

α_{1D} -adrenoceptor-induced relaxation on rat carotid artery is impaired during the endothelial dysfunction evoked in the early stages of hyperhomocysteinemia

Claudia Roberta de Andrade ^a, Sandra Yasuyo Fukada ^a, Vania Claudia Olivon ^b, Marcio A.F. de Godoy ^a, Renato Haddad ^c, Marcos Nogueira Eberlin ^d, Fernando Queiroz Cunha ^a, Heraldo Possolo de Souza ^e, Francisco R.M. Laurindo ^f, Ana Maria de Oliveira ^{b,*}

^a Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

^b Laboratory of Pharmacology, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

^c School of Medical Sciences, UNICAMP, Campinas, SP, Brazil

^d Institute of Chemistry, UNICAMP, Campinas, SP, Brazil

^e Emergency Medicine Department, School of Medicine, University of São Paulo, São Paulo, SP, Brazil

^f Vascular Biology Laboratory, Heart Institute (InCor), School of Medicine, University of São Paulo, São Paulo, SP, Brazil

Received 30 November 2005; received in revised form 27 May 2006; accepted 2 June 2006

Available online 10 June 2006

Abstract

Hyperhomocysteinemia is a known risk factor for cardiovascular diseases, but the underlying mechanisms of this pathology are complex. We aimed to evaluate the effect of hyperhomocysteinemia in vasorelaxations induced by α_{1D} -adrenoceptor agonists. Vascular reactivity of rat carotid artery to the α -adrenoceptor agonist, phenylephrine, was enhanced in hyperhomocysteinemia. Mechanical removal of endothelium did not modify the carotid responsiveness to phenylephrine, compared to control. Phenylephrine induces endothelium-dependent relaxation, in the presence of 5-methyl urapidil (α_{1A} -adrenoceptor antagonist). We hypothesised that endothelial-relaxant α_1 -adrenoceptors are impaired by hyperhomocysteinemia. Incubation with prazosin (selective α_1 -adrenoceptor antagonist) or BMY7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro [4,5]decane-7, 9-dione dihydrochloride) (selective α_{1D} -adrenoceptor antagonist), similarly inhibited phenylephrine-induced relaxations in both control and hyperhomocysteinemic carotids. Immunohistochemistry showed enhanced immunoreactivity for eNOS and iNOS in hyperhomocysteinemic rats. In carotid arteries from hyperhomocysteinemic rats there was a decrease in superoxide dismutase activity and enhanced superoxide anion production. We conclude that α_{1D} -adrenoceptors mediate endothelium-dependent relaxation triggered by phenylephrine in rat carotid artery and affect the final tone. Furthermore, the enhanced phenylephrine-induced contraction in carotid artery due to hyperhomocysteinemia is endothelium-dependent and involves a loss of the inhibitory effect of relaxant α_{1D} -adrenoceptors by reducing NO bioavailability.

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Keywords: Hyperhomocysteinemia; Carotid artery; α_{1D} -adrenoceptor; Modulation; Relaxation; Phenylephrine; Nitric oxide; Oxidative stress

1. Introduction

Hyperhomocysteinemia is a known risk factor for the development of atherosclerosis and other vascular diseases. The mechanisms by which elevated homocysteine impairs the vessel wall are likely to be multifactorial. It has been suggested that elevated concentrations of homocysteine induce direct injury of vascular endothelium (Harker et al., 1976; Rodgers and Conn, 1990; revised by Weiss, 2005). Ungvari et al. (1999)

* Corresponding author. Universidade de São Paulo, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Laboratório de Farmacologia, Avenida do Café s/n, CEP 14040-903, Ribeirão Preto, SP, Brazil. Tel.: +55 16 36024165; fax: +55 16 3633 2960.

E-mail address: amolive@usp.br (A.M. de Oliveira).

have also reported that elevations in the plasma levels of homocysteine (a sulphur-containing amino acid derived from metabolism of the essential amino acid methionine), hyperhomocysteinemia, are associated with increased norepinephrine-induced contractions, and reduced endothelium-dependent relaxations of rat skeletal muscle arterioles. The mechanism underlying these changes is most likely impairment of the endothelium-derived nitric oxide (NO) activity (Stuhlinger et al., 2001; Chen et al., 2002). Previous studies in skeletal muscle arterioles demonstrate that, upon administration of norepinephrine, there is a concomitant release of NO from the endothelium modulating the magnitude of norepinephrine-induced vasoconstriction (Kaley et al., 1992). It has been also suggested that impairment of endothelial NO synthesis is responsible for increased responsiveness to norepinephrine in arterioles from hyperhomocysteinemic rats and the removal of vascular endothelium abolishes these effects (Ungvari et al., 1999).

α_1 -adrenoceptors play critical roles in many physiological processes, including smooth muscle contraction (Ruffolo et al., 1991; Daniel et al., 1999; Hague et al., 2002; Jahnichen et al., 2004). These receptors initiate their physiological effect by activating phospholipase C in the cell membrane, resulting in production of inositol 1,4,5-triphosphate, which mobilizes intracellular Ca^{2+} , and diacylglycerol, activating protein kinase C (Theroux et al., 1996). However, increasing evidences report the existence of relaxant α_1 -adrenoceptors in different arteries such as brachial and pulmonary arteries isolated from rabbit and rat, respectively (Zschauer et al., 1997; Boer et al., 1999). According to these reports, the mechanism of action for the α_1 -adrenoceptors-induced relaxation involves the local production and release of NO from vascular endothelium (Zschauer et al., 1997; Boer et al., 1999). Studies on physiological role of this endothelial vasorelaxant adrenoceptors suggest that they may represent a local control mechanism, which is, at least in part, involved in the modulation of vasoconstrictor responses to sympathomimetic amines (Fujimoto and Itoh, 1997; Filippi et al., 2001). In fact, according to Greenberg et al. (1989) and Amerini et al. (1995) vascular response to adrenergic stimulation is enhanced by endothelium removal and nitric oxide synthase (NOS) inhibitor administration.

The aim of the present work was to study the mechanisms involved on vascular reactivity of carotid artery to phenylephrine in hyperhomocysteinemia. The second objective was study the participation of inhibitory relaxation exerted by activation of endothelial α_1 -adrenoceptors.

2. Materials and methods

2.1. Homocysteine diet-induced hyperhomocysteinemia

The experimental protocols were carried out in accordance with the standards and policies of the University of São Paulo Animal Care and Use Committee, Brazil.

Hyperhomocysteinemia was induced in male Wistar rats (80 days, weighing 350 to 400 g) by the daily administration of DL-Homocysteine-thiolactone (1 g/kg body weight) into the

drinking water for a period of 15 days (Dudman and Wilcken, 1982; Reddy and Wilcken, 1982). Homocysteine-thiolactone was used since it is converted to homocysteine by hydrolysis, which increases the homocysteine plasma levels (Jakubowski, 1997; Jakubowski et al., 2000; Ossani et al., 2004). Since DL-Homocysteine-thiolactone is easily dissolved in water, this avoids a vehicle effect during treatment. The doses given were based on the average rat daily fluid intake. All animals were weighed daily in order to adjust the homocysteine-thiolactone dosage.

2.2. Determination of plasma homocysteine levels

Blood samples (5 ml) were collected from the abdominal aorta of anesthetized rats. Plasma with EDTA 7% (200 μl /5 ml blood) was centrifuged at 3000 g for 20 min. In order to minimize the release of homocysteine from blood cells, iced tubes were used to collect blood, and centrifugation was carried out at 4 °C. Plasma was then stored at -70 °C until assayed.

Total homocysteine concentration was measured by mass spectrometry using the Q-TRAP system—Q-Trap, triple—quadrupole mass spectrometer (Applied Biosystems-Canada—Perkin-Elmer Sciex/QqQ-Trap) using positive electrospray in Multiple Reaction Monitoring mode (MRM). Separation was carried out at room temperature (22–25 °C) using a C18 precolumn and a C18 column (3.9 mm \times 150 mm i.d., 5 μm) from Symmetry coupled to a Waters HPLC Alliance. For sample preparation, the plasma was thawed at room temperature; an aliquot of 100 μl of plasma was mixed with 20 μl of homocysteine-d8 (I.S., 20 μM). The reduction of all the forms of homocysteine found in blood was accomplished by the addition of 20 μl of dithiothreitol (500 μM) and mixing in a 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, and then centrifuged for 15 min at 1300 g. For quantification, 5 μl of the supernatant was injected. The isocratic mobile phase was acetonitrile:water (60:40 — v/v) acidified with addition of 0.1% formic acid and flow rate of 0.8 ml/min. Total eluent flow from HPLC system was directed to the turbo-ion spray source through a splitter (1:10). The needle voltage was set at 5.5 kV; nebulizer gas (nitrogen) at 60 psi; curtain gas (nitrogen) at 25 psi and collision cell energy was set at 5 eV. The turbo-ion spray heater temperature was set at 200 °C and the heater gas flow rate was 75 psi. For quantification of homocysteine and homocysteine-d4 the MRM (Multiple Reaction Mode) mode was used. The instrument was optimized to monitor the parent's ion ($\text{Q1} > \text{Q3}$) of homocysteine m/z 136.1 $>$ 90.0 and homocysteine-d4 m/z 140.1 $>$ 94.0, products provided by fragmentation in q2 collision cell.

2.3. Vessel preparation

Male Wistar rats were anesthetized and sacrificed by aortic exsanguination. The carotid artery was quickly removed, cleaned of adherent connective tissues, and cut into rings. Two stainless-steel stirrups were passed through the lumen of

each ring in order to measure the isometric tension (Letica Scientific Instruments). The rings were placed in 5 mL organ chambers containing Krebs solution gassed with 95% O₂ and 5% CO₂, which was maintained at 37 °C. The composition of the Krebs solution was (mM): NaCl, 118.4; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 25.0; Glucose, 11.6; CaCl₂, 1.9.

The rings were initially stretched to a basal tension of 1 g (previously determined by doing length–tension relationship experiments), and allowed to equilibrate for 60 min in the bath fluid, which was changed every 15–20 min. In some rings, 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 (10⁻⁶ M) in the presence of contractile tone induced by phenylephrine (10⁻⁷ M). In the studies of endothelium-intact vessels, the ring was discarded if relaxation with acetylcholine was less than 80%. For studies using endothelium-denuded vessels, the rings were discarded if there was any degree of relaxation.

Homocysteine treatment induces endothelial dysfunction, decreases the bioavailability of nitric oxide (NO), and therefore impairs the response to acetylcholine. Therefore, some of the tissues obtained from homocysteine-fed animals may have an impaired relaxation response to acetylcholine due to the effect of homocysteine and not due to mechanical damage. To deal with this issue, the rings were discarded if the relaxation caused by acetylcholine was less than 50%.

2.4. Functional study of carotid rings

To delineate the effect of hyperhomocysteinemia on the phenylephrine-induced contraction, cumulative concentration–response curves (10⁻¹⁰ to 10⁻⁵ M) were obtained in endothelium-intact and endothelium-denuded rings from control and hyperhomocysteinemic groups.

The relaxation induced by phenylephrine was studied by pre-contracting rings with prostaglandin F_{2α} (PGF_{2α}, 10⁻⁶ M). To inhibit the effect of α_{1A}-adrenoceptors (inducing contractions), 5-methyl-urapidil (α_{1A}-adrenoceptor antagonist, 10⁻⁵ M) (Gross et al., 1988) was added to the organ bath 20 min before administration of PGF_{2α}.

The influence of hyperhomocysteinemia on overall arterial tone was assessed by concentration–response curves for KCl (10 to 120 mM), acetylcholine (10⁻¹⁰ to 10⁻⁵ M) and sodium nitroprusside (10⁻¹⁰ to 10⁻⁵ M), obtained in endothelium-intact and endothelium-denuded rings.

Parallel experiments were carried out under identical conditions and the following drugs were added before pre-contracting the intact rings: L-NAME (N^G-nitro-L-arginine methyl ester), a non-selective NOS inhibitor, 10⁻⁴ M, 30 min (Rees et al., 1990); indomethacin, non-selective cyclooxygenase inhibitor, 10⁻⁵ M, 30 min (Futaki et al., 1994); prazosin, selective α₁-adrenergic antagonist, 10⁻⁵ M, 20 min (Muramatsu et al., 1990; Zacharia et al., 2004); BMY7378 (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride), selective α_{1D}-adrenergic antagonist, 10⁻⁸ M, 30 min (Goetz et al., 1995) and yohimbine, selective

α₂-adrenergic antagonist, 10⁻⁵ M, 20 min) (Barbieri et al., 1998).

2.5. Levels of nitrogen oxides in vascular homogenates

Nitrite and nitrate levels were measured in supernatants from total carotid artery homogenates prepared under liquid N₂. Following a centrifugation at 3000 g for 10 min, supernatants were assayed for nitrite or nitrate through chemiluminescence, using a Nitric Oxide Analyser (Sievers, Model 280), as described (Leite et al., 2003). For nitrite, samples were reduced by NaI in acetic acid at room temperature, whereas for nitrate, samples were reduced in a saturated solution of VCl₃ in HCl at 95 °C. Data were collected for endothelium-intact rat carotid from control and hyperhomocysteinemia groups, and normalized for protein concentration, assessed through Bradford technique.

2.6. Histological analysis

Rats were anesthetized with ether, sacrificed and vessels fixed in situ by constant pressure fixation with formalin (10%) through a 22-gauge butterfly angiocatheter in the left ventricle. Carotid arteries were harvested, embedded in paraffin, and cross-sectioned (3 μm). Parallel sections were subjected to standard hematoxylin and eosin staining as well as immunohistochemistry.

2.7. Immunohistochemical analysis

Staining for eNOS, iNOS and nitrotyrosine was performed on paraffin included tissue, cut into 3 μm sections. Primary antibody incubations were for 60 min at 25 °C using rabbit monoclonal anti-eNOS, iNOS and nitrotyrosine (1:300 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). After washing with phosphate-buffered saline (PBS), sections were covered for 1 h with biotinylated secondary goat anti-rabbit IgG (1:300, Vector Laboratories) and then reacted with an avidin–biotin complex (Vecstatin Elite kit, Vector). Signal detection was performed using 3,3'-diaminobenzidine (DAB) (Vector) and counterstained with hematoxylin.

2.8. Vascular superoxide dismutase activity

Carotid artery homogenates were prepared under liquid N₂. Aliquots of the supernatant were assayed for total Superoxide dismutase (SOD) activity by monitoring spectrophotometrically inhibition of the rate of xanthine/xanthine oxidase-dependent cytochrome *c* reduction (from Sigma) at pH 7.4, as previously described (Stralin et al., 1995; Leite et al., 2003). SOD activity in each fraction was corrected for protein concentration, assessed through Bradford technique.

2.9. Lucigenin-amplified chemiluminescence assays

Lucigenin assays were performed as previously described (Brandes et al., 1997; Li et al., 1998; Oliveira et al., 2003), with

final lucigenin concentration of 5×10^{-6} M. In each fragment, signals were counted for 10 min in a luminometer (Berthold Multi Biolumat), normalized for the dry weight of each segment, and results expressed as counts per min (cpm)/mg dry tissue. Some experiments were performed following incubation of arterial segments for 5 min with MnTBAP [Mn(III)tetrakis(4-benzoic acid)porphyrin Chloride], a cell permeable SOD mimetic (5×10^{-6} M); 4,5-Dihydroxy-1,3-benzene-disulfonic acid disodium salt monohydrate (Tiron), a superoxide scavenger (10^{-3} M); or PEG-SOD (Superoxide dismutase-Polyethylene glycol, which is able to internalize into cells) (200 U/ml).

2.10. Data analysis

Contractions were recorded as changes in the displacement (grams per mg of dry tissue) from baseline. Relaxation was expressed as the percentage change from the $\text{PGF}_{2\alpha}$ -contracted levels. Agonists concentration–response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc., San Diego, CA). Agonist potencies are expressed as pD_2 (negative logarithm of the molar concentration of agonist producing 50% of the maximum response), and maximum response is expressed as E_{max} (maximum effect elicited by the agonist). For inhibition of relaxation by selective NOS inhibitors, I_{max} (maximum inhibition elicited by the inhibitor) and IC_{50} (negative logarithm of the molar concentration of inhibitor producing 50% of the maximum inhibitory response), respectively, were calculated as % maximal fall in the response induced by $\text{PGF}_{2\alpha}$. Results were expressed as mean \pm standard error of the mean (S.E.M.).

The statistical analysis of the E_{max} and pD_2 values was done using one-way analysis of variance (ANOVA). Dunnett's comparison test was used for post-hoc multiple comparisons. Two-tailed unpaired *t*-tests were used for the remaining statistical tests. The significance level was set at $P < 0.05$; *n* refers to the number of animals used.

2.11. Chemicals

The following drugs were used: DL-Homocysteine-thiolactone (Acrós Organics/Fischer Scientific, Wisconsin, USA); phenylephrine hydrochloride, acetylcholine hydrochloride, sodium nitroprusside dehydrate, Bis(*N*-methylacridinium) nitrate (Lucigenin), PEG-SOD (Superoxide dismutase-Polyethylene glycol), 4,5-Dihydroxy-1,3-benzene-disulfonic acid disodium salt monohydrate (Tiron), prazosin hydrochloride, yohimbine hydrochloride, 5-methylurapidil, L-NAME (*N*^G-nitro-L-arginine methyl ester) (Sigma, St. Louis, Mo., USA), BMY7378 dihydrochloride (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro [4,5]decane-7, 9-dione dihydrochloride) (Tocris, Avonmouth, UK); indomethacin, MnTBAP [Mn(III)tetrakis(4-benzoic acid)porphyrin Chloride] (Calbiochem, Darmstadt, Germany). Indomethacin was dissolved in Tris buffer (pH 8.4). 7-nitroindazole was prepared as stock solution in

dimethyl sulfoxide (DMSO). The other drugs were dissolved in distilled water.

3. Results

3.1. Effects of treatment with DL-Homocysteine-thiolactone on hyperhomocysteinemia plasma levels

Treatment for 15 days with the homocysteine-rich diet (1 g/kg body weight) induced a significant increase in plasma homocysteine levels (Control: 8.36 ± 0.71 versus Hyperhomocysteinemia: 79.81 ± 7.32 μM) ($P < 0.05$, Student's *t*-test).

3.2. Effects of treatment with DL-Homocysteine-thiolactone on carotid artery morphology

Carotid arteries from control and hyperhomocysteinemic rats demonstrated no morphological or pathological changes at optical microscopy. Furthermore, there were no differences in the endothelial layers in arteries from control and hyperhomocysteinemia groups (Fig. 1).

3.3. Effects of hyperhomocysteinemia on phenylephrine-induced contractions

To determine whether the dietary regimens affected smooth muscle cell responsiveness to vasoconstrictors, carotid contraction was assessed by constructing concentration–response curves to the α_1 -adrenergic receptor agonist phenylephrine. Phenylephrine induced a significant contractile response in control arteries, and mechanical removal of the endothelium significantly increased phenylephrine E_{max} (Fig. 2). Hyperhomocysteinemia caused a significant enhancement of phenylephrine-induced contractions in arteries with intact endothelium (Fig. 2). The removal of endothelium did not alter the phenylephrine-induced contractions in arteries from hyperhomocysteinemia group (Fig. 2). The pD_2 values were not altered by endothelial removal in any of the groups. In addition, L-NAME, a non-selective NOS inhibitor (10^{-4} M) induced no alterations in

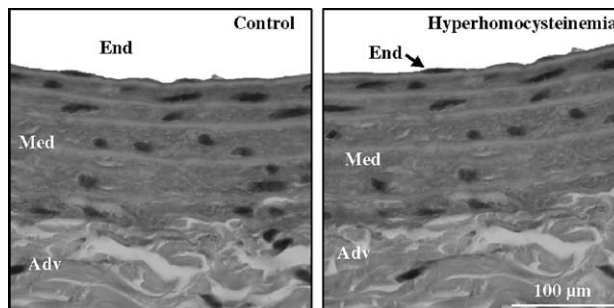


Fig. 1. Photomicrographs of representative cross-sections showing right carotid arteries from control and hyperhomocysteinemic rats. (Hematoxylin–Eosin; magnification: $\times 1000$). Adv = adventitia; Med = media; End = endothelium.

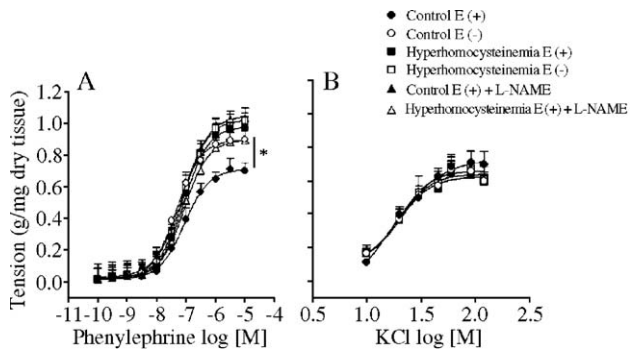


Fig. 2. Phenylephrine-induced contraction in carotid artery. (A) Graphs showing concentration–response curves for contraction induced by Phenylephrine (10^{-10} to 10^{-5} M) in carotid arteries from control and hyperhomocysteinemic rats, in the presence (E+) or absence (E-) of endothelium, and, for arteries with intact endothelium, in the presence of L-NAME (10^{-4} M). (B) Graphs showing concentration–response curves for contraction induced by KCl (10 to 120 mM) in the carotid artery from control and hyperhomocysteinemic rats in the presence (E+) or absence (E-) of endothelium. Data are mean \pm S.E.M. ($n=5-6$). $*P<0.01$ versus control group (ANOVA, Dunnett's post-hoc test).

phenylephrine E_{max} in arteries from control, compared to hyperhomocysteinemic rats (Fig. 2).

Contractile responses to KCl were not different among all studied groups (Fig. 2).

3.4. Effects of hyperhomocysteinemia on phenylephrine-induced relaxation

In ring preparations pre-contracted with $\text{PGF}_{2\alpha}$ (10^{-6} M) and in the presence of α_{1A} -adrenoceptor antagonist, 5-methylurapidil, phenylephrine induced concentration-dependent relaxations (Fig. 3), which was significantly reduced (52%) in hyperhomocysteinemia (Fig. 3).

Relaxation responses to acetylcholine in carotid arteries with intact endothelium were also impaired by hyperhomocysteinemia, however, this impairment was less pronounced (26%) than those observed with phenylephrine (Fig. 3). We repeated the experiments described above in artery rings isolated from control and hyperhomocysteinemic rats in the absence of vascular endothelium. In both groups of rats, relaxation to phenylephrine was completely absent (Fig. 3).

Endothelium-independent carotid relaxation was assessed by constructing concentration–response curves to NO donor sodium nitroprusside. Hyperhomocysteinemia induced no effect on relaxation responses to this agonist in both endothelium-intact or denuded arteries (Fig. 3).

3.5. Role of α_{1D} -adrenoceptor in phenylephrine-induced relaxation

Prazosin, a non-selective α_1 -adrenoceptor inhibitor (10^{-5} M) promoted significant inhibition of phenylephrine E_{max} in carotid arteries from control group (Fig. 4). Incubation with the selective α_{1D} -adrenoceptor antagonist, BMY7378 (10^{-8} M), signifi-

cantly inhibited the phenylephrine-induced relaxation, to the same extent as prazosin (Fig. 4). On the other hand, yohimbine (selective inhibitor of α_2 -adrenoceptors, 10^{-5} M) produced no significant effect (Fig. 4).

These results indicate that α_{1D} -adrenoceptors account for the inhibitory endothelial modulation concomitant to contraction induced by α -adrenoceptors in rat carotid rings. Hyperhomocysteinemia compromises such modulation, since it impairs the relaxation induced by α_{1D} -adrenoceptors.

3.6. Contribution of NO and endothelial cyclooxygenase metabolites in response to phenylephrine

Significant inhibition of relaxation response by L-NAME (10^{-4} M) and also by endothelium removal suggests that phenylephrine-induced relaxation is dependent upon the local production of NO from the vascular endothelium. The absence of effect by indomethacin (a non-selective COX inhibitor,

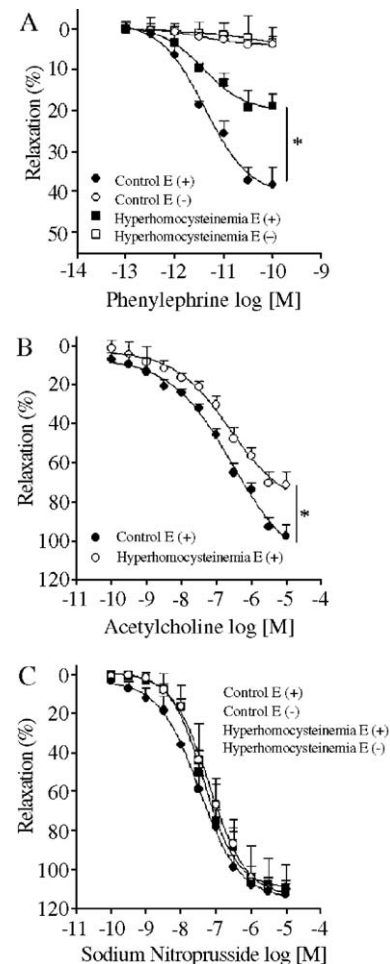


Fig. 3. Phenylephrine-induced relaxations in carotid arteries. Graphs showing concentration–response curves for relaxation induced by: (A) Phenylephrine (10^{-13} to 10^{-5} M), (B) Acetylcholine (10^{-10} to 10^{-5} M) and (C) Sodium Nitroprusside (10^{-10} to 10^{-5} M) in carotid arteries from control and hyperhomocysteinemic rats, in the presence (E+) or absence (E-) of endothelium. Data are mean \pm S.E.M. ($n=5-6$). $*P<0.01$ versus control group (ANOVA, Dunnett's post-hoc test).

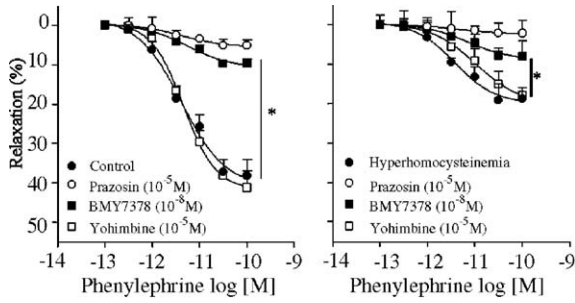


Fig. 4. Participation of α -adrenoceptors subtypes in the relaxation induced by Phenylephrine in carotid arteries. Graphs showing concentration–response curves for relaxation induced by Phenylephrine (10^{-13} to 10^{-10} M) in carotid arteries with intact endothelium, from control and hyperhomocysteinemic rats, in the absence or presence of Prazosin (10^{-5} M), BMY7378 (10^{-8} M) or Yohimbine (10^{-5} M). Data are mean \pm S.E.M. ($n=5-6$). * $P<0.01$ versus control curves (ANOVA, Dunnett’s post-hoc test).

10^{-5} M)) excludes the participation of prostanoids in the observed responses (Fig. 5).

Further evidence that the impairment of relaxation induced by α_{1D} -adrenoreceptor activation is related to decreased NO is provided in experiments which shows reduced tissue homogenate nitrite concentrations in carotid arteries from hyperhomocysteinemic rats (Control: 3.82 ± 0.53 versus Hyperhomocysteinemia: 2.03 ± 0.44 $\mu\text{mol/mg}$ protein) ($P<0.001$, Student’s t -test). We did not observe alterations in nitrate concentrations assessed in tissue homogenates (Control: 10.75 ± 4.25 versus Hyperhomocysteinemia: 11.58 ± 1.48 $\mu\text{mol/mg}$ protein). In parallel, the increased nitrotyrosine formation in arteries from hyperhomocysteinemic rats suggests that homocysteine reduces the bioavailability of NO probably by increasing its degradation by reactive oxygen species (Fig. 6). Accordingly, immunoreactivity of carotid arteries from hyperhomocysteinemic rats to eNOS (Fig. 7A) and iNOS (Fig. 7B) showed significant increase in staining intensity versus controls.

SOD activity in vessel homogenates from hyperhomocysteinemic rats was decreased versus control (6.14 ± 0.58 versus 3.82 ± 0.60 U/mg protein, respectively) ($P<0.01$, Student’s t -

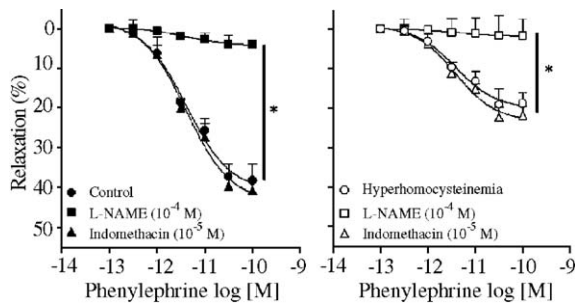


Fig. 5. Role of NO and prostaglandins in Phenylephrine-induced relaxation in carotid arteries. Graphs showing concentration–response curves for relaxation induced by Phenylephrine (10^{-13} to 10^{-10} M) in carotid arteries from control and hyperhomocysteinemic rats, in the presence or absence of L-NAME (10^{-4} M) or Indomethacin (10^{-5} M). Data are mean \pm S.E.M. ($n=5-6$). * $P<0.01$ versus control curves (ANOVA, Dunnett’s post-hoc test).

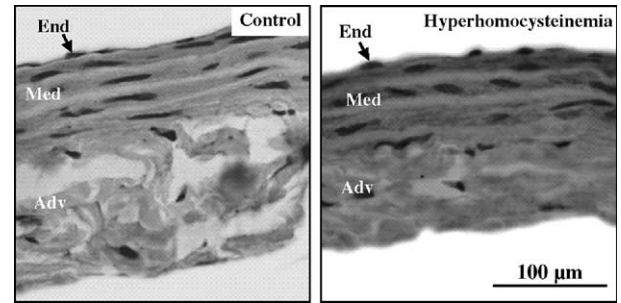


Fig. 6. Immunohistochemical staining for nitrotyrosine in carotid arteries. Transverse sections were obtained from control and hyperhomocysteinemic rats. Adv = adventitia; End = endothelium; Med = media.

test). In parallel, our data indicate increase in superoxide output from carotid segments, as assessed by chemiluminescence, in hyperhomocysteinemic rats. MnTBAP (5×10^{-6} M) and PEG-SOD (200 U/ml), cell permeable SOD mimetics and Tiron, a superoxide scavenger (10^{-3} M), abrogated superoxide generation (Fig. 8).

4. Discussion

Endothelial dysfunction is accepted as one of the earliest processes in the development of vascular diseases such as atherosclerosis (Benzuly et al., 1994; Traupe et al., 2003). Moreover, two mechanisms have been described to unleash endothelium dysfunction in hyperhomocysteinemia: injury to endothelium and reduction of NO bioavailability (Stamler et

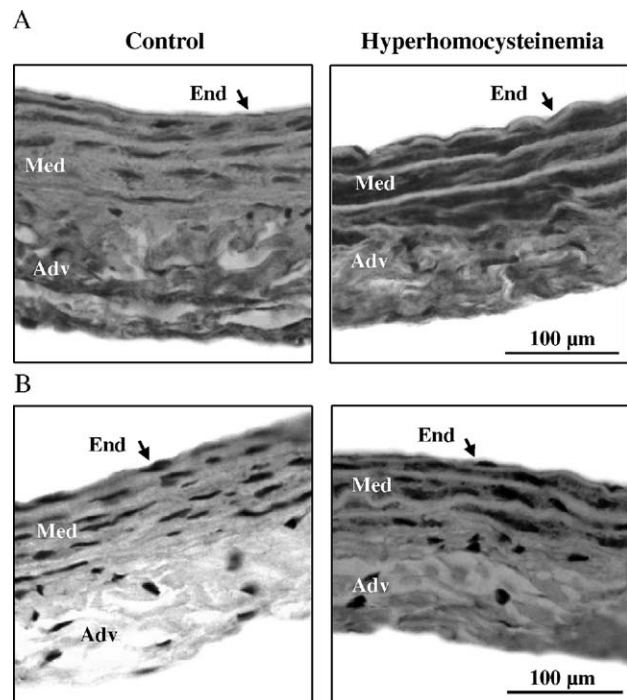


Fig. 7. Immunohistochemical staining for eNOS (Panel A) and iNOS (Panel B) in carotid arteries in control and hyperhomocysteinemic rats. Adv = adventitia; End = endothelium; Med = media.

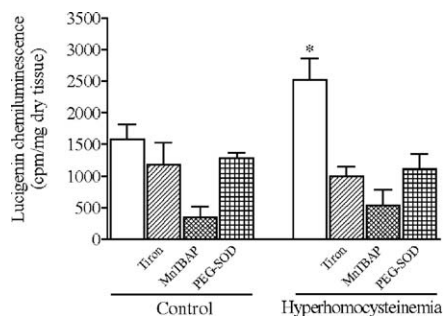


Fig. 8. Role of hyperhomocysteinemia on superoxide anion production assessed through Lucigenin-amplified-chemiluminescence. Data are shown for carotid artery rings from control and hyperhomocysteinemic rats in the absence or in the presence of MnTBAP, a cell-permeable SOD mimetic, Tiron (a superoxide scavenger) and PEG-SOD. Data are mean \pm S.E.M. ($n=5$). * $P<0.01$ versus control (ANOVA, Dunnett's post-hoc test).

al., 1993; Powers et al., 2003). In our study, gross endothelial injury is unlikely, given that morphological analysis showed that hyperhomocysteinemia did not change the structural organization of the rat carotid, either at the endothelial or the smooth muscle layer levels. Furthermore, the most significant finding in this study was that enhanced contraction of rat carotid artery to α_1 -adrenoceptors is evoked by impaired relaxation mechanism played by endothelial α_{1D} -adrenoceptors, which was markedly diminished in arteries from hyperhomocysteinemic group.

Both contraction and relaxation due to phenylephrine appear to be dependent on an intact endothelium, both in control and hyperhomocysteinemic rats. Conversely, reactivity to phenylephrine was unaltered in arteries with denuded endothelium. These results are confirmed in the presence of L-NAME, which confirms that basal release of NO in carotids from hyperhomocysteinemic group had no additional effect on phenylephrine-induced contractions. In addition, the absence of alterations on KCl-induced contraction indicates that hyperhomocysteinemia has no effect on vascular smooth muscle contraction *per se*. KCl was used in the present study to verify the consequence of hyperhomocysteinemia on smooth muscle contractile function, since KCl-induced contractions are receptor independent (Brizzolara et al., 1994).

The functional significance of different subtypes of α_1 -adrenoceptors in vascular tissues has been previously investigated, mainly with regard to their role in mediating the contractile response to sympathomimetic amines (Daniel et al., 1999; de Oliveira et al., 1998; Muramatsu et al., 1998; Yousif et al., 1998; Satoh et al., 1998). Filippi et al. (2001) also reported that two different subtypes of α_1 -adrenoceptors mediate opposite effects on vascular tone in the same preparation. In fact, although the α_{1A} -adrenoceptors mediated a contractile response, the α_{1D} -adrenoceptors were involved in endothelium-dependent relaxation (Filippi et al., 2001). Our data corroborate these studies, since we reported that the activation of endothelial α_{1D} -adrenoceptor activation induced a relaxant response on rat carotid artery and that hyperhomocysteinemia compromised such dilator response. Impairment of this modulatory mechanism

due to hyperhomocysteinemia leads to the decrease in local availability of NO, despite the increased expression of eNOS and iNOS. Decreased NO bioavailability is further impaired by the enhanced generation of O_2^- and decrease in SOD activity, both processes likely associated with the finding of increased nitrotyrosine accumulation. The role of oxidative stress in hyperhomocysteinemia is well accepted (Bellamy et al., 1998; Upchurch et al., 1997).

These results support the hypothesis that relaxation evoked by phenylephrine in the present study occurs via endothelial NO, since it was not detectable in endothelium-denuded preparations, and it was blunted in the presence of a non-selective NO synthase inhibitor, L-NAME, but not in the presence of indomethacin. Acetylcholine effect is also dependent on the release of NO from the endothelial layer; however, acetylcholine-induced relaxations were less affected than phenylephrine-induced relaxations in carotid arteries from hyperhomocysteinemic rats. The fact that relaxation responses to sodium nitroprusside were not altered in control and hyperhomocysteinemic rats indicates that it has no effect on NO-induced relaxation *per se*, which is confirmed by other previous studies in thoracic aorta from rabbits (Lang et al., 2000). Our findings showed that hyperhomocysteinemia resulted in decreased nitrite, but not nitrate generation, suggesting reduction on vascular NO production. It is well established that the reaction between superoxide and NO generates peroxynitrite or related nitrogen species, which also decays to nitrate. Thus, the lack of reduction in nitrate generation in hyperhomocysteinemia could be due to increase in such reactive nitrogen species, which in turn decay to nitrate.

Our results showed enhanced eNOS expression in carotids from hyperhomocysteinemic rats. On the other hand, Chen et al. (2002) also reported in porcine coronary arteries a reduction on eNOS expression during hyperhomocysteinemia. This difference in eNOS expression could be explained by differences in arterial beds or differences among species and experimental models.

The model of hyperhomocysteinemia used in the present study, as well as other previous experimental models (McCully and Carvalho, 1987; Hill et al., 2002; Ossani et al., 2004; Miao et al., 2005) provides important information about vascular consequences of homocysteine, although none of them mimics precisely the human disease.

The enhanced activity of iNOS may be due to an effect of oxidative stress in activation of nuclear factor- κ B (NF- κ B), which is a known transcription factor for this enzyme (Welch and Loscalzo, 1998; Desai et al., 2001). The decrease in SOD activity in carotid artery from hyperhomocysteinemic rats is at variance with results from Lang et al. (2000), who showed that homocysteine enhances SOD activity in endothelial cells. The reason for this discrepancy is unclear, but may be related to differences on complex time course of homocysteine effect on SOD activity or to distinctions between models. Impaired SOD activity likely contributes to the observed increase in superoxide (Leite et al., 2003).

In summary, our major finding is that the enhanced contractile response to phenylephrine is due, in part, to impairment of modulatory mechanism played by α_{1D} -adrenoceptor-mediated endothelial vasorelaxation in rat carotid arteries from hyperhomocysteinemic rats. Such decreased relaxation is dependent on decreased output of bioavailable NO, as well as enhanced production of superoxide radicals.

Acknowledgments

We are most grateful to Juliana A. Vercesi, Miriam C. C. de Melo, Mayara Santos Gomes (FCFRP-USP), Laura Ventura (Incor, FMUSP) and Auristela de Mello Martins (Dept Pathology, FMRP-USP), for their technical assistance. This work was supported by FAPESP-Brazil (01-05549-3 and 02/8045-9) and by FAPESP grant 04/13683-0 to FRML.

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