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ORIGINAL ARTICLE

Chronic hyperhomocysteinemia impairs vascular function in ovariectomized rat carotid arteries

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Abstract Homocysteine is an independent risk factor for coronary heart disease, as well as for cerebrovascular and peripheral vascular diseases. The purpose of this study was to investigate the effects of hyperhomocysteinemia (HHcy) on vascular reactivity within carotid artery segments isolated from ovariectomized female rats. Treatment with DL-Hcy thiolactone (1 g/kg body weight per day) reduced the phenylephrine-induced contraction of denuded rings. However, the treatment did not alter KCl-induced contractions, or relaxations induced by sodium nitroprusside or acetylcholine. We report elevated expressions of iNOS, eNOS, and nitrotyrosine in homocysteine-treated rat artery sections. Moreover, the inhibition of NOS by L-NAME, 1,400 W, or L-NNA restored phenylephrine-induced vasoconstriction in carotid artery segments from Hcy-treated rats. In conclusion, our findings show that severe HHCy can promote an acute decrease in the endothelium-independent contractile responses of carotid arteries to adrenergic agonists. This effect was restored by nitric oxide synthase inhibitors, which further supports the involvement of nitric oxide in HHcy-derived vascular dysfunction.

Keywords Homocysteine · Female rat · Carotid artery · Contraction

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Introduction

Butz and du Vigneaud (1932) described a cysteine homolog, derived from the metabolism of methionine, which they called "homocysteine" (Refsum et al. 1998). After 73 years, McCully (2005) observed that increased plasma levels of homocysteine (Hcy) correlated to the development of atherosclerosis. Since then, several studies have confirmed the association between hyperhomocysteinemia (HHcy) and premature atherosclerosis in coronary, cerebrovascular, and peripheral vascular beds (Kang et al. 1986; Israelsson et al. 1988; Malinow et al. 1989; Selhub et al. 1995).

The known mechanisms by which HHcy induces vascular disease are (a) protein homocysteinylation, (b) oxidative stress, and (c) inflammation promoted by an increased production of cytokines and chemokines (Starkebaum and Harlan 1986; Jakubowski et al. 2000; Desai et al. 2001).

During protein homocysteinylation, the conversion of homocysteine to homocysteine-thiolactone occurs inside the cell, catalyzed by methionyl-tRNA-synthetase.

Subsequently, homocysteine-thiolactone interacts with lysine residues on protein chains [i.e., albumin, hemoglobin, low density lipoprotein (LDL), membrane-associated proteins of the endothelium, and others]. This process reduces the solubility and functionality of the proteins. Additionally, these protein/thiolactone adducts can be recognized by macrophages, leading to phagocytosis of endothelial cells and damage to the endothelium (Jakubowski et al. 2000).

Normal plasma concentrations of Hcy range from 5 to 15 μmol/l (Skurk and Walsh 2004). The Physician's Health Study demonstrated that patients presenting elevated plasma Hcy levels had a threefold increase in the risk of myocardial infarction, suggesting that HHcy leads to accelerated atherosclerosis (Eikelboom et al. 1999). Plasma Hcy levels are, in part, genetically determined; but nutritional deficiencies (such as in folate, vitamin B_6, and vitamin B_{12}), enzyme abnormalities, renal failure, and postmenopausal conditions may increase Hcy levels (Ridker et al. 1999; Yen and Lau 2002).

It is well known that sex hormones influence plasma Hcy concentrations. Males have higher Hcy levels than age-matched females (Boers et al. 1983), and females tend to exhibit higher Hcy concentrations after menopause (Boers et al. 1983; Wouters et al. 1995; Hak et al. 2000). Since Hcy is a risk factor for cardiovascular disease, high levels of homocysteine in postmenopausal women can explain their susceptibility to coronary heart disease. In this study, we investigated the acute effects of HHcy upon the vascular function of carotid arteries within ovariectomized female Wistar rats.

Materials and methods

Animals and surgery

The experimental protocols used in the present study conformed to the standards and policies of the Animal Ethics Committee of the Ribeirão Preto Campus, University of São Paulo. Female mature Wistar rats, aged between 60 and 70 days (230–250 g), were subjected to a bilateral ovariectomy to simulate menopause. The rats were anesthetized intraperitoneally with ketamine (46 mg/kg) and xylazine (14 mg/kg), and both ovaries were ligated and removed. Animals were then returned to a cage with free access to water and food.

Homocysteine supplementation

Three weeks after the ovariectomy, the animals were divided into two groups (control and Hcy-treated). The Hcy-treated group received a solution of DL-Hcy thiolactone hydrochloride (Sigma-Aldrich®) (1 g/kg body weight per day) for 15 days (ad libitum) and the control group only received water. The DL-homocysteine-thiolactone (DL-HcyT) is water soluble, and is converted to Hcy by hydrolysis, thereby increasing plasma Hcy levels. The experiments were performed 15 days after the start of DL-HcyT administration.

Determination of plasma homocysteine levels

Blood samples (5 ml) from both groups were collected with 7% EDTA (200 μl/5 ml blood) and centrifuged at 3,000×*g* for 20 min. To minimize the release of Hcy from blood cells, blood collection was performed in iced tubes, and centrifugation was carried out at 4°C. Plasma was then stored at −70°C until assayed. Total Hcy concentrations were measured with a Q-TRAP triple quadrupole mass spectrometer (Applied Biosystems-Canada; Perkin-Elmer Sciex/QqQ-Trap, Perkin-Elmer Sciex, Thornhill, ON, Canada) using positive electrospray ionization in Multiple Reaction Monitoring mode (MRM). Separation was carried out by HPLC at room temperature (22–25°C), using a C18 guard column fitted to a C18 analytical column (3.9 mm × 150 mm i.d., 5 μm; Symmetry), coupled to a Waters Alliance HPLC system. For sample preparation, the plasma was thawed at room temperature. An aliquot of plasma (100 μl) was then mixed with 20 μl of homocystine-d4 (I.S., 20 μM). Oxidized forms of Hcy found in the blood were reduced by the addition of 20 μl of dithiothreitol (500 μM) and mixed by vortex for 15 s. After 20 min at room temperature, 200 μl of deproteinizing solution and 300 μl of mobile phase were added and mixed vigorously, followed by centrifugation for 15 min at 1,300×*g*. The supernatant was used for the quantification of Hcy. The isocratic mobile phase was acetonitrile:water (60:40 v/v), acidified by the addition of formic acid to 0.1%; and the flow rate was held constant at 0.8 ml/min. Total eluent flow from the HPLC system was directed to the turbo-ion spray source through a splitter (1:10). The needle voltage was set at 5.5 kV; the nebulizer gas (nitrogen) pressure was set to 60 psi; the curtain gas (nitrogen) pressure was set to 25 psi; and the collision cell energy was set at 5 eV. The turbo-ion spray heater temperature was set at 200°C, and the heater gas flow pressure was 75 psi. For quantification of Hcy and homocysteine-d4, the MRM (Multiple Reaction Mode) mode was used. The instrument was optimized to monitor the parent ions ($Q1 > Q3$) of Hcy (m/z 136.1 > 90.0) and homocysteine-d4 (m/z 140.1 > 94.0), products provided by fragmentation in q2, the collision cell. As stated earlier, the rats were administered DL-HcyT, and not Hcy; however, the former compound is converted in vivo to Hcy that can be measured in plasma.

Measurement of vascular tone

The rats were anaesthetized (ketamine, 50 mg/kg) and exsanguinated by abdominal puncture of the aorta. Then, the carotid arteries was carefully removed, placed on a Petri dish filled with Krebs solution, and cut into 5 mm-long segments. The rings were placed immediately in an organ chamber filled with 5 ml of Krebs solution with the following composition: 118 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, and 11.6 mM glucose. The vessels were kept at 37°C, and continuously gassed with 95% O_2/5% CO_2. Tension was measured isometrically with a force transducer connected to a data acquisition system (Proto 5, Letica, Spain). The rings were gradually stretched to a basal tension of 1.0 g, and then allowed to equilibrate for 60 min. In a preliminary study, a basal tension of 1.0 g was found to develop 80% tension in response to stimulation with a contracting agent (potassium chloride). In some preparations, the endothelium was mechanically removed by gently rolling the lumen of the vessel on a thin wire. Endothelial integrity was assessed qualitatively by the degree of relaxation caused by acetylcholine (Ach) (10^{-6} M) in the presence of contractile tone induced by phenylephrine (Phe) (10^{-7} M). For studies of endothelium-intact vessel rings, a ring was discarded if its relaxation with acetylcholine did not equal or exceed 80%. Cumulative concentration–response curves were generated using Phe (10^{-10} to 10^{-6} M), KCl (10–90 mM), Ach (10^{-10} to 10^{-5} M) and sodium nitroprusside (SNP) (10^{-10} to 10^{-5} M). After the vascular reactivity experiments were completed, all carotid artery segments were dried. Their dry weight was determined, and used to express the contraction induced by each drug (mm of contraction/g dry tissue). Experiments were conducted in the presence and absence of three NO-synthase inhibitors: NG-nitro-L-arginine-methyl-ester (L-NAME, 10^{-4} M), 1,400 W (10^{-6} M), and L-NNA (10^{-6} M). Arterial segments were incubated with each inhibitor for 30 min prior to constructing a concentration–response curve to Phe. We used eight arteries ($n = 8$) from different animals of each group in this protocol.

Histological analysis

Carotid arteries from the control and Hcy-treated groups were also analyzed histologically. Rats from each group were euthanized, and the arteries were fixed in situ by constant pressure fixation with formalin (10%) through a 22-gauge butterfly angiocatheter placed in the left ventricle. Both the right and left carotid arteries were harvested. Each artery was embedded in paraffin, and cross-sections (6 μm) were obtained. The sections were subjected to standard hematoxylin/eosin and Masson's trichrome staining, as well as to immunohistochemistry.

Immunohistochemical analysis

Staining for iNOS, eNOS, and nitrotyrosine were performed on paraffin-embedded tissues. A rabbit polyclonal (1:200, Transduction Laboratories) antibody was used as the primary antibody. After washing with phosphate-buffered saline (PBS), sections were incubated for 1 h with a biotinylated secondary goat anti-rabbit IgG (1:100, Vector Laboratories), and then reacted with an avidin–biotin complex (Vecstatin Elite ABC kit, Vector). Signal detection was performed using 3,3′-diaminobenzidine (DAB) (Vector) and counterstained with hematoxylin. We used ten arteries ($n = 10$) from different animals (control and Hcy-treated groups) for this protocol.

Nitrate levels in vascular homogenates

Nitrate levels were measured in supernatants obtained from total carotid artery homogenates prepared under liquid N_2. Homogenates were centrifuged at $3{,}000 \times g$ for 10 min, and 10 μl aliquots of supernatant were injected into a Sievers chemiluminescence analyzer (Model 280). Nitrate levels were analyzed by reduction with a saturated acidic solution of VCl_3 (0.8 g of VCl_3 in 1 M HCl) at 95°C. Nitrate generation experiments were performed using endothelium-intact rat carotid segments. Nitric oxide results were normalized against protein concentrations, which were separately assessed with the Bradford technique. We used eight arteries ($n = 8$) from different animals (control and Hcy-treated groups) for this protocol.

Statistical analysis

Maximal tensions generated by the agonist (Emax) and pD_2 ($-\log EC_{50}$) values were calculated using the Graph Pad Prism computer program (version 3.0). All data are reported as mean ± SEM (standard error of the mean). All experiments were repeated eight times, with the exception of immunohistochemical analyses that were performed with ten laminas per antibody per group. Statistical comparisons between the control and Hcy-treated groups were made by two-way ANOVA followed by Bonferroni testing, with the level of significance set at $P < 0.05$.

Results

Average plasma Hcy levels were found to be 4.34 ± 0.55 μM in the control group, and 207.17 ± 16.79 μM in the Hcy-treated group. Supplementation with

Hcy produced a significant increase (≈50-fold) in Hcy plasma levels, but this did not significantly change either the mean arterial blood pressure or the vessel morphology. However, the body weight of HHcy animals was reduced (Table 1).

Vascular reactivity

Neither endothelium-dependent nor endothelium-independent relaxations were altered by HHcy (Fig. 2a, b). Similarly, contractile responses to KCl did not differ between the control and Hcv-treated groups of intact and denuded rings (Fig. 1).

Contractions induced by Phe were significantly reduced in denuded rings from HHcy rats (0.76 ± 0.07*) when compared with similarly denuded rings from control animals (1.22 ± 0.11) (Fig. 3b). In contrast, in endothelium-intact rings, the efficacy of Phe in the Hcy-treated (0.68 ± 0.13) and control (0.71 ± 0.09) groups were similar (Fig. 3a).

The role of nitric oxide signaling in the Phe contractile response was evaluated in denuded arteries from HHcy animals. Three different NOS inhibitors (L-NAME, L-NNA, and 1,400 W) were found to re-establish Phe-induced vasoconstriction (Fig. 4a, b). These results suggest that HHcy leads to an increase in iNOS and eNOS activity, and consequently to an increased production of NO in vascular smooth muscle.

Homocysteine effect in the nitrate production

Nitrate was used as an indicator of nitric oxide production. Our present findings show that Hcy supplementation increased the generation of nitrate (Fig. 4c). This is an indirect but reliable measure of nitric oxide.

Effects of homocysteine on iNOS, eNOS, and nitrotyrosine immunoreactivity

In carotid artery sections from control animals, iNOS and nitrotyrosine immuno-staining was weakly detectable in endothelial cells or in the medial layer. In artery sections from the Hcy-treated group, iNOS and nitrotyrosine expression increased in the medial layer, while eNOS expression was increased in the adventitial layer (Fig. 5).

Table 1 Blood pressure, age, and body weight before and after HHcy induction

Before Hcy ingestion		After 15 days of Hcy ingestion	
Control ($n = 24$)	Hcy ($n = 24$)	Control ($n = 24$)	Hcy ($n = 24$)
Age (days)			
60–70	60–70	75–85	75–85
Body weight (g)			
337.7	334.6	377.5	335.3
Blood pressure (mmHg)			
107	106	103	105

Discussion

Yen and Lau (2002) previously showed that acetylcholine-induced relaxation of the aorta in rats fed with methionine was impaired in male, but not in female, rats; and, furthermore, that this relaxation was restored when male rats received estrogen (Yen and Lau 2002). These studies demonstrate that estrogen promotes some kind of vascular protection. Thus, in the present study, we used ovariectomized female rats to evaluate the impact of HHcy upon vascular function with minimal interference from estrogen; i.e., under conditions similar to menopause. In these rats, the average estrogen level dropped from 95 pg/ml (estrous phase) to 35 pg/ml (constant, post-surgery).

The present study examined the in vivo effects of short-term, severe HHcy on the contractility of female rat carotid artery segments. We demonstrated a reduction in adrenergically induced arterial contractions, but not in endothelial function, after 15 days of severe HHcy. Previous studies have shown that HHcy-induced endothelial dysfunction is more time-dependent than dose-dependent (Lentz et al. 1996; Tawakol et al. 1997; Woo et al. 1997; Hanratty et al. 2001). In the present study, we induced a short-term, severe HHcy, which may explain why we did not observe endothelial dysfunction in this model.

Although the average plasma concentration of Hcy chosen for our experiments was very high, it is not impossible to find patients with similar Hcy concentrations (Cipolla et al. 2000). Toxic effects of high-dose Hcy in rats include the impairment of endothelial cell growth (Chen et al. 2000), specific tissue lesions (heart, liver, and kidney), and hematologic abnormalities (Brown and Strain 1990), but these effects need chronic exposure to be detected.

Bokhari et al. showed that in hypertensive patients, HHcy predicts mortality by cardiovascular disease and a low left ventricular ejection fraction, independent of coronary artery disease (CAD) and history of myocardial infarction. These trends were not observed in normotensive patients (Bokhari et al. 2005). Marcucci et al. (2004) reported that patients with non-valvular atrial fibrillation (AF) had increased plasma Hcy levels (Hak et al. 2000). Shimano et al. (2008) presented a study in which plasma levels of Hcy were evaluated in a group of patients scheduled to undergo catheter ablation of AF (Bonaventura

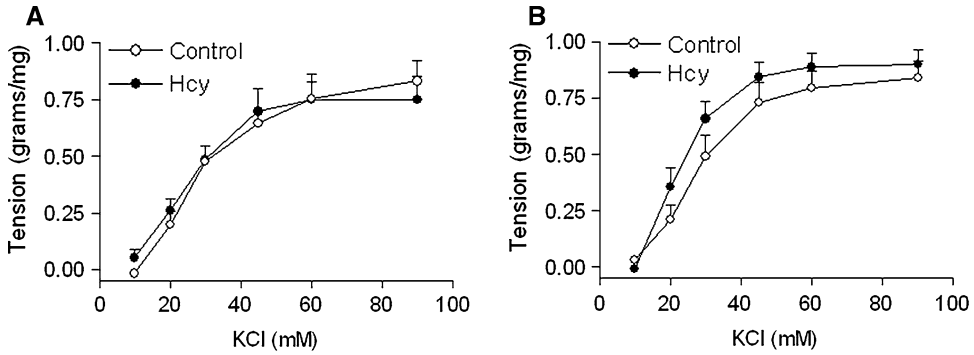

Fig. 1 KCl-induced contraction of carotid artery segments. Concentration–response curves for contractions induced by KCl (10–90 mM) in control and Hcy-treated carotid artery segments. **a** In the presence of endothelium. **b** In the absence of endothelium. Data expressed as mean ± SEM ($n = 8$)

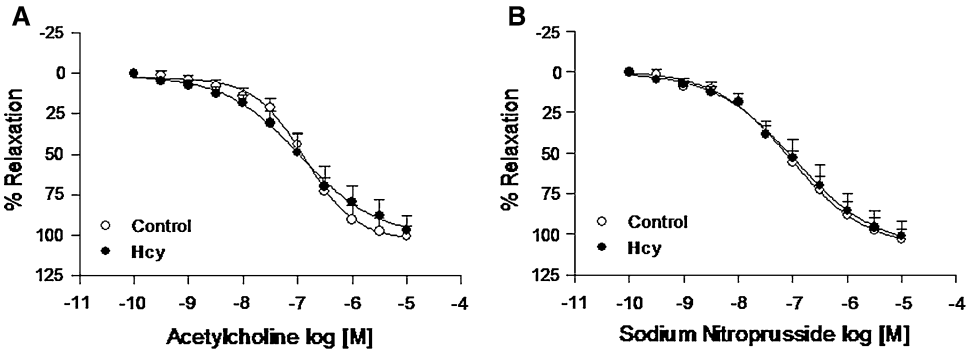

Fig. 2 Cumulative concentration–response curves of Ach and SNP. **a** Concentration–response curves for relaxation induced by Ach (10^{-10} to 10^{-5} M) in control and Hcy-treated carotid artery segments with intact endothelium. **b** Concentration–response curves for relaxation induced by SNP (10^{-10} to 10^{-5} M) in control and Hcy-treated carotid artery segments in the absence of endothelium. Data expressed as mean ± SEM ($n = 8$)

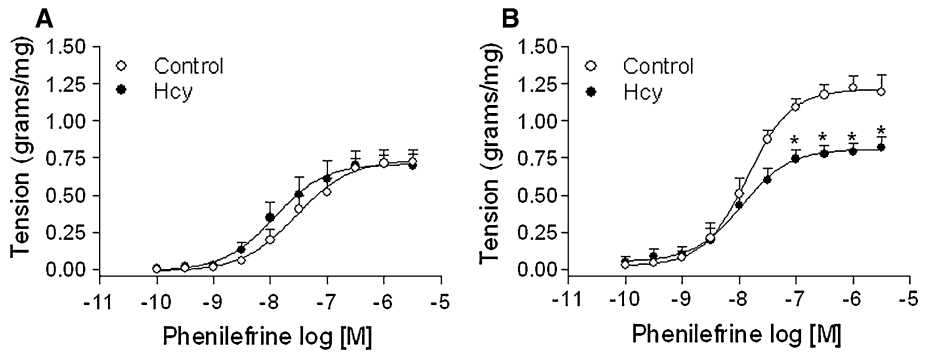

Fig. 3 Cumulative concentration–response curves of Phe. **a** Concentration–response curves for contractions induced by phenylephrine (10^{-10} to 10^{-5} M) in control and Hcy-treated carotid artery segments with intact endothelium. **b** In the absence of endothelium. Data expressed as mean ± SEM ($n = 8$). *Asterisk* indicates significant difference ($P < 0.01$) from control group

et al. 2009). Their results confirmed those of Marcucci et al., and documented a significant increase in plasma Hcy in patients with persistent (but not paroxysmal) AF.

We reasoned that the attenuation of adrenergically induced arterial contractions within HHcy rats could be due either to the specific blockage of the adrenergic stimulus, or to the release of vasodilatory factors. The latter is more likely, since the inhibitory effect of Hcy on Phe-induced contractions was abolished in denuded arterial segments by nitric oxide synthase inhibitors (L-NAME, 1,400 W, and L-NNA). This inference is strengthened by immunohistochemical staining, which demonstrates an increased expression of eNOS and iNOS in carotid arteries from HHcy rats. Moreover, increased levels of nitrate were observed in aortic tissues from HHcy rats. In agreement with our results, Wang et al. (2000b) demonstrated that

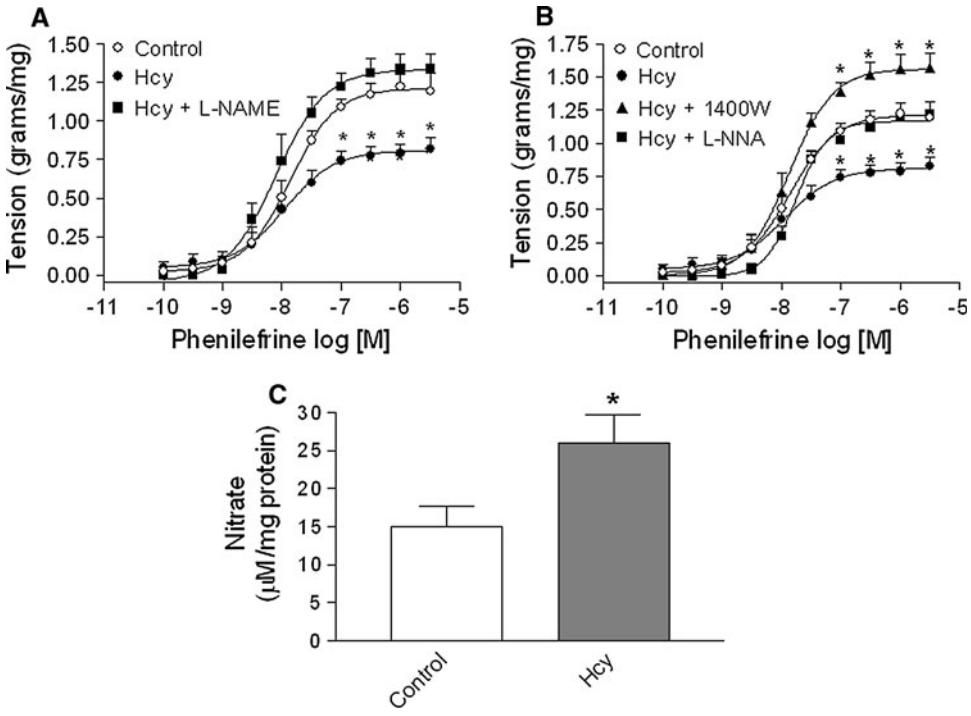

Fig. 4 Effect of nitric oxide synthase inhibitors on arterial contractions induced by phenylephrine and on nitrate levels. Concentration–response curves for contractions induced by phenylephrine (10^{-10} to 10^{-5} M) in control and Hcy-treated carotid artery segments denuded of endothelium. **a** In the presence of L-NAME. **b** In the presence of LNNA and 1,400 W. Also shown are plasma nitrate levels (**c**). Data expressed as mean ± SEM ($n = 8$). *Asterisk* indicates significant difference ($P < 0.01$) from control group ($n = 8$)

short-term exposure (4 days) to Hcy depressed rat aortic contractility to norepinephrine through an increase of NO production. The authors also showed an increase in eNOS and iNOS levels.

Moreover, our results show that the impaired contractile response of carotid arteries is relatively specific for adrenergic reactivity, since contractions induced by potassium or angiotensin II (data not shown) were not altered in HHcy aortas. This reduced sensitivity to adrenergic stimulation, but not to potassium, was also demonstrated in norepinephrine-stimulated arteries perfused with Hcy (Cipolla et al. 2000).

Another mechanism by which homocysteine could cause vasodilation is through an (*S*)-nitrosothiol metabolite with potent vasodilatation properties (Upchurch et al. 1996). Stamler et al. (1993) showed that Hcy can react with NO to form nitrosohomocysteine (*S*-NO-Hcy), and promote vasodilation through a cyclic guanosine monophosphate (cGMP)-mediated mechanism. However, we did not detect any changes to SNP or Ach sensitivity within arteries from HHcy animals.

It has been reported that HHcy is associated with endothelial dysfunction and reduced NO bioavailability (Upchurch et al. 1997; Bellamy et al. 1998; Chao et al. 2000). According to Eberhardt et al. (2000), this reduction in NO bioavailability is due to reactions of this molecule with free radicals generated by oxidative respiration (H_2O_2 and $O_2^{\cdot-}$), leading to the production of peroxynitrite, a powerful oxidant. Immunohistochemical analysis of vascular smooth muscle from HHcy animals revealed an increased staining of nitrotyrosine residues. However, this increase in oxidative stress was not enough to reduce NO bioavailability in vascular smooth muscle, and a possible explanation for this can be the increased expression of eNOS and iNOS.

The present results indicate that, despite the endothelial cell damage and death found by others, the early effects of Hcy are functionally specific, and most likely relate more to changes in cell synthesis or secretory activity than to structural or morphological damage. The increased activity and expression of iNOS in HHcy arteries, as observed by vascular reactivity and imunohistochemistry, could indicate an ability of Hcy to modulate gene expression. Recent findings that Hcy induces activation of nuclear factor-κB (NF-κB) and increases nitric oxide synthase expression in smooth muscle cells further suggest a role for Hcy in the modulation of vascular function (Welch et al. 1998; Ikeda et al. 1999). Previous reports have shown that chemokines promote atherogenesis by enhancing the production of several pro-inflammatory cytokines, such as MCP-1 and IL-8 (Ross 1999; Wang et al. 2000a, 2001; Poddar et al. 2001; Kerkeni et al. 2006). The induction of chemokine neosynthesis by Hcy was previously shown to depend on NF-κB, a transcription factor mediating downstream inflammatory processes (Collins and Cybulsky 2001; Au-Yeung et al. 2004). The Hcy-thiolactone used in this study generates reactive oxygen species more efficiently than Hcy, leading to NF-κB activation and induction of pro-inflammatory processes (Kerkeni et al. 2006).

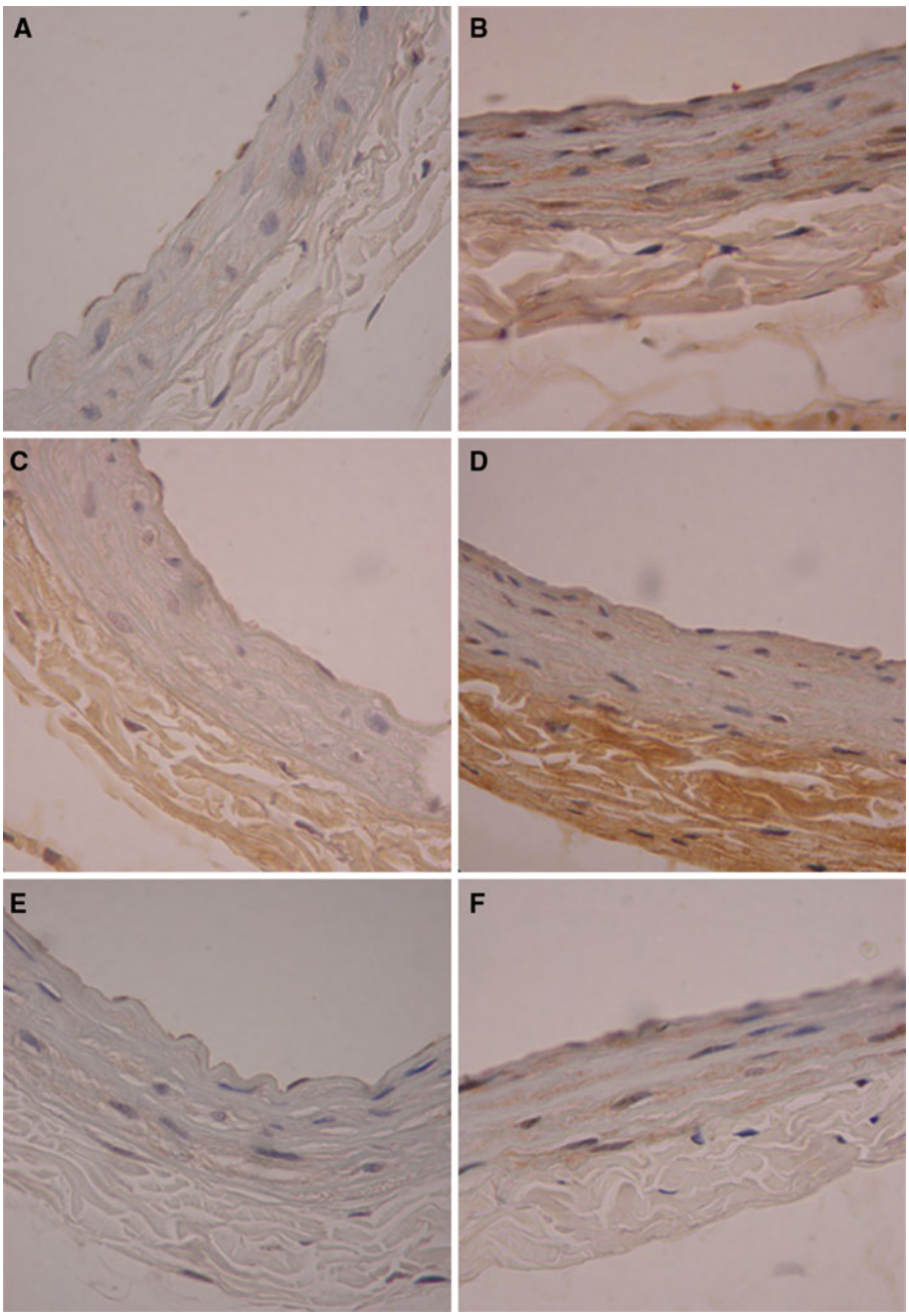

Fig. 5 Expression of eNOS, iNOS, and nitrotyrosine in carotid arteries. **a** iNOS expression, control group. **b** iNOS expression, Hcy-treated group. **c** eNOS expression, control group. **d** eNOS expression, Hcy-treated group. **e** Nitrotyrosine expression, control group. **f** Nitrotyrosine expression, Hcy-treated group. The *dark* markings around the nuclei indicates immunoperoxidase staining (100×)

In summary, our findings show that severe HHcy can promote a decrease in the endothelium-independent, adrenergic contractile response of carotid arteries. This effect was reversed by nitric oxide synthase inhibitors, which further supports the involvement of nitric oxide in HHcy-derived vascular dysfunction. Even at high plasma Hcy concentrations, we could not observe any endothelial dysfunction.

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