Homocysteine induces synthesis of a serine elastase in arterial smooth muscle cells from multi-organ donors

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Abstract

Objectives: In heart transplant recipients with diffuse coronary arteriopathy, we have previously demonstrated the prevalence of elevated homocysteinemia, also known as an independent risk factor for myocardial infarction and stroke. In hyperhomocysteinemic mini-pigs we also observed early detectable pathologic changes in the elastic laminae. We hypothesized that homocysteine causes premature breakdown in the arterial elastic fibers by activation of the elastolytic activities. Methods: We examined the effect of homocysteine on elastase-like production by smooth muscle cells from sub-inguinal arteries of multi-organ donors 23.4 ± 3.4 yr, n = 8. The freshly isolated cells were incubated for 0–72 h with homocysteine 0–250 μM, in the presence or absence of specific protease inhibitors. Results: Homocysteine was devoid of a direct effect, but after 18 h incubation the elastase-like activities increased by 5–6-fold in the extracellular medium. The enzymes were characterized as serine proteases. Incubation of cells with a nucleic acid synthesis inhibitor actinomycin D or a protein synthesis inhibitor cycloheximide suppressed the enzyme induction. Conclusions: This is the first report of serine protease induction by homocysteine in vascular smooth muscle cells. The process may require protein synthesis and account for the early alterations of the arterial elastic structures in heart transplant recipients, and in other hyperhomocysteinemic patients, as well.

Keywords: Homocysteine; Serine protease; Vascular smooth muscle; Heart transplantation; Protein synthesis; Human arteries

1. Introduction

High plasma levels of homocysteine (Hcys), are believed to cause premature occlusive vascular disease and thrombosis. Excessive Hcys diminishes endothelium thrombo-resistance processes and induces endothelial injury. In vascular smooth muscle cells (VSMC), Hcys increases DNA synthesis, induces cyclin A gene expression and favors the release of mitogenic factors, leading to cell proliferation and migration [1–6]. Men and women with coronary artery disease [7], and young myocardial infarction patients [8], have significantly higher Hcys plasma levels than controls and this elevation is independent of traditional risk factors. In heart transplant recipients, we have shown the prevalence of high homocysteinemia prior to the development of obstructive coronary arteriopathy [9]. The lesions due to Hcys are clinically silent, and presumably in the presence of atherosclerosis-associated risk factors, they progress to advanced atherosclerotic lesions, the major cause of late mortality in the patients, through mechanisms which remain unidentified.

In hyperhomocysteinemic patients and mini-pigs, the lesions of the vascular wall are characterized by fibrous thickening of the intima, fragmentation of the internal elastic lamina, fraying and splitting of muscle and elastic fibers of the media, without alteration of the de novo elastin synthesis [1,3,10–13]. The Hcys-induced alterations are currently thought to result from impairment of the action of lysyl oxidase responsible for native collagen and elastin cross-linkings which in turn causes elastin and...
collagen fragmentation [1]. The morphological assessment of ‘breaks’ in the elastic lamina increased in the human aorta, as the elastolytic activity increases [14], linearly with the grade of atheroma and exponentially with age [15,16].

In vivo and in vitro, serine protease inhibitors reduced the extent of the disease with a limitation of elastic laminae fragmentation [17,18]. Finally, an inverse causal correlation was postulated between serine elastase activity and elastin fragmentation [14,19,20]. From these observations, we hypothesized that Hcys activates the degradation pathway of elastin [14].

2. Methods

2.1. Isolation of native vascular cells

The sub-inguinal arteries were obtained from donor heart transplants (22 ± 5 yr, n = 8) after cerebral death, under strict compliance with ethical recommendations. The donors were without previously identified vascular disease, and gross and microscopic pathologic observations revealed that the samples were from unscathed arteries [21]. The investigation conforms with the principles outlined in the Declaration of Helsinki. The arteries were extensively washed with ice-cold RPMI 1640, to remove contaminating blood cells. As previously described [22], endothelial cells were removed within 20 min by percolation with collagenase D–hyaluronidase mixture. Then, the arterial fragments were cut into small pieces, and within 3 h the vsmc were entirely dispersed into single cells. Tissue fragments were placed into 3 mg/ml Boehringer collagenase D in RPMI 1640 under gentle shaking at 37°C for 1 h, followed by 0.5 mg/ml elastase (pancreatopeptidase E, Sigma) for a further hour. The incubation mixture was filtered and the remaining fragments were again placed in the collagenase D–elastase mixture for 1 h. Suspensions of VSMC were pooled, washed twice (1000 × g for 5 min), resuspended, counted and finally diluted to 10⁶ cells/ml in RPMI 1640 containing penicillin–streptomycin antibiotics. VSMC had a typical appearance and more than 95% excluded trypan blue. In late experiments, the final VSMC were pooled, washed twice, and resuspended in RPMI at the usual concentrations.

2.2. VSMC incubations

In 6-well tissue culture plates (Beckton-Dickinson), 2 × 10⁶ cells were incubated for 18 h at 37°C under controlled atmospheric conditions, with 0–250 μM Hcys (Sigma Chemicals, St. Louis, MO, USA). FCS was omitted to prevent inhibition of elastolytic activities. Therefore the VSMC kept their microscopic appearance and did not attach. When required and before Hcys addition, 10 μl of RPMI containing cycloheximide (10 μg/ml final concentration) or actinomycin D (3 μg/ml final concentration) (Sigma Chemicals) were added to the incubation suspension. At the end of the incubation period, the suspensions were collected from the dishes (observed under the microscope), centrifuged at 1000 × g for 15 min at 4°C. Supernatants were collected, and pellets were washed once in cold RPMI. The final pellet was resuspended in 0.8 ml buffer (0.3 M sucrose, 1 mM EDTA, pH 7.4), and treated in a Dounce homogenizer prior to adding 0.4 ml buffer (0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, 0.05% Brij). Extracellular medium and homogenates were processed for measurement of elastase-like activities within 1 h following collection.

2.3. Assay of elastase-like activities

Assays with N-succinyl trialanyl-Nitroanilide (SANA) and N-methoxy succinyl-Ala–Ala–Pro–Val-Nitroanilide (NMSN) were performed according to the method of Bieth et al. [23], as modified by Osborne-Pellegrin et al. [24]. Briefly, 50 and 100 μl aliquots of VSMC extracellular medium and homogenate were incubated at 37°C in triplicate, in 1 ml final volume of 0.1 M Tris-HCl, pH 8.0, 0.5 M NaCl, 0.05% Brij with either 20 μl of SANA (125 mM in N-methylypyrrolidone) or 10 μl NMSN (1 mM in N-methylypyrrolidone). At different incubation times (1, 3, 6, 9, 12, or 24 h) the substrate hydrolysis was measured at 410 nm using a Uvicod 860 spectrophotometer (Roche-Kontron). The linearity of the reaction was checked for time, substrate and protein concentrations. When required, a protease inhibitor (100 mM PMSF in DMSO, 10 mM E64 in water, 100 μg/ml pepstatin in DMSO, 100 mM 1,10-phenanthroline in DMSO) was added (10 μl) to the assay medium. Proteins were determined using the Bio-Rad protein assay, with BSA as a standard.

2.4. Statistics

The results were expressed as the mean ± s.d. of nmol hydrolyzed per hour per 10⁶ cells. Statistical analysis were performed by using the t-test. A value of P < 0.05 was considered to be statistically significant.

3. Results

3.1. Cell viability and purity

Preliminary experiments showed that VSMC viability, as evaluated by a dye exclusion test, phase-contrast and scanning electron microscopy, was not significantly affected by incubations up to 24 h at moderate Hcys concen-
trations (data not shown). In contrast, viability decreased by 5 and 10% after 48 h incubation with and without Hcys, respectively, and was further lowered by 25% for concentrations up to 100 μM. The purity of VSMC were attested by α-actin labelling (data not shown). In addition, contamination by endothelial cells is not expected to contribute to elastolytic activity because there is relatively little, if any, elastase in these cells [25].

3.2. Elastase-like activity in human VSMC

We measured the elastase-like activity from human VSMC kept in a survival medium devoid of FCS to limit the presence of plasma protease inhibitors. We performed an enzymatic assay at pH 8.0 with extracellular medium or homogenate from VSMC suspensions obtained under conditions which did not allow the cell to enter into a proliferating state [22]. Since a specific synthetic substrate is not ascribed to the VSMC serine elastase, we therefore used two synthetic substrates: SANA specific for pancreatic serine elastase and NMSN specific for leukocyte serine. In this study, the elastase-like activity of human VSMC cleaved SANA (100%) more efficiently than NMSN (80%) (data not shown). Immediately after the isolation procedure, VSMC exhibited low elastase-like activity (4.0 ± 1.7 and 0.66 ± 0.41 μmol/h/10^6 cells in extracellular medium and homogenate, respectively). Linear time-dependent production of elastase occurred from 18 to 72 h (Fig. 1). This argues for enzyme production by the VSMC instead of contamination by the enzymes used during the isolation procedure.

To characterize the enzymes, the activities were measured in the presence of specific inhibitors of the principal protease classes. As shown in Fig. 2, both cellular and extracellular activities from control VSMC were almost completely inhibited by PMSF, indicating that the elastase-like activity was mostly due to serine protease.

3.3. Hcys and elastase-like activity

To determine whether Hcys acts directly on the enzyme, elastase-like activity was measured immediately after addition of Hcys to the assay medium. Results in Fig.
Fig. 3. Dose-dependent activation of elastase-like activity by homocysteine. The elastase-like activity was measured in a suspension of human vascular smooth muscle cells either after immediate addition of homocysteine to the extracellular medium (○), or in the extracellular medium (□) and the cellular homogenate (■) after 18 h incubation of cells with increasing concentrations of homocysteine.

3 indicate a moderate direct activation. In contrast, the enzyme activity peaked after 18 h of incubation, specifically with 50 μM Hcys with a 6-fold increase from basal value. This elastase-like activity had the same characteristics as in the control situation, serine protease in both the homogenate and the extracellular medium (Fig. 2).

The present findings that Hcys induced elastase-like activity of VSMC only after 18 h incubation, whereas the direct effect remained at a low level, strongly support the view that de novo protein synthesis was involved. This hypothesis was confirmed by the total loss of enzyme induction by Hcys when the nucleic acid synthesis inhibitor, actinomycin D, or the protein synthesis inhibitor, cycloheximide, was added to the incubation medium (Fig. 4). On the other hand, freshly isolated VSMC incubated in the presence of trypsin were no longer able to respond to Hcys, indicating the possibility of a membrane step in the induction processes.

4. Discussion

VSMC are thought to contribute to the physiological turnover of elastic fibers by synthesizing elastin, and elastinolytic enzymes characterized mostly as serine proteases [16,19], and also as metallo-proteases [26]. In the present study, low elastase-like activity was observed in the VSMC suspensions during the first few hours after cell isolation. Harvested cells often require an adaptation period [27] which resulted in minimal elastolytic activity within the first 18 h. However, release of a matrix metallo-protease was not detectable in this study, but our cells were kept for at least 48 h and metallo-protease secretion by cells requires a 3-day waiting period before being measurable [28,29].

We investigated immediate and delayed Hcys effects on the elastase-like activity of VSMC freshly isolated from sub-inguinal arteries of multi-organ donors. Hcys was devoid of a significant direct effect, but after an 18 h incubation period the cells exhibited marked induction of elastase-like activity which was due in major part to serine proteases. Hcys concentrations as low as 0.01 μM were effective and the peak effect was observed for the 50 μM plasma concentration, currently encountered in hyperhomocysteinemic patients [1] and in heart transplant recipients [9]. Serine elastase induction endows Hcys with a pathogenic role in mild homocysteinemic heart transplant recipients prior to obstructive coronary arteriopathy [9]. The development of neointima formation in aorta organ culture is associated with an increasing elastase activity and is prevented by serine protease inhibitors [30]. The Hcys-induced elastase activation may account for rupture of the internal elastic lamina observed in the arterial wall of mild and hyperhomocysteinemic patients. In the piglet experimental model, Oho and Rabinovitch reported a 10-fold increase in serine elastase activity correlated with a 5-fold increase in the break in the internal elastic lamina in the donor as compared to the host coronary artery [14]. These disorders also may amplify the inhibitory effect of Hcys upon elastin build-up mediated by lysine-linking lysyl oxidase [1].

The absence of a direct effect of Hcys on the enzymes, and the 18 h incubation time requirement to induce increased elastase-like activity, suggested de novo synthesis of enzyme. This hypothesis was confirmed by the suppression of the Hcys-inducing effect when blocking DNA transcription with actinomycin D, or protein synthesis with cycloheximide. That Hcys induction of elastase-like activities requires protein synthesis is in line with the acknowledged DNA synthesis and cyclin A mRNA activation by low Hcys concentrations in human and rat VSMC [2,6]. In human VSMCs, it therefore remains to be established
whether Hcys activates (several) regulatory elements and cognate DNA-binding proteins important for serene elastase gene transcription.

When priming VMSC with trypsin prior to Hcys activation of the cells, very low elastolytic activity was found either in the intracellular or extracellular compartments. It is usually acknowledged that trypsin does not penetrate the cells. A mechanism whereby induction of elastase in VMSC was associated with increased elastin adhesion to the 67 kDa elastin-binding protein on the VSMC surface [24].

The present results provide new insights into the mechanism of Hcys-induced atherosclerosis, and highlight the potential of Hcys to regulate protease synthesis, although the mechanisms by which elevated Hcys levels induce the synthesis of serene elastase remain to be elucidated. Through serum elastase activation, Hcys increases degradation of the extracellular matrix [31] and release of chymotryptic elastin peptides [32] which induce VSMC proliferation [2,6] and migration into the subendothelium, leading to neointima formation and progressive vascular occlusion. Inhibition of serum elastase prevents these VSMC alterations and the associated progressive vascular disease [18,29,31]. Finally, the cumulative induction of cell proliferation and of elastin fragmentation by Hcys provides interesting opportunities for therapeutic measures related to the development of obstructive coronary arteriopathy in hyper-homocysteinemic heart transplant recipients.

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References

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