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Induction of Oxidative Stress by Homocyst(e)ine Impairs Endothelial Function

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Abstract Previous studies have demonstrated a relationship between hyperhomocysteinemia and endothelial dysfunction, reduced bioavailability of nitric oxide, elastinolysis, and vascular muscle cell proliferation. In vivo decreased nitric oxide production is associated with increased matrix metalloproteinase (MMP) activity and formation of nitrotyrosine. To test the hypothesis that homocysteine neutralizes vascular endothelial nitric oxide, activates metalloproteinase, causes elastinolysis and vascular hypertrophy, we isolated aortas from normotensive Wistar rats and cultured them in medium containing homocysteine, and calf serum for 14 days. Homocysteine-mediated impairment of endothelial-dependent vasodilatation was reversed by co-incubation of homocysteine with nicotinamide (an inhibitor of peroxinitrite and nitrotyrosine), suggesting a role of homocysteine in redox-mediating endothelial dysfunction and nitrotyrosine formation. The Western blot analysis, using anti-nitrotyrosine antibody, on aortic tissue homogeneates demonstrated decreased nitrotyrosine in hyperhomocysteinemic vessels treated with nicotinamide. Zymographic analysis revealed increased elastinolytic gelatinase A and B (MMP-2,-9) in homocysteine treated vessels and the treatment with nicotinamide decreases the homocysteine-induced MMP activation. Morphometric analyses revealed significant medial hypertrophic thickening (1.4±0.2-fold of control, $P = 0.03$) and elastin disruption in homocysteine-treated vessels as compared to control. To determine whether homocysteine causes endothelial cell injury, cross-sections of aortas were analyzed for caspase activity by incubating with Ac-YVAD-AMC (substrate for apoptotic enzyme, caspase). The endothelium of homocysteine treated vessels, and endothelial cells treated with homocysteine, showed marked labeling for caspase. The length-tension relationship of homocysteine treated aortas was shifted to the left as compared to untreated aortas, indicating reduced vascular elastic compliance in homocysteine-treated vessels. Co-incubation of homocysteine and inhibitors of MMP, tissue inhibitor of metalloproteinase-4 (TIMP-4), and caspase, YVAD-CHO, improved vascular function. The results suggest that alteration in vascular elastin/collagen ratio and activation of MMP-2 are associated with decreased NO production in hyperhomocysteinemia. J. Cell. Biochem. 82: 491–500, 2001. © 2001 Wiley-Liss, Inc.

Key words: vascular compliance; ECM remodeling; hypertrophy; aorta; hypertension; elastin; collagen; smooth muscle; fibrosis; organ culture; caspase; vasospasm; apoptosis

Acute homocysteine infusion impairs endothelial-dependent vasodilation [Chambers et al., 1998]. The role of chronic homocysteinemia in endothelial-dependent vascular structure and function is unclear. Nitric oxide in conjunction with superoxides forms peroxinitrite. In the presence of thiol, the peroxinitrite transforms into nitration of tyrosine residues in proteins [Beckman et al., 1990; Huie and Padjama, 1993; Simon et al., 1996]. Accumulation of plasma homocysteine reduces the levels of cysteine [Wollesen et al., 1999] and glutathione peroxidase activity [Upchurch et al., 1997a]. In hyperhomocysteinemia, homocysteine may be the primary thiol in regulating redox reactions. The levels of Cu^{2+} ions are increased in hyperhomocysteinemia [Dudman and Wilcken, 1983; Yoshida et al., 1992] and

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catalyzes the formation of peroxinitrite. These studies, however, did not relate the levels of homocysteine to nitrotyrosine formation. Because tetrahydrobiopterin (BH_4) is a cofactor for endothelial nitric oxide synthase (eNOS) activity [Milstein and Katusic, 1999], the treatment of BH_4 may increase levels of NO and improve vascular function. Homocysteine also induces eNOS [Upchurch et al., 1997b]. The nitro-derivatives of arginine, L-NAME, and other antagonists of substrate, L-arginine, have been shown to inhibit NO formation [Schmidt et al., 1993]. These studies may suggest that in the presence of high plasma homocysteine NO inhibits its own production and/or the levels of BH_4 are dwindled by homocysteine. The nitration may induce conformational changes in the proteins and cause inactivation of enzymes and form intermolecular crosslinks. In aortas of rats with pressure overload hypertrophy by aortic coarctation, and two kidney one clip Goldblatt hypertension, increased levels of nitrotyrosine has been observed [Bosse and Bachmann, 1997; Bouloumie et al., 1997]. The inhibition of peroxinitrite, an intermediate of nitrotyrosine, by nicotinamide prevented cardiovascular dysfunction [Cuzzocrea et al., 1998]. However, the mechanism by which nitrotyrosine is generated in aortas of these rats is unclear. We hypothesized that homocysteine reduces bioavailability of endothelial nitric oxide by forming nitrotyrosine.

Elastin turnover is lower as compared to collagen. The degraded elastin is replaced by stiffer collagen. Diminution of vascular elastic content is one of the hallmarks of increased vascular stiffness. Studies suggest that high levels of homocysteine are associated with atherosclerotic lipid rich lesions [Matthias et al., 1996; Hofmann, 1999; Jovin, 1999] as well as arteriosclerotic and thrombotic lesions [Matthias et al., 1996; Hofmann, 1999; Jovin, 1999]. The reasons of this controversial and dual role of homocysteine are not clear. Jourdheuil-Rahmani et al. [1997] have demonstrated that homocysteine leads to elastin breakdown by increasing elastase activity in vascular smooth muscle cells (SMCs) [Rolland et al., 1995; Jourdheuil-Rahmani et al., 1997]. In vitro, homocysteine activates latent resident metalloproteinases (MMPs) [Tyagi et al., 1998] and degrades elastin and collagen. Elastin and collagen peptides induce a hypertrophic phenotype in vascular cells [Tummalapalli and Tyagi, 1999] and facilitate vascular contractile dysfunction [Faury et al., 1995]. In culture conditions inhibition of cytokine-induced NOS reduced both expression and activity of MMPs [Sasaki et al., 1998]. In contrast, cytokine inducible MMPs in immortalized cells were not modified by NOS inhibition [Horton et al., 1998]. The reasons for such diverse effects of NO on MMPs are not clear. However, a differential regulation of MMPs, release and activation in vivo vs. in vitro may account for this discrepancy. Oxygen species stimulate MMPs [Rajagopalan et al., 1996] and in vivo inhibition of NO production increases MMP activity in other tissue [Radomski et al., 1998]. The mechanism of NO-mediated MMP activation and ECM disruption is unclear. We hypothesized that reduction of nitric oxide by homocysteine increases MMP activity.

MATERIALS AND METHODS

Aortic Organ Culture and Treatment of Vascular Tissue

Aortas from normotensive Wistar rats, 6–8 weeks of age, weighing 225–275 g were isolated. Rats were anesthetized by intraperitoneal injection of Nembutal (50 mg/kg) and a thoracotomy was performed. The full length of the thoracic aorta was aseptically removed and placed in cold physiological salt solution (PSS) containing NaCl 131.5 mM, KCl 1.25 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.25 mM, $NaHCO_3$ 23.5 mM, and glucose 11.2 mM. The solution was perfused with 95% O_2-5% CO_2 (pH 7.4). The vessel was placed into a tissue culture dish containing PSS where adherent periadventitial fat and connective tissue were removed using fine forceps. The vessel was then transferred to another dish containing fresh PSS and trimmed of intercostal arteries using iris scissors. The aorta was irrigated with 20 ml of PSS to remove blood particles. In order to avoid injury to the endothelium, all vessel manipulations were performed using the cut ends.

The remaining aorta was then cut into ring segments (2–4 mm) using a sharp scalpel blade. Approximately 6–8 rings were prepared from the thoracic aorta. Rings were placed into multiwell plates (24 well/plate) containing DMEM with 10% FCS under 95% air and 5% CO_2 atmosphere at 37°C. Cultures were monitored daily using phase contrast microscopy and fresh media was added every day. The

treatment with homocysteine (100 μM) was performed in 0.4% serum-DMEM. Similar treatments were performed with cysteine, glutathione, and homocysteine + nicotinamide. Control vessels remained in 0.4% serum-DMEM for 14 days. Similar conditions were employed for the treatment and co-treatment with TIMP-4 (40 μM) [purified in our laboratory from rat hearts], caspase inhibitor, YVAD-CHO, (100 μM) [Oncogene Research Product]. The purified cardiac MMP-2 [Tyagi and Cleutjens, 1995], gelatinase-a (10 μM) was used to treat the normal aortas in serum free medium for 30 min prior to the measurements. The fresh aortic rings were treated with L-NAME (300 μM) for 30 min prior to measurements. The endothelium was denuded by gentle treatment with 0.01% Triton X-100 for 5 sec [Mebazaa et al., 1995]. The vessel was quickly equilibrated in PSS after the treatment of Triton X-100.

Vascular Contractility

The contraction in fresh or 14 day cultured aortic segments were carried out within 5 min after removal of the ring from animal or culture conditions. The tissue was aerated with 95% O_2 and 5% CO_2 (pH 7.4) and equilibrated at 37°C continuously. The rings were prepared and mounted in an isometric tension measurement tissue myograph (World Precision Instrument, Inc., FL). The rings were mounted in between the two stainless steel wires, one connected to a force transducer (World Precision Instrument, Inc., FL) and the other connected to a micrometer. The signal from the ring under experimentation was digitized by on-line analysis using PICKUP-95 software. The PSS containing NaCl 131.5 mM, KCl 0.2 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 0.5 mM, $NaHCO_3$ 23.5 mM, and glucose 11.2 mM was used. To evaluate the viability of the aortic rings, the rings were contracted three times by inducing active muscle tone using 20 mM $CaCl_2$, rinsed and re-equilibrated before the treatment.

After in vitro stabilization, each aortic ring was systematically stretched to the optimum of its length-active tension relation. A known amount of stretch was placed on the ring, and a contraction was induced with 20 mM $CaCl_2$ PSS. This procedure was repeated at progressive levels of stretch until the increase in active tension was 2. Acetylcholine and nitroprusside were used as endothelial-dependent and -independent vasodilators, respectively. The generated tension in grams was normalized by weight of the tissue in grams.

Preparation of homocysteine, cysteine, glutathione, L-NAME, TIMP-4, and caspase inhibitor solutions: 1 M DL-homocysteine (Sigma Chemical Co., St. Louis, MO) was solubilized in 0.1 M PBS buffer (pH 7.4). The precise concentration of homocysteine, cysteine, and glutathione were determined by spectrophometric titration with dithio-bis-nitrobenzoate with absorption measured at 412 nm, using $\varepsilon_{412\ nm}$ of 13,600/M/cm [Tyagi et al., 1998]. The concentrations of TIMP-4, Ne-nitro-L arginine methylester (L-NAME) [Sigma] and Ac-YVAD-AMC were determined by weight measurements. All dilutions from stock solutions were made freshly prior to experiment. Buffer was used as vehicle control.

To assess passive mechanical and stiffness properties of aortas, the force–tension relationship was measured. The unloaded vessel diameter was measured under minimal resting load. The vessel was then stretched by twisting the knob of micrometer and force/length developed was recorded. A force/length analysis was then performed on the data obtained above. Force in grams was normalized by the weight of the ring (y). The y values for different rings were not superimposed, partly because of a difference in lumen diameter. In order to adjust at the same point, the offset value at the y-axis was corrected in individual plot by adding or subtracting the offset from each value. The percent increase in initial circumference of the aorta was determined by adding the incremental stretch to the initial circumference and dividing by the initial circumference and multiplying with 100. To compare vessels of different diameter, all length measurements have been expressed as a percentage of the initial circumference. This does not alter the shape of the curves, but merely changes their position relative to one another. A stress-strain curve was then generated for each tissue ring by fitting a second-order polynomial to the resulting data [Hodgkin et al., 1992]. Stress,

$$(y) = a + b.x + c.x^2$$

where a, b, and c are constants. The slope of the first derivative of this equation was considered to be the coefficient of aortic stiffness.

Histology and Morphometry

The aortas were stained with van Gieson for elastic labelling as described in [Tyagi et al., 1995a]. Elastin breakdown in homocysteine treated aortas was identified and compared with control aortas. The medial thickness between inner and outer elastic laminae was measured by a digital micrometer and compared with control aortas.

Total Protein, Electrophoresis, Western-Blot, and Zymographic Analysis of Aortic Homogenates

A Bio-Rad dye binding assay was applied to estimate total protein concentration in the tissue extracts according to the method of Bradford [1976]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with and without reduction following the method of Laemmli [1970]. The gelatinase A and B (MMP-2) activity in control and homocysteine-treated aortic homogenates was measured by elastin zymography, using 50 μg/ml elastin in SDS–PAGE [Senior et al., 1991; Tyagi et al., 1995a]. Western-blot analysis was performed as described [Tyagi et al., 1995b] using anti-nitrotyrosine antibody (Upstate Biotechnologies, Inc).

In Situ Caspase Labelling

Tissue sections of 10 μm thickness were cut at the same time of histological section preparation. The sections were washed 2–3 times with PBS containing 0.01% Tween-20. The tissue sections were incubated with 1 mM florogenic substrate, Ac-YVAD-AMC, of caspase at 37°C for 2 h. The florescence in the inner lining of the aortic segments treated with homocysteine was determined using florescence microscope and compared with control without the treatment. The caspase labelling in the isolated endothelial cells was performed. The endothelial cells were isolated by gentle scapping of the lumen surface of aortas in DMEM. The endothelial cells were characterized by positive labelling of factor VIII and by their cobblestone appearance [Tyagi et al., 1995b; Tyagi, 1998]. The cells treated with homocysteine for 3 days in serum-free DMEM and control cells in DMEM alone were grown on coverslips in monolayer. The cells were permeabilized with 10% methanol for 30 min prior to incubating with caspase substrate. The florescence was visualized under florescence light.

Statistical Analysis

All reported values are mean±SD. Two-way analysis of variance was used for determination of differences among groups. A value of $P < 0.05$ was considered significant.

RESULTS

Homocysteine, Cysteine, Glutathione, and Vascular Function

The 14-day culture condition preserves the vascular contractile response to $CaCl_2$ and acetylcholine (Fig. 1). However, 14-day culture with homocysteine, compared to non-culture aortic rings, reduces vascular function greater than glutathione and cysteine (Figs. 1 and 2). The dose-response curve to acetylcholine shifted to right in 14-day homocysteine treated vessel as compared to control (Fig. 3). The effect of homocysteine was reversed by co-treatment of homocysteine with nicotinamide (Fig. 2). The response to nitroprusside was preserved in both the homocysteine-treated and control vessels (Fig. 4).

Vascular ECM Remodeling by Homocysteine

Severe elastin breakdown and significant medial hypertrophy were observed in homocysteine treated vessels as compared to control vessels (Fig. 5A and B). The medial thickness was 66±12, 95±14, and 74±11 μm, for control, homocysteine treated vessel, and homocysteine + nicotinamide treated vessel, respectively. The homocysteine treated vessel was 1.4±0.2-

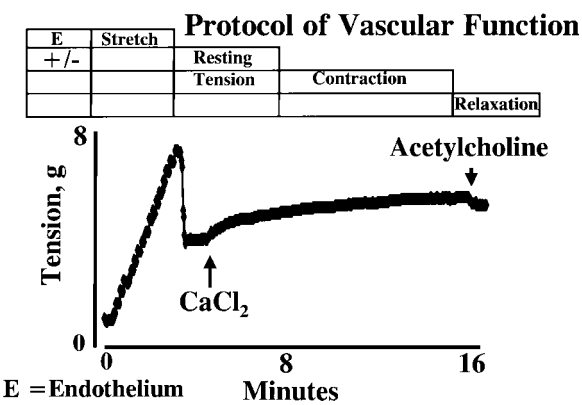

Fig. 1. Typical protocol of aortic contraction and relaxation: Aortic rings with or without endothelium (+/− E) stretch to maximum length. The aorta was brought to resting tension at ~20% of total length. The vasoactive agent (20 mM $CaCl_2$) was added (arrow up). At maximum contraction vasorelaxant, acetylcholine (10^{-8} M) was added (arrow down).

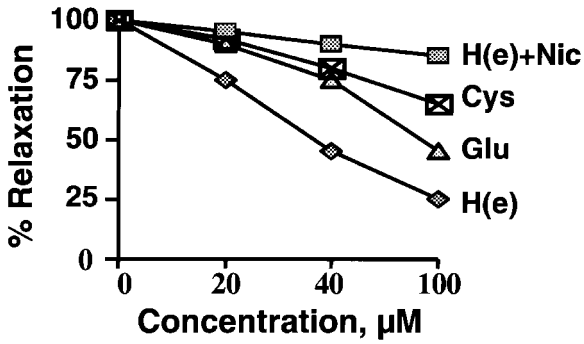

Fig. 2. Dose-response of (thiol containing reagents) homocysteine, glutathione, and cysteine on vascular contractile function: the aortic rings were incubated for 14 days in different dose of thiols. The percent relaxation to 1 mM acetylcholine of 20 mM $CaCl_2$ contracted ring was measured. Each experimental point is an average of five independent observation. ×, aortas cultured in medium containing 0.4% serum + cysteine (Cys) for 14 days; △, glutathione (Glu); □, homocysteine in the presence of 300 μM nicotinamide (H(e) + Nic); ◆, homocysteine [H(e)] alone. The medium was replaced every day with fresh reagents. The x-axis, concentration (μM) is referred to all the agents used.

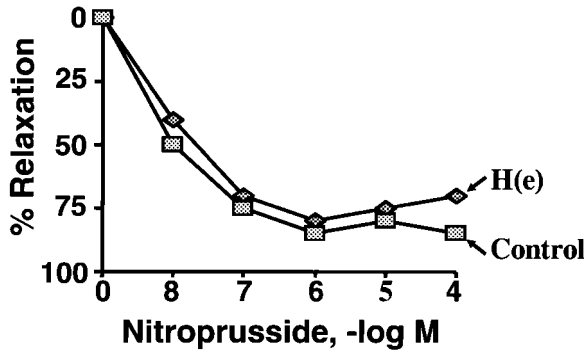

Fig. 4. Nitroprusside-dependent response in control and homocysteine treated aortas: ◆, aortas were cultured in medium containing 0.4% serum + 100 μM homocysteine [H(e)] for 14 days; □, control aorta 14 days in 0.4% serum condition. The medium was replaced every day. The tension in grams was normalized by weight in grams of ring. The percent relaxation to 20 mM $CaCl_2$ concentration is reported. Each experimental point is an average of five independent observation.

fold thicker, $P = 0.03$ when compared with control. The treatment with nicotinamide decreased elastin disruption. Western blot analysis revealed decrease nitrotyrosine in the vessel co-treated with homocysteine + nicotinamide (Fig. 6A). Elastin zymographic analysis on aortic tissue homogenates demonstrated increased constitutively expressed MMP-2, 72 kDa, gelatinase A as well as induction of gelatinase B, 92-kDa in the homocysteinemic vessels (Fig. 6B). This induction was inhibited by nicotinamide.

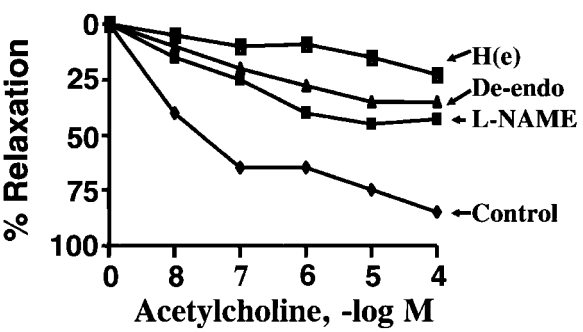

Fig. 3. Dose-dependent vascular relaxation by acetylcholine: ×, aortas were cultured in medium containing 0.4% serum + 100 μM homocysteine [H(e)] for 14 days; △, endothelial-denuded (De-endo) aorta by treatment with 0.01% Triton X-100 for 5 sec; ■, L-NAME (300 μM) treated aorta for 30-min; ◆, control aorta 14 days in 0.4% serum condition. The medium was replaced every day. The tension in grams was normalized by weight in grams of ring. The percent relaxation to 20 mM $CaCl_2$ concentration is reported. Each experimental point is an average of five independent observation.

Role of Metalloproteinase in Vascular Tissue Distensibility

Figure 7 depicts the experimental data on length vs. tension generated in the aortas cultured for 14 days with homocysteine, control aorta and aorta treated with active MMP-2. The curves for the fresh and control vessel in serum condition for 14 days were identical. The control vessel was practically linear until it had been stretched to more than 140%. In contrast, homocysteine or gelatinase treated vessel immediately become steep. To determine whether the treatment of active exogenous gelatinase produces elastin breakdown similar to homocysteinemic aortas, we incubated aortas with 10 μM purified active rat heart gelatinase A. The aortas were stained with van Gieson for elastin. The results suggested that 30 min incubation with active MMP-2 decreased vascular elastic compliance (Fig. 7).

Endothelial Injury

To determine whether endothelial dysfunction is associated with endothelial injury, we measured intracellular caspase activity in situ and in primary vascular endothelial cell monolayer. Caspase is an intracellular interleukin converting enzyme and a marker of cell death

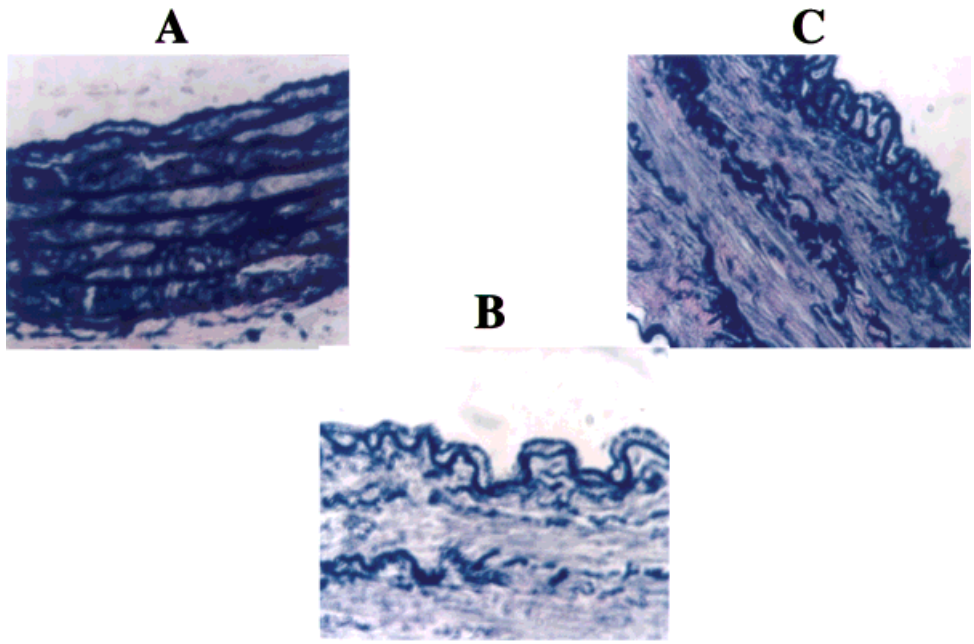

Fig. 5. Histological analysis of elastin-breakdown of aortas cultured with or without 100 μM homocysteine for 14 days. The control aortas **(A)**; aortas cultured with homocysteine **(B)**; aortas cultured with homocysteine + (300 μM) nicotinamide **(C)**. The aortas were stained with van Gieson for elastin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

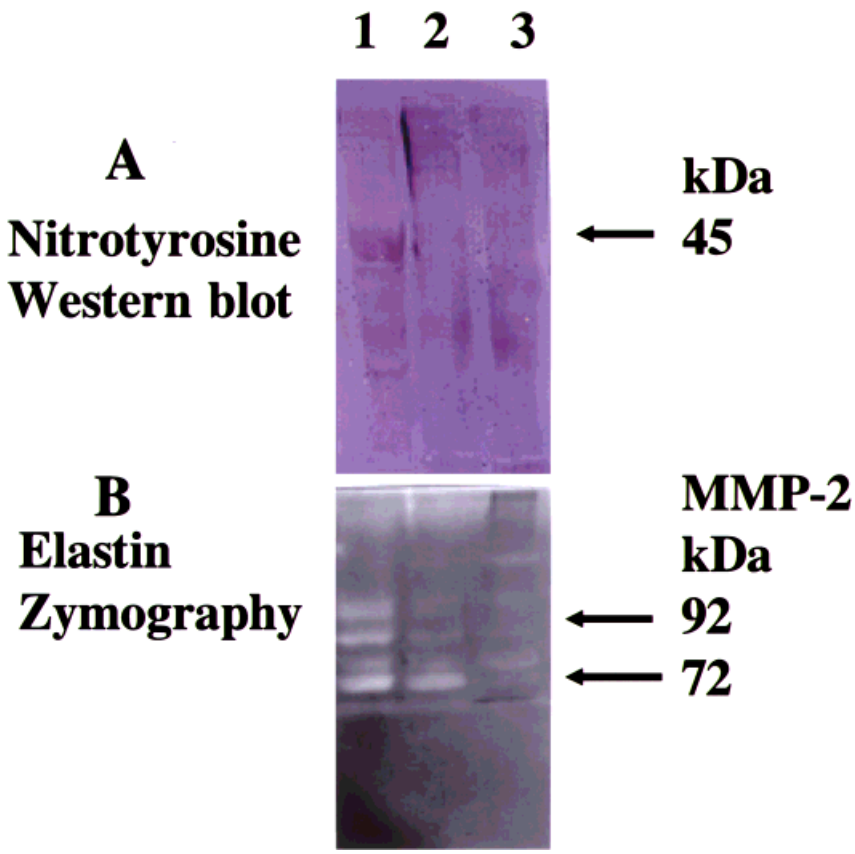

Fig. 6. A: Western-blot analysis for nitrotyrosine: tissue homogenates were prepared from cultured control, homocysteine, homocysteine + nicotinamide treated aortas. The SDS–PAGE and Western-blot were performed by loading same amount of total protein in each lane. **Lane 1**, aorta cultured with homocysteine; **lane 2**, cultured with homocysteine + nicotinamide; **lane 3**, control aorta. **B:** Elastin-zymographic analysis of the samples used in A. The elastin zymography was performed by loading same amount of total protein in each lane. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

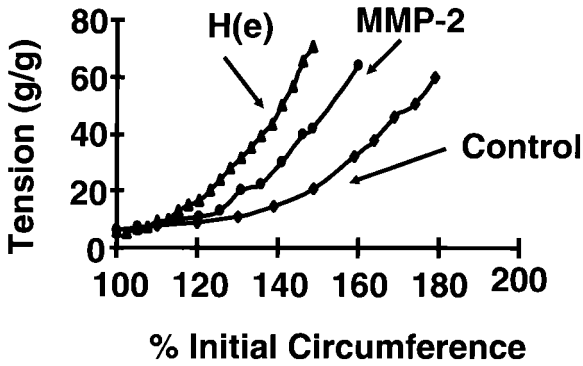

Fig. 7. Length-tension relationships of aortas control (◆), cultured with homocysteine [H(e)] (△) and control aortas treated with gelatinase A (10 μM, MMP-2) (●) prior to the measurements of stretch-strain: Each aortic ring was systematically stretched to the optimum of its length-active tension relation. A known amount of stretch placed on the ring, the tension was measured in physiological salt solution. The increment in length was divided by initial length and percent initial circumference of the rings are reported on x-axis. Tension was normalized by weight of the tissue and reported on y-axis. Each point is an average of five rings from five different rats.

and injury. Labelling was carried out using a florogenic substrate for caspase, Ac-YVAD-AMC. This substrate produces florescence after cleavage by caspase. The serial aortic tissue sections similar to histology were incubated with caspase substrate. The florescence in control and homocysteinemic aortas was specifically increased in the endothelial lining of the homocysteinemic aortas as compared to the control vessels. To support that endothelial cells were activated and injured by homocysteine, freshly isolated endothelial cells (cobblestone appearance) from normal rat aortas were incubated with homocysteine and then were labelled with caspase substrate (Fig. 8). Results suggested that homocysteine-treated cells were elongated and indicated strong labelling for caspase activity than controls.

Role of Proteinase Inhibition in Vascular Function

To determine whether inhibition of gelatinase and caspase improves vascular function in homocysteinemic aortas, the aortas were cultured with homocysteine in the presence of TIMP-4 or YVAD-CHO, inhibitor of gelatinase and inhibitor of caspase, respectively. The vascular reactivity to acetylcholine was measured.

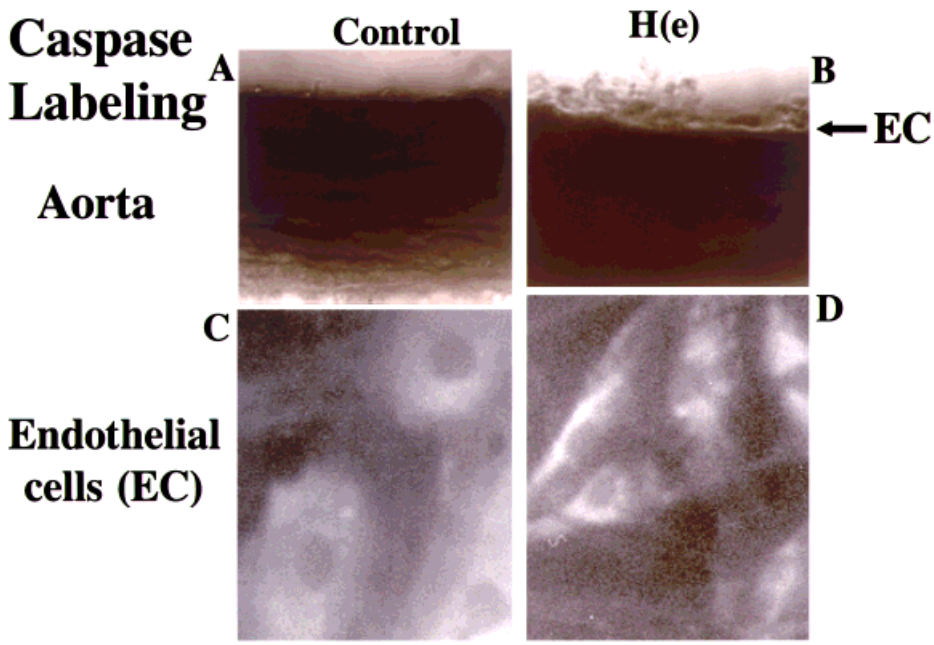

Fig. 8. Endothelial injury: caspase activity in the aortas **(A** and **B)** and isolated rat aortic endothelial cells **(C** and **D)** cultured without (Control) or with 100 μM homocysteine, [H(e)], respectively, was measured using florogenic substrate (Ac-YVAD-AMC) for caspase. Thin (~10 μm) aortic segments were cultured for 14 days in condition medium. The segments were incubated at 37°C with 1 mM caspase substrate in phosphate buffered saline (PBS) containing 0.01% Tween-20. After washing with PBS the florescence was visualized under florescence microscope. Arrow indicates marked labelling in the endothelium of homocysteine-treated aortas. The endothelial cells were grown on cover slip and permeabilized by 10% methanol. After washing coverslips were incubated with caspase substrate for 1 h and visualized under florescence microscope. Note: cobblestone appearance of endothelial cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

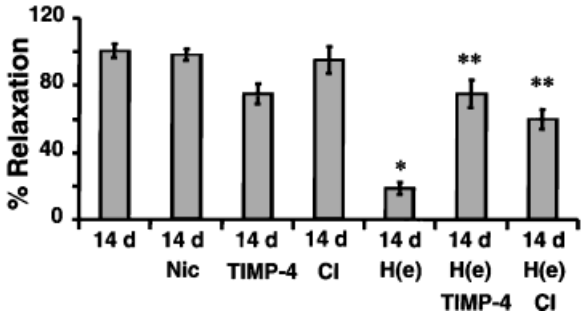

Fig. 9. Effect of prior treatment of 40 μM TIMP-4 or an caspase inhibitor (100 μM, YVAD-CHO) on vascular function 14 days without or with homocysteine treatment: Aortas were cultured in the presence of 100 μM nicotinamide (Nic), or 100 μM homocysteine [H(e)]; or in the presence of 40 μM TIMP-4; or 100 μM caspase inhibitor (CI). The rings were contracted with 20 mM $CaCl_2$ prior to relaxing by 1 mM acetylcholine. The percent relaxation was reported. Each bar is an average of five rings from five different rats. $^{*}P$ value < 0.01 when compared with aortas cultured without homocysteine; $^{**}P$ value < 0.02 when compared with aortas cultured with homocysteine.

The data suggested that TIMP-4 and YVAD-CHO preserve vascular function and reverse the injury response of homocysteine (Fig. 9).

DISCUSSION

Previously, we have demonstrated increased intracellular calcium in vascular SMCs by homocysteine in order of homocysteine > glutathione > cysteine [Mujumdar et al., 2000]. Here, we show by dose–response curves generated for homocysteine, glutathione, and cysteine that homocysteine impairs significantly greater vasodilatory function than glutathione and cysteine. The order of impairment was homocysteine > glutathione > cysteine. All thiol reagents reduced vascular function at a concentration 100 μM. The treatment of nicotinamide reverses impairment of aortas by homocysteine. Similar reversal was observed with glutathione and cysteine (Fig. 2). The results suggested that thiols decrease nitric oxide availability by generating peroxinitrite and this decrease is reversed by nicotinamide.

Aortic explants in culture have been shown to maintain a well-formed sheet of endothelial cells positioned around the explant upto $1-1\frac{1}{2}$ weeks [Diglio et al., 1989]. We demonstrated that the aortic organ culture condition allows not only ECM homeostasis like in vivo around the SMCs but also maintains viable endothelium upto two weeks (Figs. 3 and 4). In the presence of homocysteine, the aortas demonstrated decrease in the availability of viable endothelium. The decreased acetylcholine response in homocysteine treated vessels was similar to denuded or L-NAME treated vessels (Fig. 3). However, the response to endothelial-independent vasodilator, nitroprusside, was not altered significantly by homocysteine as compared to control (Fig. 4). Our results may suggest that treatment of homocysteine or removal of endothelium or treatment with L-NAME reduces endothelial-dependent vasodilatation and exposes smooth muscle to exogenous stimuli. Removal of endothelium in vivo has been shown to stimulate DNA synthesis in medial cell and migration of SMC [De Mey et al., 1991; Tsai et al., 1994].

Morphometric analysis demonstrated degradation of elastic fibers in homocysteine treated vessels as compared to control (Fig. 5A and B). This may suggest migration and hypertrophy of underlying SMCs, leading to increase vascular medial thickness. The gradual loss of elastin and its replacement with collagen, that accompanies endothelial damage may lead to amplify the increase in blood pressure as observed in homocysteinemic pigs [Rolland et al., 1995]. The treatment of nicotinamide reduces elastin breaks (Fig. 5C) as well as reduces nitrotyrosine formation in aortas treated with homocysteine (Fig. 6A). This may suggest a role of peroxinitrite and nitric oxide in elastinolysis. In vivo decreased nitric oxide production leads to increase MMP-2 activity [Rodomski et al., 1998]. The MMP-2 degrades elastin [Tyagi et al., 1995]. Our results suggest increased elastinolytic MMP-2 in aortas cultured with homocysteine. This increase was reduced by the co-treatment of aortas with homocysteine + nicotinamide (Fig. 6B). However, it is difficult to know that indeed the reduction in acetylcholine-induced dilation in response to homocysteine is due to the lack of NO and reversing of the response by nicotinamide is due to regaining NO-mediation. This question is especially important, since relaxation to acetylcholine, in addition to NO, involves prostaglandins and endothelial derived hyperpolarizing factors (EDHF), factors that may be affected by high homocysteine levels. These studies are in progress.

The length–tension curves shifted to left in aortas cultured with homocysteine and/or ex vivo MMP-2 treatment (Fig. 7). These results suggested reduced elastic resistance in homo-

cysteine or MMP-2 treated vessels as compared to control. The elastic properties of aortas depend largely on the presence of a long-lived elastin in the vessel wall. Elastin accounts for 40–50% of the dry weight of the tissue of aortas and a major component in the coronary and carotid arteries [Martyn and Greenwald, 1997]. The Young's stiffness module constant for elastin is 3×10^6 dynes/cm^2 and for collagen is 1×10^9 dynes/cm^2 [Roach and Burton, 1957]. Collagen is about 1,000 times stiffer than elastin and the gradual loss of elastin is replaced by collagen and is inevitably accompanied by a reduction in vascular elastic compliance [Martyn and Greenwald, 1997].

Homocysteine injures endothelium [Harker et al., 1976; Lentz, 1996]. We demonstrated that the endothelium of aortas treated with homocysteine increase caspase activity (Fig. 8), an enzyme induced during apototsis [Enari et al., 1998]. To determine the cause and effect relationship between elastin breakdown, endothelial damage, and vascular contractile function, we measured acetylcholine response in homocysteine treated vessels in the presence of metalloproteinase inhibitor, TIMP-4, and a caspase inhibitor, YVAD-CHO. The treatment with TIMP-4 and caspase inhibitor reverses the vascular dysfunction by homocysteine (Fig. 9). In aortic aneurysms MMPs are activated and elastin is degraded [Boutouyrie et al., 1999]. TIMPs inhibit gelatinase A and B [Knox et al., 1997]. The inhibition of proteinases may improve vascular structure and function in hyperhomocysteinemia.

PERSPECTIVE

Homocysteine injures endothelium and proliferate underlying SMCs. This leads to contractile abnormalities in the vessel wall. The elastin disruption is mediated by activation of gelatinase, leading to impairment of vascular function. The inhibition of elastin breakdown and endothelial injury improve vascular function. The decrease in elastin content in the vessel wall is directly proportional to a decreased vascular compliance. Inhibition of NO production increases elastin degradation. It would be of great interest to determine whether the in vivo increased NO production increases elastin content and improves vascular elastic compliance by inhibiting metalloproteinase production.

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