinases (MMPs), MMP-2, MMP-9, MMP-3, and MMP-7 [12–14] as well as on urokinase plasminogen activator (uPA) and human tissue kallikrein (hK1), arterial serine proteases known to be involved in the activation of MMPs [15,16]. We demonstrated that homocysteine increases the secretion of MMP-2 and MMP-9 and hK1. These results support the involvement of endothelium in the metalloproteinase-dependent degradation of the arterial elastic structure in hyperhomocysteinemia.

Materials and methods

Cell culture, viability, and proliferation assay. Human umbilical vein endothelial cells (HUVECs) were prepared according to the method of Jaffe et al. [17]. They were maintained in RPMI-1640 medium containing 20% heat-inactivated fetal calf serum (FCS) (Life Technologies, Paisley, United Kingdom), 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 50 IU/ml heparin, and 50 μg/ml endothelial growth supplement (Sigma). HUVECs were selected for experimental use at the first passage and cultured into gelatin-coated culture six-well plate.

Cells were grown to 90% confluency and incubated for 24 h in serum-free RPMI-1640 medium supplemented with homocysteine (DL-homocysteine, Sigma) and 0.5% bovine serum albumin (BSA, Sigma) to protect cells from serum-free apoptosis [18]. Homocysteine was used at 10 μM as control corresponding to physiological homocysteinemia, and 50, 100, and 250 μM corresponding to mild and severe hyperhomocysteinemia. After 24 h, condition medium (CM) was collected and centrifuged. Cells were washed with phosphate-buffered saline and harvested by scraping into 0.1 ml of 0.2% Triton X-100 in 0.1 M Tris–HCl. Cell lysates (CL) and CM were centrifuged for 5 min at 1000 g. Protein content was measured in CM and CL using Bradford method [19].

Cell viability was checked using Annexin-V assay according to the manufacturer's instruction (Annexin-V FITC Kit, Beckman). HUVECs cultured without homocysteine were used as positive controls. HUVECs cultured in a medium starved of FCS and BSA were used as reference for cell apoptosis. Cell proliferation was assessed by BrdU incorporation ELISA assay according the manufacturer's instruction (Roche Molecular Biochemicals, Applied Science).

Zymography. CM was submitted to gelatin–Sepharose purification procedure according to the method of Mazzieri et al. [20]. Proteolytic activities were checked in CM and CL by gelatin, casein, and casein/plasminogen zymography as previously described [21–23]. Gelatin type A (1 mg/ml, Sigma), β-casein (1 mg/ml, Sigma) and α-casein (1 mg/ml, Sigma)/plasminogen (20 μg/ml, generous gift from Victor Gurewich, Boston, MA) were, respectively, embedded as substrate in 8.5%, 12%, and 10% SDS–polyacrylamide gel. Prestained rainbow molecular weight markers (Amersham–Pharmacia Biotech) were also run on each gel. Conditioned medium of HT1080 cells and uPA (generous gift from Victor Gurewich, Boston, MA) were used as standard. The nature of the proteases was assessed by using protease inhibitors: 1,10-phenanthroline (PHE), 1 mM, phenylmethylsulfonyl fluoride (PMSF), 1 mM, iodoacetamide (IA), 100 μM, and pepstatin A (PEPST), 10 μM (Sigma) as MMP-, serine-, cysteine-, and aspartate-protease inhibitors, respectively. After staining, gelatinolytic activities were quantified by densitometry and normalized relatively to HT1080 activities to allow the quantitative comparison between gels. Activities were expressed relatively to those found in HUVECs cultured in the absence of homocysteine.

Western blotting. CM and CL were electrophoresed under non-reducing conditions as previously described [24]. Proteins were transferred onto PVDF membranes (Millipore) in transfer buffer containing 192 mM glycine, 25 mM Tris, 0.01% SDS, and 10% methanol. Non-specific binding sites were blocked overnight at 4 °C in 3% non-fat milk. Membranes were incubated for 1 h with mouse anti-human monoclonal primary antibodies directed against MMP-2 (Ab-3) (1/200) (Oncogene Research Products) and rabbit antiserum monospecific for human true tissue kallikrein (anti-hK1, 1/2000) (Calbiochem) in 0.5% non-fat milk and then with goat anti-mouse (1/2000) (Chemicon International), anti-rabbit (1/4000) (Santa Cruz, sc-2004), respectively, peroxidase conjugated IgG for a further 1 h. After washing, blots were incubated with Supersignal chemiluminescent substrate (Pierce) and exposed to Kodak Biomax Light-1 film for 1–5 min. Multiple exposures were examined to ensure that the results reflected those produced in the linear range of the film. Primary antibodies were omitted for negative control experiments.

Statistical analysis. Data were analyzed by one-way ANOVA followed by Student–Newman–Keuls test for multiple comparisons. Results are expressed as means ± SD. A value of $p < 0.05$ was considered significant.

Results

Cell viability and proliferation

The viability of HUVECs cultured with homocysteine (10–250 μM) for 24 h was over 95% and the cells remained in a quiescent state (data not shown).

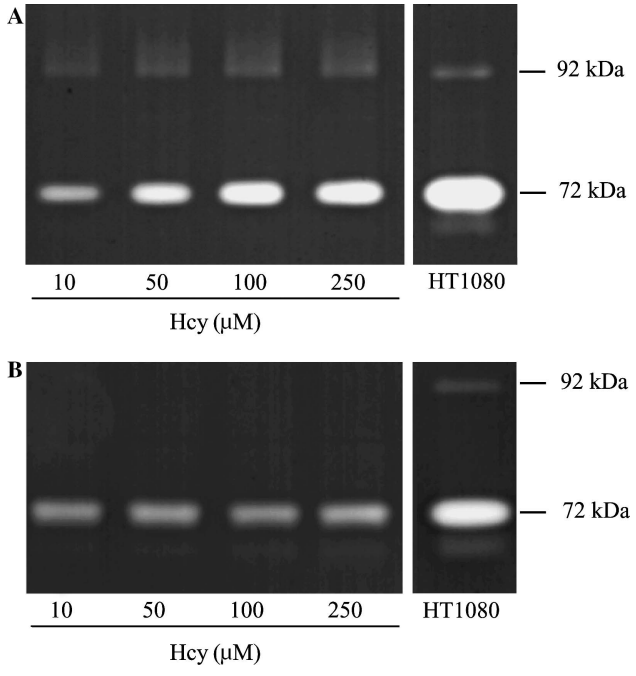

Fig. 1. Gelatin zymography of purified conditioned medium (A) and cell lysate (B) from HUVECs cultured with 10, 50, 100, and 250 μM homocysteine. Conditioned medium of HT1080 cells was used as internal standard. Latent forms of MMP-2 (72 kDa) and MMP-9 (92 kDa) were detected in conditioned medium while only latent form of MMP-2 was detectable in cell lysate. Each zymogram is representative of six independent experiments.

These results indicate that physiopathological concentrations of homocysteine had no significant apoptotic or proliferative effect on HUVECs after 24 h of culture.

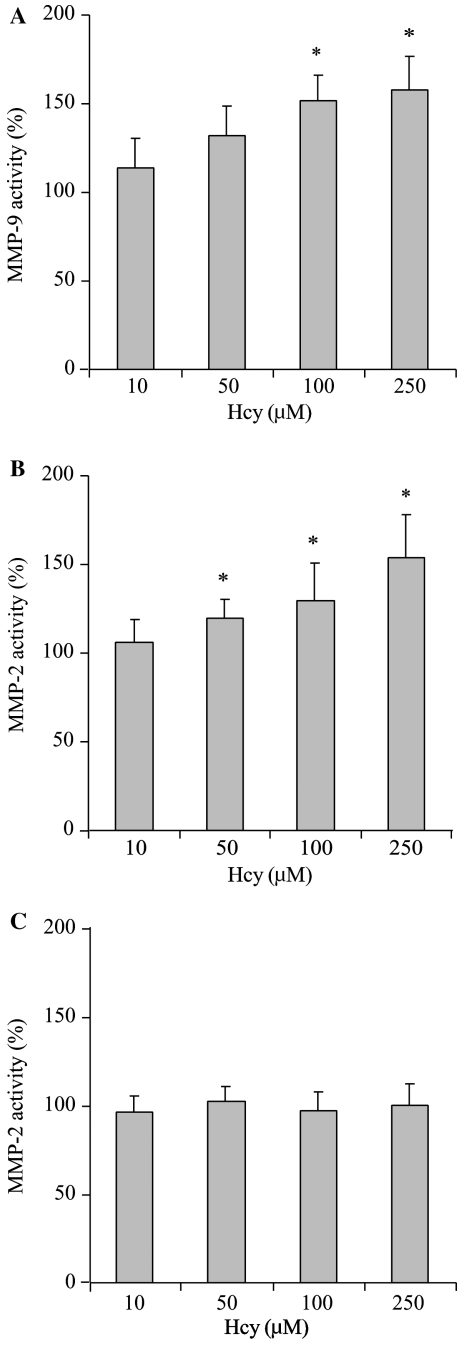

Fig. 2. Homocysteine increases MMP-2 and MMP-9 produced by HUVECs. Gelatinolytic bands were quantified by densitometry analysis. Specific gelatinolytic activities of MMP-9 (A) and MMP-2 (B,C) were expressed as a percentage of the specific activities of HUVECs cultured in absence of homocysteine. Values are means ± SD of six independent experiments. Both MMP-9 (A) and MMP-2 (B) activities were significantly increased in conditioned medium from HUVECs cultured with 100 and 250 μM homocysteine; $*p < 0.05$ versus control HUVECs (10 μM). In cell lysate (C) MMP-2 activity was unchanged.

Homocysteine altered MMP pattern produced by HUVECs

MMP-2 and MMP-9 were examined by gelatin zymography in both CM and CL. Two major gelatinolytic bands were detected at molecular weights corresponding to the latent forms of MMP-9 (92 kDa) and MMP-2 (72 kDa) in CM (Fig. 1A) whereas only one gelatinolytic band at 72 kDa was detected in CE (Fig. 1B). These gelatinases were confirmed as metalloproteinases by inhibition with PHE but not with PMSF, IA or PEPST (results not shown). Densitometric quantification showed a significantly increased expression of the 92 kDa (Fig. 2A) and 72 kDa (Fig. 2B) gelatinases in CM from HUVECs cultured with 50, 100, and 250 μM homocysteine compared to

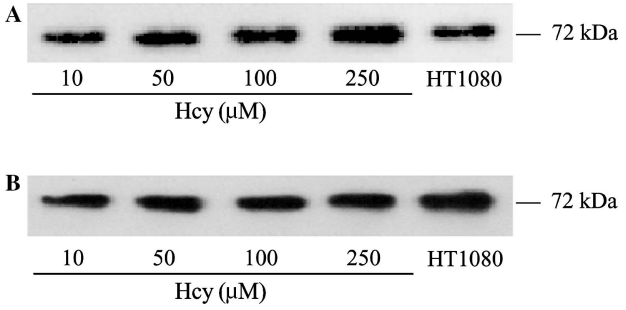

Fig. 3. Western blot analysis of conditioned medium (A) and cell lysate (B) from HUVECs cultured with 10, 50, 100, and 250 μM homocysteine. Conditioned medium of HT1080 cells was used as internal standard. MMP-2 levels were increased in conditioned medium from HUVECs cultured with 50, 100, and 250 μM homocysteine compared to control HUVECs (10 μM), but were unchanged in cell lysate. Each Western blot is representative of four independent experiments.

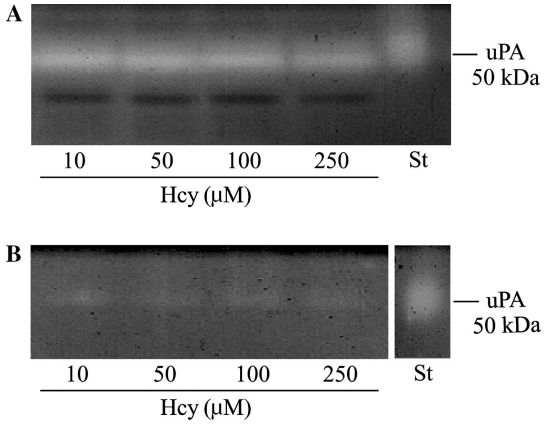

Fig. 4. Homocysteine does not alter uPA produced by HUVECs. Casein-α/plasminogen zymography of cell lysates (A) and conditioned medium (B) from HUVECs cultured with 10, 50, 100, and 250 μM homocysteine was performed. An urokinase standard (St) was run in each zymogram. Each zymogram is representative of four independent experiments.

control HUVECs. In contrast, 72 kDa gelatinase expression was not significantly altered in CL (Fig. 2C) and 92 kDa gelatinase remained undetectable (Fig. 1B). The expression of 72 kDa latent MMP-2 was confirmed by Western blotting in CM and CL from HUVECs cultured with 50, 100, and 250 μM homocysteine and control HUVECs (Fig. 3).

No caseinolytic metalloproteinase activity was detected in casein zymography at any molecular mass corresponding to active or latent form of MMP-3 or MMP-7 either in CM or CL from HUVECs cultured with homocysteine (data not shown).

Homocysteine altered serine-protease pattern in HUVECs

Both CL (Fig. 4A) and CM (Fig. 4B) from HUVECs cultured with 50, 100, and 250 μM homocysteine exhibited similar levels of uPA to those observed in control HUVECs cultured with 10 μM homocysteine as determined by casein-α/plasminogen zymography.

Casein zymography showed in CL a caseinolytic band at 27 kDa corresponding to human tissue kallikrein (Fig. 5A). This caseinolytic activity was confirmed as a serine protease by inhibition with PMSF but not with PHE, IA or PEPST (Fig. 5B) and characterized as hK1 by Western blotting (Fig. 6). A second immunoreactive band was detected at 75 kDa using the monospecific anti-hK1 antibody. This predominant 75 kDa form of hK1 was not detectable in casein zymography and was likely an inactive complex form of hK1 with an inhibitor (Fig. 6). Both active 27 kDa form and inactive 75 kDa complex forms of hK1 were increased in CL from HUVECs cultured with 50, 100, and 250 μM of homocysteine relative to control HUVECs (Figs. 5A and 6).

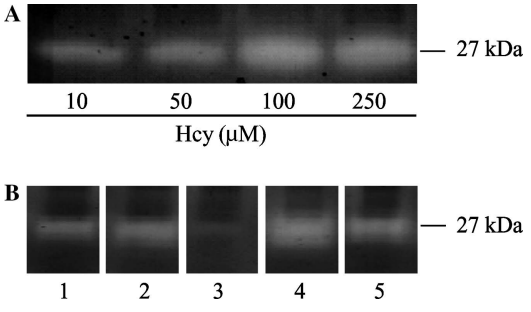

Fig. 5. Casein-β zymography of cell lysate from HUVECs cultured with 10, 50, 100, and 250 μM homocysteine. (A) A caseinolytic band at 27 kDa corresponding to hK1 was detected in cell lysate from control HUVECs and was increased in cell lysate from HUVECs cultured with 50, 100, and 250 μM homocysteine. (B) 27 kDa caseinolytic protease was inhibited by PMSF and confirmed as a serine protease. Identical gels were incubated in the presence of protease inhibitor: (1) absence of inhibitor, (2) MMP inhibitor (PHE, 1 mM), (3) serine-protease inhibitor (PMSF, 1 mM), (4) cysteine-protease inhibitor (IA, 100 μM), and (5) aspartate-protease inhibitor (PEPST, 10 μM). Each zymogram is representative of six independent experiments.

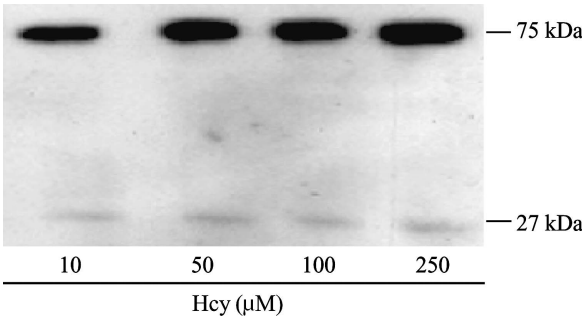

Fig. 6. Homocysteine increases hK1 expressed by HUVECs. Western blot analysis of cellular extracts from HUVECs cultured with 10, 50, 100, and 250 μM homocysteine was performed. Antibody monospecific for human hK1 recognized a 27 kDa form of hK1 as well as a predominant 75 kDa complex form of hK1. Both 27 and 75 kDa forms were increased in cell lysate from HUVECs cultured with 50, 100, and 250 μM homocysteine compared to control HUVECs (10 μM). Western blot is representative of four independent experiments.

Discussion

In this study, we showed that homocysteine at pathological levels altered the proteolytic potential of endothelial cells (HUVEC). HUVECs were cultured with homocysteine at concentrations matching plasma levels encountered in human hyperhomocysteinemia (50–250 μM). In these conditions, HUVECs remained in a quiescent state whereas their proteolytic pattern was altered. High levels of homocysteine significantly increased the secretion of the latent forms of MMP-9 (92 kDa) and MMP-2 (72 kDa) as well as the expression of human tissue kallikrein (hK1).

An intense extracellular degradation was reported in arteries from homocystinuric human [7,9] as well as from animals with mild hyperhomocysteinemia [11,25]. This remodeling was dominated by a severe metalloproteinase-dependent elastolytic process [8,26], particularly marked toward the internal elastic lamina and in the first layers of the media, nearby the endothelium [7,8]. We suggest that endothelial cells could participate in this subendothelial matrix degradation induced by homocysteine through the increased expression of MMPs as well as through MMP-activating serine proteinases.

ProMMP-2 was constitutively expressed in control endothelial cells cultured with a physiological level of homocysteine (10 μM). In the same conditions, proMMP-9 was weakly expressed while the active forms of both enzymes were undetectable. Neither latent nor active forms of MMP-7 and MMP-3 were detected in control HUVECs. This MMP pattern closely matched

the basal pattern previously reported in non-atherosclerotic arteries [27] as well as in culture of endothelial cells derived from macrovasculature [28]. Pathological levels of homocysteine (100 and 250 μM) markedly increased the secretion of proMMP-9 and proMMP-2, while MMP-3 and MMP-7 were not altered and remained undetectable.

It has been previously reported that high experimental concentrations of homocysteine (5 mM) decreased the secretion of MMP-2 by bovine aortic endothelial cells (BAEC) in culture [29]. In addition, homocysteine was shown to inhibit the gelatinolytic activity of both APMA-preactivated purified MMP-2 and APMA-preactivated purified MMP-9 at high molar ratio homocysteine:MMP in the range 10^3:1–10^4:1 [30,31]. Together, these results support the antiangiogenic effect of high levels of homocysteine described in endothelial cells in culture [29]. In contrast, we showed that pathology-matching levels of homocysteine (less than 250 μM) increased the expression of both proMMP-2 and proMMP-9 in cultured human umbilical vascular endothelial cells (HUVECs). Moreover, homocysteine was shown to activate proMMP-2 through a non-proteolytic process at low molar ratio homocysteine:MMP 10:1 [30]. Such discrepancy in homocysteine effects is not surprising since other divergent effects have already been reported for prostacyclin synthesis by endothelial cells or for DNA synthesis in human vascular smooth muscle cells according to the concentrations of homocysteine used in culture. Thus, the present results are in favor of an increase of the MMP-dependent proteolytic potential of endothelial cells by pathological levels of homocysteine.

The overexpression of MMP-2 and MMP-9 by endothelial cells is likely one of the major processes participating in the homocysteine-induced arterial elastolysis. In arteries cultured with pathological levels of homocysteine, an increased expression of MMP-2 and MMP-9 was associated with a dramatic degradation of the elastic structures in the subendothelial part of the media (unpublished data). Both MMP-2 and MMP-9 are well known to degrade elastin in vitro [12,14] and were also reported to degrade the tissue elastic fibbers in human skin ex vivo [32]. Moreover, elastin can activate proMMP-2 (contact autoactivation) resulting in its own elastolysis [33]. Therefore, endothelial cells could directly participate, through elastolytic MMP-2 and MMP-9, in the metalloproteinase-dependent elastolytic process occurring in the subendothelial areas of hyperhomocysteinemic arteries.

On an other hand, endothelial cells could participate in the driving of the homocysteine-induced metalloproteinase-dependent damages by modulating the elastolytic MMP activity through other proteases, especially serine proteases. Urokinase plasminogen activator (uPA) is known to generate active forms of MMP-2 and MMP-9 through the proteolysis of their latent forms [15,34]. Nevertheless, the constitutive basal expression of uPA by HUVECs was not altered by pathological levels (50–250 μM) of homocysteine. In addition, Rodriguez-Nieto et al. [29] reported that uPA expression by BAEC was inhibited by high experimental concentrations of homocysteine (5 mM). Thus, uPA is unlikely to act in the driving of the homocysteine-induced metalloproteinase dependent arterial elastolysis.

Human tissue kallikrein was detected in control HUVECs cultured with 10 μM homocysteine. It consisted of a 27 kDa form exhibiting proteolytic activity against casein, and a dominant 75 kDa form undetectable by casein zymography. It is now well established that kallikreins are post transcriptionally modulated by endogenous tissue kallikrein inhibitors, such as kallistatin [35], so that this predominant high molecular weight band was likely a covalent complex form of hK1. Recent works reported that human endothelial cells constitutively expressed tissue kallikrein in culture as well as in arteries [36,37]. Both active 27 kDa and complexed-75 kDa forms of hK1 were markedly increased in HUVECs cultured with pathological levels of homocysteine (100 and 250 μM). In a model of porcine endothelial cell in culture, Desrivieres et al. [16] demonstrated that hK1 is an efficient activator of MMP-9 and suggested that hK1 could be an important physiological activator of MMP-9 in vivo. In addition, hK1 was suggested to be involved in tissue activation of MMP-2 through the release of bradykinin, since a decrease of MMP-2 activity has been described in mice knockout for bradykinin B2 receptors [38]. Thus, hK1 could be a driver of the metalloproteinase-dependent elastolytic process occurring in the subendothelial areas of hyperhomocysteinemic arteries through the activation of MMP-2 and MMP-9.

In this study we showed that pathological levels of homocysteine altered the proteolytic potential of endothelial cells in culture. These results support the involvement of endothelium in the metalloproteinase-dependent degradation of the arterial elastic structures in hyperhomocysteinemia through the increased expression of elastolytic MMP-2 and MMP-9 as well as of hK1 as potential activator of MMP-2 and MMP-9. Further experiments are required to confirm the overexpression of these endothelial proteases in hyperhomocysteinemic arteries in vivo and to shed light on their respective role in the homocysteine-induced degradation of the elastic structure.

Acknowledgments

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