

# Homocysteine modulates the proteolytic potential of human arterial smooth muscle cells through a reactive oxygen species dependant mechanism

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**Abstract** Pathological levels of homocysteine induce a dramatic degradation of arterial elastic structures. This severe metalloproteinase-dependant elastolysis affects elastic structures all over the media suggesting that smooth muscle cells (SMC) may participate to this process induced by homocysteine. Therefore, we investigated the effect of physiological (10  $\mu$ M) and pathological (50, 100, and 500  $\mu$ M) concentrations of homocysteine on the metalloproteinase-dependant proteolytic potential of human arterial SMC in culture. Pathological levels of homocysteine increased concomitantly the secretion of latent MMP-2 and TIMP-2 while the secretion of other elastolytic matrix metalloproteinases (MMPs) and expression of MT1-MMP were not altered. The increased secretion of latent MMP-2 induced by homocysteine was associated with an increased production of reactive oxygen species (ROS). Moreover, the increased secretion of latent MMP-2 induced by homocysteine was inhibited by antioxidant superoxide dismutase alone or in combination with catalase. These results suggest that SMC could participate, through an oxidative stress dependant secretion of elastolytic MMP-2, to the metalloproteinase-dependant degradation of arterial elastic structures induced by homocysteine.

**Keywords** Homocysteine · Matrix metalloproteinases · Oxidative stress · Endothelial cell · Smooth muscle cell · Elastin

## Introduction

High plasma levels of homocysteine, a physiological sulfur-containing amino acid, are well known as an independent risk factor for atherosclerosis on the basis of many epidemiological studies [1, 2]. In vivo studies using genetic [3] and dietary [4] animal models of hyperhomocysteinemia support the concept that homocysteine contributes to and accelerates atherogenesis. According to a wide range of in vitro experiments and, to a lesser extent, in vivo observations, the mechanism by which homocysteine promotes atherogenesis are multiple and likely interrelated, including endothelial dysfunction [5], proliferation of vascular smooth muscle cells (SMC) [6], and extracellular matrix (ECM) remodeling [7].

Among the ECM alterations, it has been clearly ascertained that a dramatic degradation of arterial elastic structures is one of the major features of the arterial lesions associated with hyperhomocysteinemia [8]. The degradation of elastic structures was shown to be due to an elastolytic process supported by an increase in metalloproteinase-dependant elastolytic activity [4, 7, 9]. It was previously shown that endothelial cells could participate to the subendothelial degradation of arterial elastic structures induced by homocysteine through elastolytic matrix metalloproteinases (MMPs) [10]. Interestingly, a well-established feature of this elastolytic process is that it affects elastic structures throughout the media [8, 9]. However, little is known about the participation of SMC to this elastolytic process. Therefore, we investigated the effect of homocysteine on the metalloproteinase-dependant proteolytic potential of human arterial SMC. We cultured SMC with pathological levels of homocysteine matching those encountered in human, focusing our study on elastolytic matrix metalloproteinases MMP-2, MMP-9, MMP-3, and MMP-7 [11, 12].

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Furthermore, we investigated whether homocysteine effects involved reactive oxygen species (ROS), which is a key point of MMP synthesis and secretion induced by many stimuli [13] and is responsible for other homocysteine-induced effects on vascular SMC [14]. We showed that homocysteine altered the MMP-2 dependant proteolytic potential of SMC through a mechanism involving ROS. These results suggest that SMC could participate, through the ROS dependant secretion of elastolytic MMP-2, to the metalloproteinase-dependant degradation of arterial elastic structures induced by homocysteine.

## Materials and methods

### Cell culture

Human arterial SMC were isolated from umbilical arteries as previously described [15]. Briefly, umbilical arteries were isolated from whole cord, opened longitudinally, gently scrap on their luminal face to remove endothelial cells and dissected into 5 mm explants. Explants were laid lumen-side down onto plastic culture plates. Dulbecco Modified Eagle Medium (DMEM, Gibco) supplemented by 20% Fetal Calf Serum (FCS, Gibco), penicillin, and streptomycin (50 U/ml) was added to the culture plates and placed at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Media were replaced with fresh media twice per week. Cell started growing from the explants within 1 week. At confluence, explants were removed and SMC were identified by their typical hill and valley morphology and indirect immunofluorescent positive staining for  $\alpha$ -actin. The absence of endothelial cell was confirmed by indirect immunofluorescent negative staining for VE-cadherin.

### Cell treatment

SMC from passage second to fifth were plated on 6-well plates (Nunc), grown to confluence in DMEM containing antibiotics and 10% FBS and changed to basal medium (serum free medium containing 0.1% bovine serum albumin) for 4 h before exposure to experimental treatments. SMC were incubated for 24 h in a basal medium alone or supplemented with physiological (10  $\mu$ M) or pathological (50, 100, 500  $\mu$ M) concentrations of homocysteine (D,L-homocysteine, Sigma). At the same time, SMC were incubated for 24 h with medium supplemented with 10 and 100  $\mu$ M of methionine (D,L-methionine, Sigma) or cysteine (D,L-cysteine, Sigma) to ascertain the specificity of the effect of homocysteine. To assess the involvement of ROS, SMC were incubated for 24 h in basal medium alone or supplemented with 100  $\mu$ M homocysteine in the absence or presence of superoxide

dismutase (SOD, 1,000 U/l, Sigma) and catalase (1,000 U/l, Sigma) alone or in combination.

At the end of the incubation time, conditioned medium were collected and centrifuged, and cells were used to measure ROS production (see below). Thereafter, cells were washed with phosphate-buffered saline and harvested by scrapping into lysis buffer containing 50 mM Tris-HCl, 1% Triton X-100, and anti-protease cocktail inhibitors (Roche Diagnostics). Cell lysates and conditioned medium were centrifuged for 5 min at 1,000 $\times$ g to remove cell debris. Total cellular protein was quantified using the Bicinchoninic Acid Protein assay reagent (BCA, Sigma).

### Zymography

Cell lysates and conditioned medium of HT1080 cells as standard were submitted to gelatin and casein zymography as previously described [16]. The metalloproteinase nature of the detected proteases was confirmed by incubating identical gels in the presence of a 1,10-phenanthroline (1 mmol/l). Proteolytic activities were quantified by densitometry and normalized relatively to HT1080 activities to allow the quantitative comparison between zymograms. Activities were expressed as a percentage of specific activities of SMC cultured in basal medium.

### Western blotting

To identify MMPs and TIMP-2, conditioned medium and cell lysates extracts were electrophoresed under non-reducing conditions as previously described [10]. Proteins were transferred onto PVDF membranes and 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 antibodies directed against MMP-2 (Calbiochem, Ab-3), MMP-9 (Calbiochem, Ab-8), MMP-3 (Calbiochem, Ab-5), MMP-7 (Interchim, Ab-1), TIMP-2 (Calbiochem, Ab-4), or polyclonal goat anti-human MT1-MMP (Tebu-bio, L-15). Then, membranes were incubated for 1 h with anti-goat or anti-mouse IgG horseradish peroxidase conjugated (1/2,000 dilution) and peroxidase activity was revealed by Super-signal<sup>®</sup> chemiluminescent substrate (Pierce). For negative control experiments, primary antibodies were omitted.

### MMP gelatinase activity assay kit

Gelatinolytic activity in conditioned medium was quantified using a MMP gelatinase assay kit (Chemicon) based on a biotinylated gelatinase specific substrate of active MMP-2 and MMP-9. Conditioned medium were incubated with biotinylated gelatinase specific substrate and remaining biotinylated fragments were added to a biotin-binding

96-well plate and detected with streptavidin–enzyme complex. The addition of enzyme substrate resulted in a colored product quantified by its optical density at 450 nm. The color intensity was inversely proportional to gelatinolytic activity. APMA-activated MMP-2 and basal medium were used as positive and negative control, respectively. Gelatinase activities were expressed relatively to negative control.

#### Measurement of ROS production

The production of ROS was measured by 6-carboxy-2', 7'-dichlorofluorescein diacetate (DCFH-DA) fluorescence as previously described [17]. This assay is based on the oxidation of DCFH-DA to fluorescein by oxygen and nitrogen reactive species, whose fluorescence can be measured at 522 nm. Briefly, after 24 h of culture with the different stimuli conditions and collection of conditioned medium (see above), SMC were washed with medium and incubated with 5  $\mu\text{M}$  of DCFH-DA in PBS for 2 h at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ . Thereafter, the cells were washed with PBS to remove the excess probe and the fluorescence of oxidized DCFH-DA was measured by a Cytofluor<sup>®</sup> Series 4000 Fluorescence multi-well plate reader (PerSeptive Biosystems, Framingham, MA, USA). The excitation filter was a 20-nm bandwidth centered at 485 nm and the emission filter was a 25-nm bandwidth centered at 530 nm. Fluorescence intensities reflecting ROS production were expressed as a percentage of fluorescence intensity found in SMC cultured in basal medium alone.

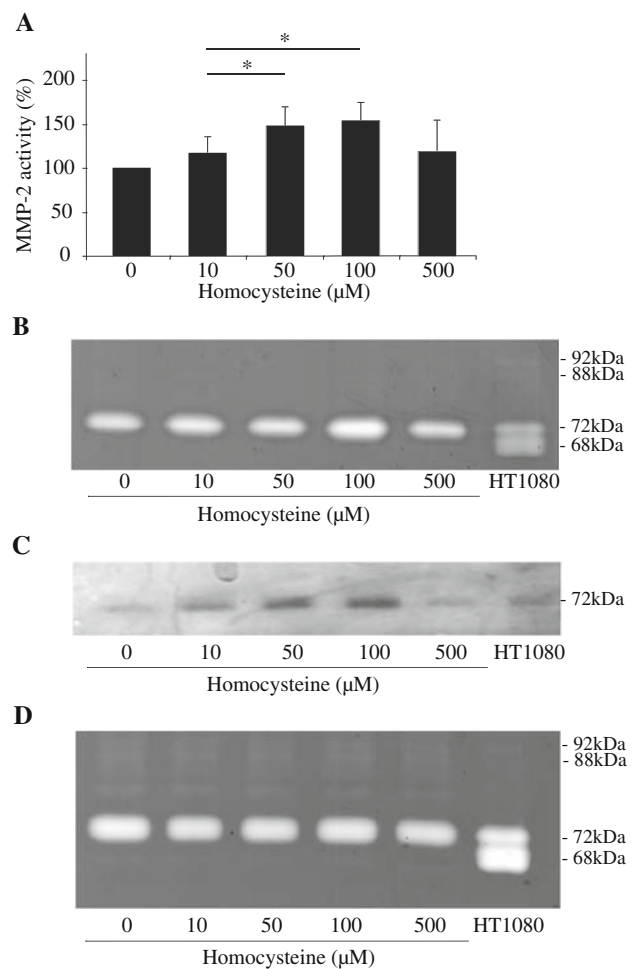
#### Statistical analysis

Data were analyzed by non-parametric Wilcoxon paired test. Results are expressed as means  $\pm$  standard deviation. A value of  $P < 0.05$  was considered significant.

## Results

#### Homocysteine modulates the MMP-2 dependant proteolytic potential of human arterial SMC

MMP-2 and MMP-9 were examined by gelatin zymography in both conditioned medium and cell lysates. In conditioned medium from SMC cultured in basal medium, one major gelatinolytic band was detected at molecular weight corresponding to latent MMP-2 (72 kDa) while no band was detected at molecular weight corresponding to active MMP-2 as well as latent or active MMP-9 (Fig. 1b). Gelatinolytic activities were confirmed as metalloproteinase by inhibition with PHE (results not



**Fig. 1** Homocysteine increases the secretion of latent MMP-2. Representative gelatin zymography of conditioned medium (**b**) and cell lysates (**d**) and western blot analysis of conditioned medium (**c**) from SMC cultured in basal medium alone or supplemented with 10, 50, 100, and 500  $\mu\text{M}$  of homocysteine. Conditioned medium of HT1080 cells was used as internal standard. Each zymogram and western blot is the representative of four independent experiments. The 92, 72, and 68 kDa gelatinolytic band are, respectively, the latent form of MMP-9, the latent form of MMP-2 and the active form of MMP-2. In **a**, gelatinolytic bands corresponding to latent MMP-2 were quantified by densitometry analysis and specific activities were expressed as a percentage of specific activities of SMC cultured in basal medium. Values are means  $\pm$  standard deviation of six independent experiments. \*  $P < 0.05$  vs. 10  $\mu\text{M}$  homocysteine

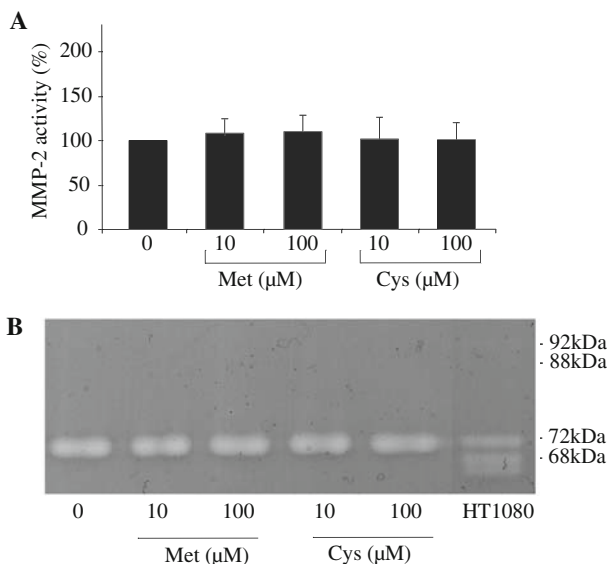
shown). In conditioned medium, densitometric quantification showed a significantly increased secretion of the 72 kDa gelatinase with 50 and 100  $\mu\text{M}$  homocysteine, while its secretion was not significantly increased with 500  $\mu\text{M}$  homocysteine when compared to 10  $\mu\text{M}$  homocysteine (Fig. 1a). This result was confirmed by western blotting (Fig. 1c). At the same time, active MMP-2 (68 kDa) and both latent (92 kDa) and active (88 kDa) MMP-9 remained undetectable in conditioned medium whatever the concentration of homocysteine used in culture (Fig. 1b). In cell lysates from SMC cultured in basal

medium, significant amount of latent MMP-2, no active MMP-2 and very low amount of latent and active MMP-9 were detected (Fig. 1d). However, none of these gelatinolytic bands was altered by homocysteine whatever the concentration used in culture (Fig. 1d).

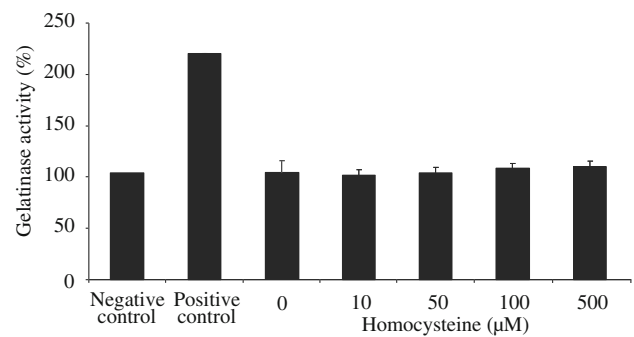
Culture of SMC with methionine or cysteine did not alter the secretion of latent MMP-2 and confirmed the specificity of the inducing effect of homocysteine on the secretion of latent MMP-2 by SMC (Fig. 2).

Gelatinase activities in conditioned medium were measured against a specific substrate of active MMP-2 and MMP-9. In conditioned medium from SMC cultured in basal medium, no gelatinase activity was detected when compared to a negative control free of gelatinase activity (Fig. 3). Gelatinase activities were not altered in conditioned medium from SMC cultured with 50, 100, and 500  $\mu\text{M}$  of homocysteine compared to 10  $\mu\text{M}$  homocysteine.

Weak caseinolytic bands corresponding to latent MMP-3 (57 kDa) and MMP-7 (28 kDa) were detected in conditioned medium by casein zymography. Both caseinolytic activities were not altered in conditioned medium from SMC cultured with 50, 100, and 500  $\mu\text{M}$  homocysteine compared to 10  $\mu\text{M}$  homocysteine (data not shown).



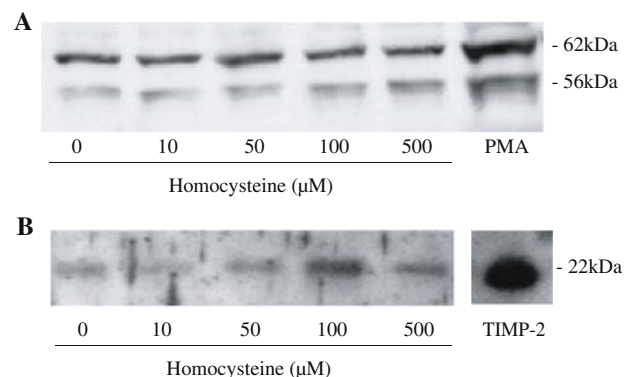
**Fig. 2** Methionine and cysteine have no effect on the secretion of latent MMP-2. **b** Representative gelatin zymography of conditioned medium from SMC cultured in basal medium alone or supplemented with 10 and 100  $\mu\text{M}$  of methionine (MET) or cysteine (CYS). Conditioned medium of HT1080 cells was used as internal standard. The zymogram is representative of four independent experiments. In **a**, gelatinolytic bands corresponding to latent MMP-2 were quantified by densitometry analysis and specific activities were expressed as a percentage of specific activities of SMC cultured in basal medium. Values are means  $\pm$  standard deviation of six independent experiments



**Fig. 3** Homocysteine does not alter gelatinase activity in conditioned medium from cultured SMC. Gelatinase activity in conditioned medium was measured against a specific substrate cleaved by active MMP-2 and MMP-9. APMA-activated MMP-2 and basal medium were, respectively, supplied as positive and negative control. Gelatinase activities were expressed as a percentage of negative control. Values are means  $\pm$  standard deviation of four independent experiments

#### Homocysteine altered the secretion of TIMP-2 but not the expression of MT1-MMP

We determined the effect of homocysteine on the expression of MT1-MMP in cell lysates by western blotting. A major and a weak band were, respectively, detected at molecular weight corresponding to the latent (62 kDa) and active (56 kDa) forms of MT1-MMP in cell lysates from SMC cultured in basal medium (Fig. 4a). The expression of both forms was not altered in cell lysates from SMC cultured with 50, 100, and 500  $\mu\text{M}$  of homocysteine compared to 10  $\mu\text{M}$  homocysteine. The secretion of TIMP-2 in conditioned medium was determined by western blotting. As show in Fig. 4b, a weak band at molecular weight of 22 kDa equivalent to standard of TIMP-2 was detected in conditioned medium from control SMC. The secretion of

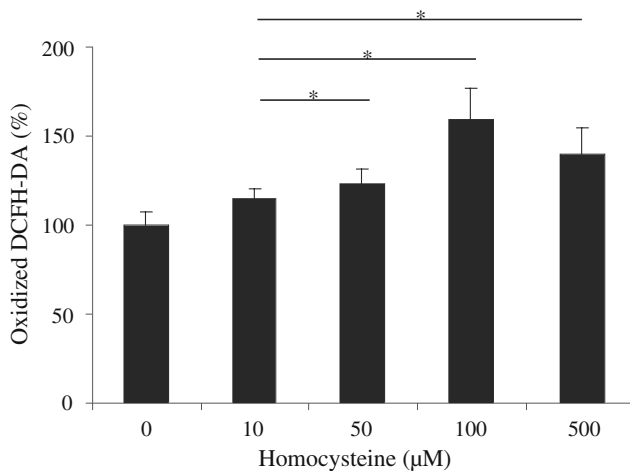


**Fig. 4** Western blot analysis of **a** MT1-MMP in cell lysates and **b** TIMP-2 in conditioned medium from SMC cultured in basal medium alone or supplemented with 10, 50, 100, and 500  $\mu\text{M}$  of homocysteine. Purified TIMP-2 and cell lysates from SMC stimulated with phorbol myristyl acetate (PMA) were used as standard. Each western blot is representative of four independent experiments

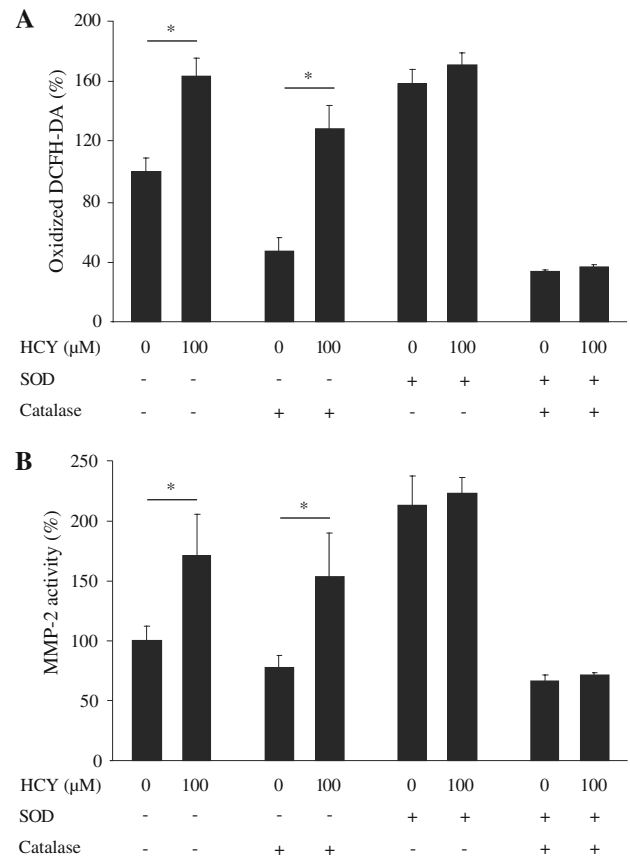
TIMP-2 was increased in conditioned medium from SMC cultured with, 50, 100, and 500  $\mu\text{M}$  of homocysteine compared to 10  $\mu\text{M}$  homocysteine.

ROS are involved in the increased secretion of MMP-2 induced by homocysteine

To determine whether ROS are involved in the increased secretion of MMP-2 induced by homocysteine, we first examined whether the increased secretion of latent MMP-2 induced by homocysteine was associated with an increased production of ROS in SMC using DCFH-DA assay. The production of ROS was significantly increased in SMC cultured with 50, 100, and 500  $\mu\text{M}$  homocysteine compared to 10  $\mu\text{M}$  homocysteine (Fig. 5). Moreover, we studied the effects of free oxygen radical scavengers SOD and catalase alone or in combination on the production of ROS and on the secretion of latent MMP-2 in presence or absence of homocysteine. In SMC cultured in absence of homocysteine, catalase alone or in combination with SOD decreased concomitantly the production of ROS (Fig. 6a) and the secretion of latent MMP-2 (Fig. 6b), while SOD alone increased both production of ROS (Fig. 6a) and secretion of latent MMP-2 (Fig. 6b). These effects of SOD and catalase on ROS production in cultured SMC are in agreement with previous in vitro study [18, 19]. Catalase alone had no effect on increased secretion of latent MMP-2 and increased production of ROS induced by 100  $\mu\text{M}$  homocysteine, while SOD alone or in combination with catalase both completely inhibited the increased secretion



**Fig. 5** Homocysteine increases the production of ROS in SMC. Oxidation of DCFH-DA to fluorescein was measured in SMC cultured in absence or with 10, 50, 100, and 500  $\mu\text{M}$  of homocysteine. Fluorescence intensities reflecting ROS production were expressed as a percentage of fluorescence intensity found in SMC cultured in basal medium. Values are means  $\pm$  standard deviation of six independent experiments. \*  $P < 0.05$  vs. 10  $\mu\text{M}$  homocysteine



**Fig. 6** Effect of SOD and catalase on the production of ROS and the secretion of latent MMP-2 induced by homocysteine (HCY) in SMC. SMC were cultured in basal medium alone or supplemented with 100  $\mu\text{M}$  homocysteine in the absence or presence of SOD (1,000 U/l) and catalase (1,000 U/l) alone or in combination. **a** ROS production was determined by the oxidation of DCFH-DA to fluorescein in SMC. Fluorescence intensities reflecting ROS production were expressed as a percentage of fluorescence intensity found in SMC cultured in basal medium. **b** Secretion of latent MMP-2 was determined by gelatin zymography analysis of conditioned medium. Gelatinolytic bands corresponding to latent MMP-2 were quantified by densitometry analysis and specific activities were expressed as a percentage of specific activities of SMC cultured in basal medium. Values are means  $\pm$  standard deviation of five independent experiments. Effect of homocysteine was considered significant (\*) when  $P < 0.05$

of latent MMP-2 (Fig. 6b) and the increased production of ROS (Fig. 6a) induced by 100  $\mu\text{M}$  homocysteine.

## Discussion

In this study, we showed that pathological levels of homocysteine increased the MMP-2 dependant proteolytic potential of arterial SMC through a ROS dependant process. SMC were cultured with homocysteine at concentrations matching plasma levels encountered in human hyperhomocysteinemia (50–500  $\mu\text{M}$ ). In these conditions, high levels of homocysteine increased the secretion of

latent MMP-2. Extracellular free-radical inhibitors markedly reduced SMC secretion of latent MMP-2 induced by homocysteine.

A dramatic degradation of arterial elastic structures throughout the media is one of the major features of the arterial lesions associated with hyperhomocysteinemia [8, 9]. It was shown to be due to an elastolytic process supported by an increase in metalloproteinase-dependant elastolytic activity [9]. Our results suggest that SMC could participate, through the secretion of latent elastolytic MMP-2, to the metalloproteinase-dependant degradation of arterial elastic structures induced by homocysteine.

Latent MMP-2 was constitutively secreted by SMC, while neither latent nor active forms of MMP-9 were detectable. Concomitantly, latent MMP-3 and MMP-7 were weakly secreted while their active forms were undetectable (data not shown). This elastolytic MMP pattern closely matched the basal pattern previously described in non-atherosclerotic arteries [20] as well as in cultured SMC derived from macrovasculature [21]. Pathological levels of homocysteine (50 and 100  $\mu\text{M}$ ) increased SMC secretion of latent MMP-2 alone whereas secretion of its active form and secretion of latent and active forms of MMP-3, MMP-7, and MMP-9 were not altered. Homocysteine at 500  $\mu\text{M}$  did not significantly increase MMP-2 secretion, which is in accordance with previous reports in which such levels of homocysteine were shown to have cytotoxic effect on cultured SMC [22]. In conditioned medium, the increased secretion of latent MMP-2 was associated with an increased secretion of TIMP-2, while specific gelatinase activity revealed by gelatinase activity assay was not altered. Actually, the gelatinase activity assay can only reveal the activity of active MMP-2 (68 kDa) free of TIMPs whereas gelatin zymography reveals also the activity of latent MMP-2 due to the SDS-induced dissociation of latent MMP-2/TIMP-2 complexes and the further activation of latent MMP-2 by SDS-induced conformational change [23]. Together, these results clearly evidence that pathological levels of homocysteine increased the MMP-2 dependant proteolytic potential of human arterial SMC in culture.

We have previously reported that homocysteine was able to activate purified latent MMP-2 through a non-proteolytic process which led to an increased proteolytic activity of the enzyme [24]. Although it has not yet been clearly demonstrated, the absence of increased gelatinase activity in our conditioned medium suggest that homocysteine may not be able to process such a non-proteolytic activation when latent MMP-2 is complexed with TIMP-2, which is the predominant form of MMP-2 secreted by cells [25]. In previous studies, Doronzo et al. found that constitutive secretion of only active form of MMP-2 was increased by homocysteine in cultured human aortic SMC

[26], whereas Guo et al. reported that homocysteine increased only latent MMP-2 constitutively secreted by rat aortic SMC [27]. One of the main activating processes of MMP-2 is a cell surface MT1-MMP dependant proteolytic process that also involves TIMP-2. Indeed, it is well known that TIMP-2 is unique as a member of the TIMP family in that, in addition to inhibiting active forms of MMPs, it can selectively interact with MT1-MMP to trigger or inhibit the cell-surface activation of MMP-2 according to the molecular ratio of TIMP-2/MMP-2 [28]. In our study, the increased secretion of latent MMP-2 by pathological levels of homocysteine was associated with an increase of TIMP-2 secretion, while MT1-MMP expression was not altered in cell lysates. Together, our results are in accordance with the absence of active MMP-2 in the conditioned medium from SMC cultured with pathological levels of homocysteine. Therefore, although it is difficult to reconcile these studies, many factors such as SMC heterogeneity in molecular control of SMC gene expression and in response to numerous stimuli, phenotypic state, and grade of confluence may explain this discrepancy [29].

We suggest that SMC could participate, through the secretion of latent elastolytic MMP-2, to the metalloproteinase-dependant degradation of arterial elastic structures induced by homocysteine. MMP-2 is well known to degrade elastin *in vitro* [11] and the elastic fibers network *ex vivo* in human skin [30]. We previously observed an increased immunoreactivity of MMP-2 (latent and active forms) with medial SMC that colocalized with the degradation of elastic structures in arteries cultured with pathological levels of homocysteine (unpublished data). Moreover, in the arterial wall, once secreted by SMC, latent MMP-2 could exert its elastolytic activity against elastic structures since: (i) elastin was previously shown to activate latent MMP-2 by contact autoactivation which results in its own elastolysis [31]; (ii) latent MMP-2 can be activated *in vivo* by endothelial serine-proteases such as human tissue kallikrein [32] which expression was shown to be increased by homocysteine [10].

An important finding in this study is that ROS are involved in the increased secretion of MMP-2 induced by homocysteine in SMC. Indeed, the increased secretion of latent MMP-2 induced by homocysteine was associated with an increased production of ROS. Furthermore, inhibition of ROS production by SOD alone or in combination with catalase inhibited the increased secretion of latent MMP-2 induced by homocysteine. This finding is consistent with previous observations showing that increased expression of MMP-2 in SMC in response to proatherogenic stimuli is dependant on ROS [33]. Moreover, since we used SOD and catalase that are large molecules and only act as extracellular free oxygen radical inhibitors, the increased expression of MMP-2 induced by homocysteine

involved extracellular ROS. This is in agreement with previous *in vitro* study that clearly demonstrated that extracellular generation of ROS increased MMP-2 expression in human dermal fibroblasts [34]. Recently, Doronzo et al. showed that phosphatidyl inositol 3-kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK) are involved in increased expression of MMP-2 induced by homocysteine [26]. Since MAPK and PI3K pathway are ROS-sensitive downstream pathways [35], these previous data together with the complete inhibition by antioxidant SOD and catalase of the increased expression of MMP-2 induced by homocysteine suggest that ROS may be upstream activators of MAPK and PI3K pathway leading to increased expression of MMP-2 in SMC stimulated by pathological levels of homocysteine.

In summary, we have shown that pathological levels of homocysteine increased the MMP-2 dependant proteolytic potential of SMC through a mechanism involving ROS. These results are in favor of an active participation of SMC to the metalloproteinase-dependant arterial elastolysis induced by homocysteine. Further experiments *in vivo* are required to confirm the active participation of MMP-2-dependant elastolytic potential of SMC in the degradation of arterial elastic structures associated with hyperhomocysteinemia. This study reinforces the key role of oxidative stress in the deleterious effect of homocysteine on arterial wall, suggesting antioxidant therapy may have a beneficial effect on cardiovascular complications of hyperhomocysteinemia.

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