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# Hyperhomocysteinemia induced by feeding rats diets rich in DL-homocysteine thiolactone promotes alterations on carotid reactivity independent of arterial structure

Claudia Roberta de Andrade [a], Carlos Renato Tirapelli [b], Renato Haddad [c], Marcos N. Eberlin [c], Leandra N.Z. Ramalho [d], Mamie M. Iyomasa [e], Sergio A. Uyemura [f], Ana Maria de Oliveira [g,*]

[a] Department of Pharmacology, FMRP-USP, Ribeirão Preto, SP, Brazil
[b] Department of Psychiatry Nursing and Human Sciences, Laboratory of Pharmacology, EERP-USP, Ribeirão Preto, SP, Brazil
[c] Institute of Chemistry, UNICAMP, Campinas, SP, Brazil
[d] Department of Pathology, FMRP-USP, Ribeirão Preto SP, Brazil
[e] Department of Morphological Sciences, FORP-USP, Ribeirão Preto, SP, Brazil
[f] Department of Clinical, Toxicological, and Food Science Analysis, FCFRP-USP, Ribeirão Preto, SP, Brazil
[g] Department of Physics and Chemistry, Laboratory of Pharmacology, FCFRP-USP, Ribeirão Preto, SP, Brazil

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ABSTRACT

We aimed to investigate whether hyperhomocysteinemia (HHcy) interferes on carotid vascular reactivity, and how morphological and functional aspects are related. With this purpose male Wistar rats received a solution of DL-homocysteine-thiolactone (1 g/kg body weight/day) in the drinking water for 4, 15 and 30 days. Lipid profile, carotid artery-morphology and -responsiveness to acetylcholine, phenylephrine and endothelin-1 were analyzed. Similar increase on homocysteine plasmatic levels occurred in rats treated for 4, 15 and 30 days. High levels of serum cholesterol and triglycerides were observed after HHcy 30 days. Vascular reactivity experiments using standard muscle bath procedures showed that HHcy induced a time-dependent reduction on acetylcholine-induced-relaxation at 4, 15 and 30 days. HHcy enhanced the contractile response of endothelium-intact, but not denuded carotid rings to phenylephrine and endothelin-1, despite the treatment time. Morphometric analysis showed that intimal/medial area ratio was enhanced only at 30 days of HHcy, despite its reduced cell density. The major new finding of the present study is that it establishes a time–course relationship for the events involved on vascular effects associated with HHcy. We demonstrated that alterations on vascular responsiveness precede alterations on arterial structure. Based on such findings it is possible to suggest that vascular dysfunction occurs in early stages while alterations on vessel morphology take place in latest stages of HHcy.



## 1. Introduction

Homocysteine is a sulphur-containing amino acid that is derived from methionine, an essential amino acid found in abundance in proteins of animal origin, which is the only source of homocysteine in man (Perry, 1999). An elevated level of plasma homocysteine has long been suspected as a metabolic risk factor for the development of atherosclerotic vascular diseases (McCully, 1969). Hyperhomocysteinemia (HHcy) is defined as an elevation of total plasma homocysteine (Mudd et al., 2000), and its association to atherothrombosis was first recognized in children with severe HHcy (plasma homocysteine >100 μM) caused by inborn errors of the methionine cycle (McCully, 1969). Furthermore, numerous clinical studies have established a positive correlation between elevated homocysteine levels and recurrent cardiovascular events (Papatheodorou and Weiss, 2007).

It has been reported that homocysteine evokes endothelial dysfunction and impairment of nitric oxide (NO) biodisponibility in animal models (Harker et al., 1976; de Andrade et al., 2006), and cell culture studies (Wall et al., 1980; Blundell et al., 1996). One of the possible mechanisms involved with the homocysteine effects is the generation of hydrogen peroxide (Starkebaum and Harlan, 1986) and superoxide anion, which increases the oxidative degradation of NO (Lang et al., 2000; de Andrade et al., 2006). Interestingly, the increase of superoxide anion production triggered by homocysteine in endothelial cells is time-dependent (Lang et al., 2000). The patho-physiological consequences include alterations in vascular function and the possible premature development of atherosclerosis.

The compromising of the endothelial-dependent regulatory function of NO caused by HHcy is associated with increased contraction for vasoconstrictor agents such as phenylephrine (de Andrade et al., 2006), angiotensin II (Bonaventura et al., 2004) and to a decreased vasorelaxation induced by acetylcholine (de Andrade et al., 2006) or histamine

*Abbreviations:* HHcy, hyperhomocysteinemia; NO, nitric oxide; ET-1, endothelin-1; PHE, phenylephrine; ACh, acetylcholine.

* Corresponding author. Laboratory of Pharmacology, FCFRP, USP; Av. do Café s/no, 14040-903, Ribeirão Preto, SP, Brazil. Tel.: +55 16 3602 4706; fax: +55 16 3633 4880.
*E-mail address:* amolive@usp.br (A.M. de Oliveira).

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(Ungvari et al., 1999). Also, alterations of smooth muscle cells are thought to contribute to the pro-atherogenic action associated with HHcy, since an enhanced collagen synthesis and accumulation in cultured aortic smooth muscle cells was observed (Majors et al., 1997). Finally, some studies have demonstrated that homocysteine is mitogenic for arterial smooth muscle cells (Tsai et al., 1994; Dalton et al., 1997; Majors et al., 1997). Whether the mitogenic effects of homocysteine are time-dependent remains to be determined.

It has been suggested that vascular structural adaptations can induce alterations on vascular responsiveness (Accorsi-Mendonça et al., 2004; de Andrade et al., 2007), which is associated with increased response to vasocontractile agents. However, there is a lack of evidence describing a time-dependent correlation between functional dysfunction and alterations of the vascular morphology induced by HHcy. Moreover, the majority of the experiments designed to study the vascular effects of HHcy on vascular functionality and morphology, analyze these two parameters independently and use a single period of treatment with homocysteine.

Based on the above-mentioned studies, we can conclude that the simultaneous effects of homocysteine on arterial morphology and function remain largely unknown. The present work aimed to investigate whether there is a relation between the carotid morphological and functional alterations associated with elevated level of plasma homocysteine and the time–course for the development of these alterations. With this purpose we studied the effects of HHcy on carotid structure and vascular responsiveness to acetylcholine, phenylephrine and endothelin-1 at 4, 15 and 30 days after treatment with DL-homocysteine thiolactone (1 g/kg body weight/day).

## 2. Materials and methods

### 2.1. Experimental design

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. HHcy was induced in male Wistar rats (80 days old, weighing 350 to 400 g) by the daily administration of DL-homocysteine thiolactone (1 g/kg body weight/day) into the drinking water for 4, 15 and 30 days, as previously described (de Andrade et al., 2006).

### 2.2. Blood homocysteine measurements

Blood samples from control and HHcy rats were collected with EDTA and 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 in the Q-TRAP system—Q-Trap, triple-quadrupole mass spectrometer (Applied Byosystems-Canada—Perkin-Elmer Sciex /QqQ-Trap) using positive electrospray in Multiple Reaction Monitoring mode (MRM), as previously described by de Andrade et al. (2006).

### 2.3. Serum cholesterol and triglycerides measurements

Blood samples were collected from abdominal aorta for measurement of cholesterol and triglycerides concentration using non-heparinized syringes. Samples were centrifuged at 8000–10,000 *g* for 10 min at room temperature. The serum was analyzed (in triplicate) for cholesterol and triglycerides content using commercially available kits (Labtest Diagnóstica, São Paulo, Brazil) and an auto-analyzer (ABAA model, Abbott, VP, USA).

### 2.4. Vessel ring preparation

Animals were anaesthetised and sacrificed by aortic exsanguination. The carotid was quickly removed, cleaned of adherent connective tissues and cut into rings. Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer (Letica Scientific Instruments, Barcelona, Spain) to measure tension in the vessels. The rings were placed in a 5 ml organ chamber containing Krebs solution gassed with 95% $O_2$/5%$CO_2$, and maintained at 37 °C. The composition of Krebs solution was as follows (mM): NaCl, 118.4; KCl, 4.7; $KH_2PO_4$, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; Glucose, 11.6; $CaCl_2$, 1.9. The rings were stretched until a basal tension of 1.0 g, which was determined by length-tension relationship experiments and then were allowed to equilibrate for 60 min with the bath fluid being changed every 15–20 min. In some rings, the endothelium was removed mechanically 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 (1 μM) in the presence of contractile tone induced by phenylephrine (0.1 μM). High homocysteine levels are described to induce endothelial dysfunction, and therefore impair 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 40%. For studies of endothelium-denuded vessels, the rings were discarded if there was any degree of relaxation.

#### 2.4.1. Experimental protocols

Cumulative concentration–response curves for endothelin-1 ($10^{-12}$ to $3\times10^{-7}$ M) or phenylephrine ($10^{-10}$ to $10^{-5}$ M) were performed in endothelium-intact or -denuded rings by a stepwise increase in the concentration of the agonists. Additions were made as soon as a steady response was obtained from the preceding concentration. In another set of experiments, endothelium-intact rings were pre-contracted with phenylephrine (0.1 μM) and after they reached a stable and sustainable contraction, acetylcholine ($10^{-10}$ to $10^{-5}$ M) was added cumulatively to the organ bath. The vascular responsiveness to these agonists was studied in carotid rings from homocysteine-treated rats (4, 15 and 30 days) and their respective age-matched controls.

### 2.5. Histological analysis

Rats were anaesthetized, sacrificed and the 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–eosin staining or Masson trichrome. Hematoxylin–eosin and Masson trichrome staining were used to determine the morphology and thickness in the carotid wall, respectively. Stained sections were examined with light microscopy and the image was captured at 100× or 400×. The cell density and the number of vascular smooth muscle cells were evaluated by using ImageJ Program (NIH-National Institute of Health) software. The slides were examined using a Nikon microscope and photographed with a digital camera (Nikon Coolpix 4500) coupled to an imaging system.

### 2.6. Data analysis

Contractions were recorded as changes in the displacement (grams per mg dry tissue) from baseline. Relaxation was expressed as the percentage change from the phenylephrine-contracted levels. Agonist concentration–response curves were fitted using a nonlinear