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# Adult rats are more sensitive to the vascular effects induced by hyperhomocysteinemia than young rats

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ABSTRACT

We aimed to investigate the vascular effects of hyperhomocysteinemia (HHcy) on carotid arteries from young and adult rats. With this purpose young and adult rats received a solution of DL-homocysteine-thiolactone (1 g/kg body weight/day) in the drinking water for 7, 14 and 28 days. Increase on plasma homocysteine occurred in young and adult rats treated with DL-homocysteine-thiolactone in all periods. Vascular reactivity experiments using standard muscle bath procedures showed that HHcy enhanced the contractile response of endothelium-intact, carotid rings to phenylephrine in both young and adult rats. However, in young rats, the increased phenylephrine-induced contraction was observed after hyperhomocysteinemia for 14 and 28 days, whereas in adult rats this response was already apparent after 7 day treatment. HHcy impaired acetylcholine-induced relaxation in arteries from adult but not young rats. The contraction induced by phenylephrine in carotid arteries in the presence of Y-27632 was reversed to control values in arteries from young but not adult rats with hyperhomocysteinemia. HHcy did not alter the contraction induced by $CaCl_2$ in carotid arteries from young rats, but enhanced $CaCl_2$-induced contraction in the arteries from adult rats. HHcy increased the basal levels of superoxide anion in arteries from both groups. Finally, HHcy decreased the basal levels of nitrite in arteries from adult but not young rats. The major new finding of the present work is that arteries from young rats are more resistant to vascular changes evoked by HHcy than arteries from adult rats. Also, we verified that the enhanced vascular response to phenylephrine observed in carotid arteries of DL-homocysteine thiolactone-treated rats is mediated by different mechanisms in young and adult rats.



## 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). Hyperhomocysteinemia (HHcy) is a known risk factor for the development of atherosclerosis and other vascular diseases. Elevated concentrations of homocysteine induce direct vascular dysfunction (Harker et al., 1976; Rodgers and Conn, 1990; Weiss, 2005) and increase the contraction induced by vasoconstrictor agents such as phenylephrine (de Andrade et al., 2006, 2009b), angiotensin II (Bonaventura et al., 2004) and endothelin-1 (de Andrade et al., 2009a). Moreover, HHcy is associated with decreased vasorelaxation induced by acetylcholine (de Andrade et al., 2006), histamine (Ungvari et al., 1999) and bradykinin (Bonaventura et al., 2009). The mechanisms by which homocysteine alters the vessel functionality are most likely to involve direct injury of the vascular endothelium (Harker et al., 1976; Rodgers and Conn, 1990; Weiss, 2005). Impairment of the endothelium-derived nitric oxide (NO) activity (Stuhlinger et al., 2001; Chen et al., 2002) is also described to be associated with elevated plasma homocysteine levels.

Aging is associated with a number of changes in the cardiovascular system. One of the most consistent and well-studied changes is the gradual dilatation and hardening of the arteries, the process of arteriosclerosis (McEniery et al., 2007). This age-related endothelial dysfunction has been characterized by reduced agonist-induced vasodilatation (Taddei et al., 2001). In addition, several studies showed that flow-induced dilation, the most physiologically relevant measure of endothelium-dependent regulation of vascular tone, was reduced in healthy elderly humans and aged animals (Celermajer et al., 1994; Csiszar et al., 2002; Muller-Delp et al., 2002; Woodman et al., 2003). Interestingly, HHcy is considered an age-related risk factor

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for cardiovascular disease that might be related to several physiologic changes in the cardiovascular system, which are associated with the aging process (Hirsch et al., 2004). Indeed, an elevated plasma homocysteine concentration is a risk factor for the development of vascular disease and acute ischemic events such as myocardial infarction and stroke, as well as deep venous thrombosis (Nygard et al., 1997). Ungvari et al. (1999) reported that elevations in the plasmatic levels of homocysteine in old animals are associated with increased noradrenaline-induced contractions; and reduction of endothelium-dependent relaxations of rat skeletal muscle arterioles. The mechanism underlying these changes in vascular reactivity is most likely the impairment of the endothelium-derived NO activity (Stuhlinger et al., 2001; Chen et al., 2002).

Although HHcy is well established as a causative factor for cardiovascular dysfunction in old animals and humans, little information describing the effects of HHcy on the young is available. It was demonstrated that elevated levels of homocysteine induce direct noxious consequences on vascular function of young individuals with peripheral arterial occlusive disease (Huemer et al., 2006) and primates with absence of structural vascular disease (Lentz et al., 1996). The damage induced by HHcy was related to its concentration in plasma and is being hypothesized to be mediated, at least in part, by oxidative phenomena (Welch et al., 1998; Verhaar et al., 2002). In fact, non-atherosclerotic factors, such as hypercoagulability, are the main factors involved in the pathogenesis of myocardial infarct at a younger age (Hamsten et al., 1987; Thompson et al., 1995; Choudhury and Marsh, 1999; Zimmerman et al., 1995).These data may suggest that HHcy does not play a pivotal role in the development of cardiovascular dysfunctions in youths.

A variety of age-related changes occur in the responses of vascular smooth muscle. However, whether homocysteine plays a role in cardiovascular diseases in youth remains elusive. Based on the above-mentioned observations we hypothesized that the effects of HHcy in the cardiovascular system is age-dependent, being old animals more susceptible to the effects of homocysteine. Therefore, the aim of the present study was to investigate and compare the effects of elevated levels of homocysteine in carotid arteries from young and old rats. With this purpose we studied the impact of age in the effects of HHcy on vascular responsiveness to acetylcholine and phenylephrine and generation of superoxide anions.

## 2. Materials and methods

### 2.1. Homocysteine diet-induced hyperhomocysteinemia

Male Wistar rats were housed under standard laboratory conditions (12-h light–dark cycle at 24 °C) with free access to food and water. The housing conditions and experimental protocols were in accordance with the Ethical Animal Committee of the Campus of Ribeirão Preto (University of São Paulo).

HHcy was induced in young (21 days old) and adult (80 days old) rats by daily administration of DL-homocysteine thiolactone (1 g/kg body weight) (de Andrade et al., 2006, 2009a,b) in the drinking water for 7, 14 or 28 days. The doses administered were based on average daily fluid intake. The animals were weighed daily to allow the adjustment of homocysteine-thiolactone dosage. Control animals had free access to water.

### 2.2. Determination of plasma homocysteine levels

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 using the Q-TRAP system (de Andrade et al., 2006).

### 2.3. Vessel ring preparation

Rats were anaesthetized and killed by aortic exsanguination in accordance with the standards and policies of the University of Sao Paulo's Animal Care and Use Committee. The carotid artery was quickly removed, cleaned of adherent connective tissues. Two stainless-steel stirrups were passed through the lumen of each ring. One stirrup was connected to an isometric force transducer (Letica Scientific Instruments, Spain) to measure tension in the vessels. The rings were placed in 5 mL organ chambers containing Krebs solution, pH 7.4, gassed with 95% $O_2$/5% $CO_2$, and maintained at 37 °C. The composition of the Krebs solution was as follows (mmol/l): NaCl, 118.4; KCl, 4.7; $KH_2PO_4$, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25.0; Glucose, 11.6; and $CaCl_2$, 1.9.

The rings were initially stretched until basal tensions of 0.75 (young) and 1 g (adult), which were previously determined by length–tension relationship experiments. The rings were allowed to equilibrate for 60 min in the bath fluid, which was 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 μmol/l) in the presence of contractile tone induced by phenylephrine (0.1 μmol/l). 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, for studies of endothelium-intact vessels, the rings were discarded if the relaxation caused by acetylcholine was less than 50%. For studies of endothelium-denuded vessels, the rings were discarded if there was any degree of relaxation.

### 2.4. Concentration–response curves for phenylephrine, KCl and acetylcholine

After 60 min of equilibration, each carotid ring was exposed three times to phenylephrine (0.1 μmol/l) to attain its maximum contractility. Each ring was sequentially washed and re-equilibrated and was allowed to relax to baseline. After 30 min, cumulative concentration–response curves for phenylephrine ($10^{-10}$–$10^{-5}$ mol/l) or KCl (10–120 mmol/l) were determined in intact and denuded rings. In another set of experiments, endothelium-intact rat carotid rings were pre-contracted with phenylephrine (0.1 μmol/l). After reaching a stable and sustainable contraction, acetylcholine ($10^{-10}$–$10^{-5}$ mol/l) was added cumulatively to the organ bath. The vascular relaxation evoked by acetylcholine was expressed as percentage change from the phenylephrine-contracted levels. To avoid a possible influence of the pre-contracting levels induced by phenylephrine on acetylcholine-induced relaxation, the contraction was evoked with a dose of phenylephrine of 0.1 μmol/l, which was seen to contract with similar magnitude tissues from control and DL-homocysteine thiolactone-treated rats (Bonaventura et al., 2009). The concentration–response curves for phenylephrine, KCl and acetylcholine were performed in carotid rings from rats treated with DL-homocysteine thiolactone (1 g/kg body weight) for 7, 14 or 28 days and their respective age-matched controls.

### 2.5. Contribution of intracellular and extracellular $Ca^{2+}$ in the enhanced reactivity of the rat carotid arteries to phenylephrine

To further analyze the relative contribution of the release of intracellular $Ca^{2+}$ on the enhanced reactivity to phenylephrine, contractile

response to this agonist was obtained in calcium-free medium. With this purpose, the normal Krebs' solution was replaced by a $Ca^{2+}$-free solution. The rings were exposed to this solution for 1 min and then were stimulated with 1 μmol/l phenylephrine. In addition, the role of extracellular $Ca^{2+}$ mobilization was investigated by performing concentration–response curves to $Ca^{2+}$ in the presence of phenylephrine. In addition, the role of extracellular $Ca^{2+}$ mobilization was investigated by $CaCl_2$-induced contraction in the presence of phenylephrine. Endothelium-denuded rings were first contracted with phenylephrine (0.1 μmol/l) to deplete the intracellular $Ca^{2+}$ stores in $Ca^{2+}$-free solution (approximately 90 min) containing EGTA (1 mmol/l) and then rinsed in $Ca^{2+}$-free solution (without EGTA) containing phenylephrine (0.1 μmol/l). The contraction induced by $CaCl_2$ was obtained in rings from control and 14-day DL-homocysteine thiolactone-treated rats.

### 2.6. Contribution of Rho-kinase and cyclooxygenase arachidonic acid metabolites in the enhanced reactivity of the rat carotid arteries to phenylephrine

Cumulative concentration–response curves for phenylephrine were obtained in endothelium-intact carotid rings from control and DL-homocysteine thiolactone-treated rats in the absence or in the presence of the Y-27632 (Rho-kinase selective inhibitor, 0.1 μmol/l, 30 min) (Chitaley and Webb, 2002). The participation of cyclooxygenase-arachidonic acid metabolites was investigated by obtaining the cumulative concentration–response curves for phenylephrine, in endothelium-intact carotid rings, in the presence of the cyclooxygenase inhibitor indomethacin (10 μmol/l, 30 min). The curves for phenylephrine in the presence of the inhibitors were obtained in rings from the control and 14-day-treated rats.

### 2.7. Detection of superoxide anion

Hydroethidine, an oxidative fluorescent dye, was used to evaluate the levels of superoxide in situ. Cells are permeable to hydroethidine, and in the presence of superoxide anion, hydroethidine is oxidized to fluorescent ethidium bromide, in which form it is trapped by intercalation with DNA. This method provides sensitive detection of superoxide anion levels in situ. Unfixed frozen ring segments were cut into sections 30 μm thick and placed on glass slides. Hydroethidine (5 μmol/l) was applied to each tissue section and coverslipped. Slides were incubated in a light-protected humidified chamber at 37 °C for 20 min. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Fluorescence was detected with a 585-nm long-pass filter. Measurements were performed using the program Image J (version 1.43; NIH).

### 2.8. Nitrite and nitrate levels in vascular homogenates

Nitrite and nitrate levels were measured in supernatants from total carotid artery homogenates prepared under liquid $N_2$. 10 ul aliquots were injected into a Sievers chemiluminescence analyzer (model 280) and pelleted by centrifugation with $VCl_3$ (Vanadium chloride III) and HCl (at 95 ° C) as reductants for nitrate and NaI and acetic acid as reductants for nitrite. The experiments for nitrite and nitrate generation were performed in endothelium-intact rat carotid arteries from control and HHcy groups. NO results were normalized for protein concentration assessed with the Bradford technique (de Andrade et al., 2006).

### 2.9. Drugs

The following drugs were used: DL-homocysteine-thiolactone (Acrós Organics/Fischer Scientific, USA), phenylephrine hydrochloride, acetylcholine hydrochloride (Sigma, USA), L-NAME, (Sigma/RBI, USA) and indomethacin (Calbiochem). Indomethacin was dissolved in Tris buffer (pH 8.4). The other drugs were dissolved in distilled water.

### 2.10. Data analysis

Contractions were recorded as changes in the displacement (g/mg tissue) from baseline. Relaxation was expressed as the percentage change from phenylephrine-contracted levels. Agonist concentration-response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 3.0; GraphPad Software Inc.). Agonist potencies were expressed as $pD_2$ (negative logarithm of the molar concentration of agonist producing 50% of the maximum response), and maximum response was expressed as $E_{max}$ (maximum effect elicited by the agonist). The results were reported as mean ± standard error of the mean (SEM). Statistically significant differences were calculated by one-way analysis of variance (ANOVA) or Student's *t*-test. $P<0.05$ was considered as statistically significant.

## 3. Results

### 3.1. Body weight and plasma homocysteine levels

The values of HHcy in the mentioned groups, as well the body weight of animals, are given in Table 1. Homocysteine-rich diet for 7, 14 and 28 days increased plasma homocysteine concentrations when compared to their respective age-matched control groups, in both, young and adult animals. However, the values of homocysteine in young animals were greater when compared to those obtained in adult rats.

### 3.2. Concentration–response curves for phenylephrine, KCl and acetylcholine

In young rats, the $E_{max}$ of the concentration–response curve for phenylephrine was significantly higher in endothelium-intact arteries from DL-homocysteine thiolactone-treated rats (14 and 28 days) than in arteries from control, without there being significant differences among $pD_2$ values. In adult rats, treatment with DL-homocysteine thiolactone for 7, 14 or 28 days increased the $E_{max}$ values for phenylephrine in endothelium-intact rings (Fig. 1). In arteries from control and DL-homocysteine thiolactone-treated rats, mechanical removal of the endothelium significantly increased the maximal contraction induced by phenylephrine (data not shown). The magnitude of the enhancement observed after DL-homocysteine thiolactone treatment on phenylephrine-induced contraction in endothelium-denuded rings did not differ among the three different periods of treatment employed in the present study in either young or adult rats.

**Table 1**
Values of plasmatic homocysteine (μmol/L), and body weight in control and HHcy rats (DL-HcyT, 1 g/kg/day).

| | Young | | Adult | |
|---|---|---|---|---|
| | Control | HHcy | Control | HHcy |
| *Plasmatic homocysteine (umol/L)* | | | | |
| 7 days | 1.41 ± 0.14 | 95.17 ± 9.42[a] | 5.48 ± 0.34 | 67.01 ± 4.83[a,b] |
| 14 days | 2.10 ± 0.20 | 105.70 ± 5.58[a] | 5.31 ± 0.82 | 63.22 ± 6.88[a,b] |
| 28 days | 2.27 ± 0.37 | 117.56 ± 15.25[a] | 5.59 ± 1.21 | 63.78 ± 3.62[a,b] |
| *Body weight (g)* | | | | |
| 7 days | 113.37 ± 3.34 | 105.37 ± 2.62 | 324.48 ± 4.50 | 314.74 ± 4.37 |
| 14 days | 186.64 ± 4.61 | 163.17 ± 3.94[a] | 337.92 ± 5.72 | 311.92 ± 5.15 |
| 28 days | 331.58 ± 7.15 | 250.75 ± 6.39[a] | 353.28 ± 6.07 | 317.95 ± 5.46 [a] |

Values are means ± SEM of $n = 8–9$ preparations.
[a] Compared to respective control.
[b] Compared to young rats ($P<0.05$; one-way ANOVA).

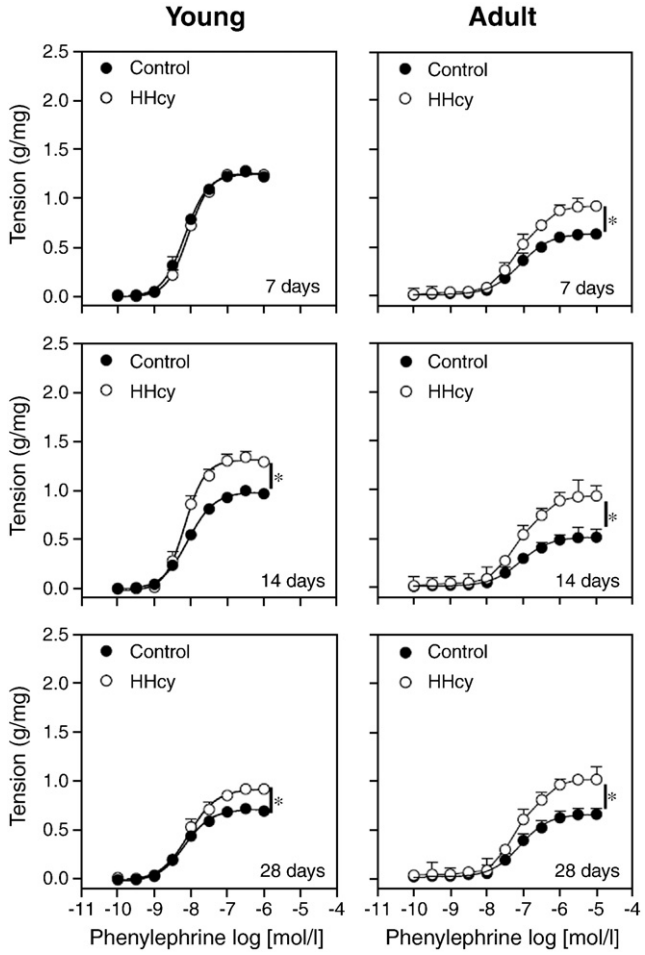


**Fig. 1.** Effect of DL-homocysteine thiolactone treatment on phenylephrine-induced contractile response in carotid rings from young and adult rats. Concentration–response curves for phenylephrine were determined in endothelium-intact carotid rings from control or DL-homocysteine thiolactone-treated rats. *Compared to respective control ($P<0.05$; ANOVA followed by Bonferroni's multiple comparison test).

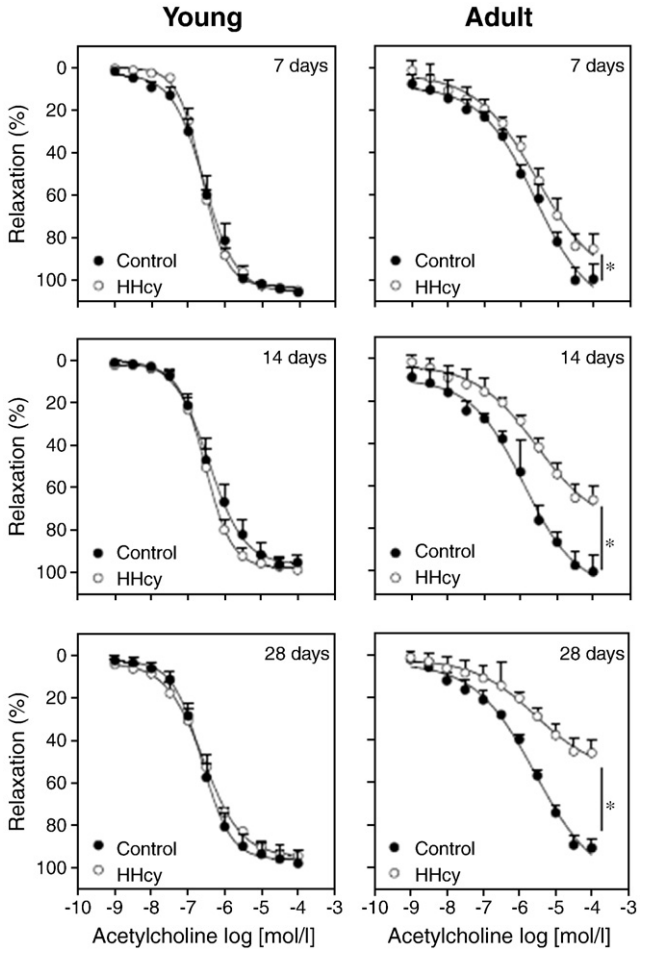


**Fig. 2.** Effect of DL-homocysteine thiolactone treatment on acetylcholine-induced relaxation in carotid rings from young and adult rats. Concentration–response curves for acetylcholine were determined in endothelium-intact carotid rings from control or DL-homocysteine thiolactone-treated rats. *Compared to respective control ($P<0.05$; ANOVA followed by Bonferroni's multiple comparison test).

KCl-induced contraction in endothelium-intact or denuded rat carotid rings was not altered at any time after treatment with DL-homocysteine thiolactone in either young or adult rats (data not shown).

In young rats, acetylcholine-induced relaxation was not altered at any time after treatment with DL-homocysteine thiolactone. On the other hand, treatment with DL-homocysteine thiolactone for 7, 14 or 28 days reduced the relaxation induced by acetylcholine in arteries from adult rats (Fig. 2).

Based on the functional findings, the following experiments designed to investigate the mechanisms underlying the effect induced by DL-homocysteine thiolactone treatment on phenylephrine-induced contraction were obtained in endothelium-intact carotid rings from 14-day-treated rats and its respective age-matched control animals (Table 2).

### 3.3. Contribution of Rho-kinase and cyclooxygenase arachidonic acid metabolites in modulating the response to phenylephrine

In adults and young animals, pre-incubation with Y-27632 did not produce changes in the concentration–response curves for phenylephrine in rings from control rats (Fig. 3). In the presence of Y-27632, the contraction induced by phenylephrine in rings from young DL-homocysteine thiolactone-treated rats was reduced to values similar to the ones observed in control carotid arteries. On the other hand, Y-27632 did not affect the contraction induced by phenylephrine in carotid arteries from adult rats treated with DL-homocysteine thiolactone. Incubation with indomethacin did not alter phenylephrine-induced contraction in arterial segments from both control and HHcy rats (Fig. 3).

### 3.4. Contribution of intracellular and extracellular $Ca^{2+}$ in the enhanced reactivity of the rat carotid arteries to phenylephrine

In $Ca^{2+}$-free medium, phenylephrine (1 μmol/l) induced a rapid phasic contraction that reached a peak and then returned close to baseline levels. In young rats, the magnitude of the peak phasic response was similar in control and DL-homocysteine thiolactone-treated rats. On the other hand, treatment with DL-homocysteine thiolactone increased the contraction induced by phenylephrine in

**Table 2**
Participation of intracellular and extracellular $Ca^{2+}$ (g/mg tissue) on Phenilephrine contraction in arteries from control and HHcy rats.

| | Young | | Adult | |
|---|---|---|---|---|
| | Control | HHcy | Control | HHcy |
| Intracellular $Ca^{2+}$ | 0.47 ± 0.04 | 0.45 ± 0.05 | 0.19 ± 0.05 | 0.53 ± 0.03[a] |
| Extracellular $Ca^{2+}$ | 0.87 ± 0.04 | 0.91 ± 0.06 | 0.14 ± 0.03 | 0.28 ± 0.03[a] |

Values are means ± SEM of $n = 6–7$ preparations.
[a] Compared to respective control ($P<0.05$; one-way ANOVA).

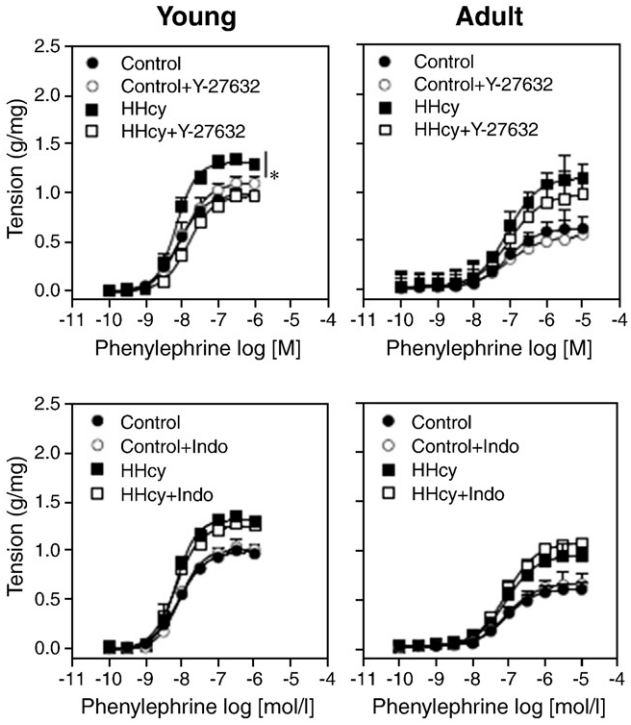


**Fig. 3.** Effect of Y-27632 or indomethacin on phenylephrine-induced contraction of rat carotid rings. Concentration–response curves for phenylephrine were determined in endothelium-intact carotid rings from control or 14 day-treated DL-homocysteine thiolactone rats. The curves were determined in the absence or after a 30-min period of incubation with 0.1 μmol/l Y-27632 or 10 μmol/l indomethacin. *Compared to respective control ($P<0.05$; ANOVA followed by Bonferroni's multiple comparison test).

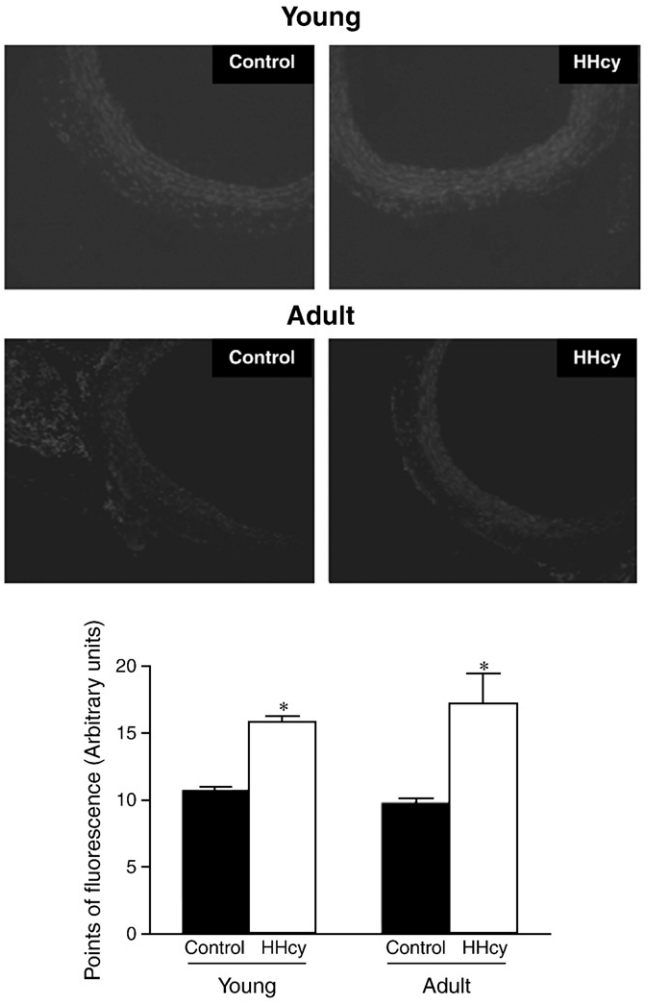


**Fig 4.** Effect of DL-homocysteine thiolactone treatment on superoxide anion generation in carotid arteries from adult and young rats. Hydroethidine, an oxidative fluorescent dye, was used to evaluate levels of superoxide in carotid arteries from control or 14-day DL-homocysteine thiolactone-treated rats. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with a krypton/argon laser. Fluorescence was detected with a 585-nm long-pass filter. The bars represent the mean ± SEM of $n = 5$ experiments. *Compared to respective control ($P<0.05$; ANOVA followed by Bonferroni's multiple comparison test).

arteries from adult rats (Table 1). Similarly, $CaCl_2$-induced contraction was greater in carotid rings from adult DL-homocysteine thiolactone-treated rats when compared with control. Conversely, no differences in $CaCl_2$-induced contraction were found between control and HHCy carotid arteries in young rats (Table 1).

### 3.5. Effect of HHcy on basal superoxide anion generation

Basal generation of superoxide anion did not differ in control tissues from young and adult rats. HHcy induced enhancement on superoxide anion levels in carotid arteries from both young and adult rats (Fig. 4).

### 3.6. Effect of HHcy on basal nitrate and nitrite levels

HHcy did not alter the levels of nitrate in carotid arteries from both young and adult rats. On the other hand, reduced levels of nitrite were found in arteries from adult, but not young rats treated with DL-homocysteine thiolactone (Fig. 5).

## 4. Discussion

Chronic DL-homocysteine thiolactone intake produced a time-dependent enhancement on phenylephrine-induced contraction in endothelium-intact carotid arteries. Our observations are in agreement with previous studies showing that HHcy is associated with an increase in noradrenaline-induced constriction and a reduction in the endothelium-dependent dilation of skeletal muscle arterioles (Ungvari et al., 1999). Moreover, we have previously described that HHcy increased the contraction induced by angiotensin II (Bonaventura et al., 2004) and endothelin-1 (de Andrade et al., 2009a) in the rat carotid artery and decrease the endothelium-dependent relaxation induced by bradykinin in this same blood vessel (Bonaventura et al., 2009). Interestingly, endothelium denudation of arterial rings from control but not DL-homocysteine thiolactone-treated rats enhanced the contractile response of these arterial vessels to phenylephrine, indicating that the

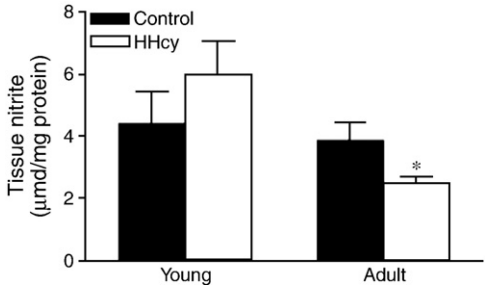


**Fig. 5.** Effect of DL-homocysteine thiolactone treatment on nitrate and nitrite levels in carotid arteries from adult and young rats. The experiments for basal nitrite and nitrate generation were performed in endothelium-intact carotid arteries from control or 14-day DL-homocysteine thiolactone-treated rats. The results were normalized for protein concentration assessed with the Bradford technique. *Compared to respective control ($P<0.05$; ANOVA followed by Bonferroni's multiple comparison test).

endothelium partially counteracts the phenylephrine-mediated vasoconstriction. This result suggests that HHcy impairs the modulatory activity of the endothelium; this alteration could partly contribute to the hyperreactivity of the carotid artery to phenylephrine observed in DL-homocysteine thiolactone-treated animals. Some reports suggested that the period of exposure to elevated levels of homocysteine is the major factor in the development of cardiovascular abnormalities (Bonaventura et al., 2009; de Andrade et al., 2009a,b). Results presented here demonstrate that HHcy produced an enhanced responsiveness to phenylephrine in carotid arteries, although evidencing no relation between the period of treatment and the magnitude in the enhancement of phenylephrine-induced contraction. However, it is important to note that, in adult animals, the increased vascular responsiveness to phenylephrine was already observed in 7-day DL-homocysteine thiolactone-treated animals. On the other hand, in young animals, this response was only apparent after treatment for 14 and 28 days. Moreover, treatment with DL-homocysteine thiolactone did not alter the endothelium-dependent relaxation induced by acetylcholine in young animals but significantly reduced this response in adult rats after treatment for 14 and 28 days. Taken together, these results support the notion that young animals are less sensitive than adult rats to the cardiovascular alterations induced by HHcy.

Elevated plasma levels of homocysteine, a naturally occurring thiol-containing amino acid, constitute a recognized risk factor in cardiovascular disease (Boushey et al., 1995). Normal levels of homocysteine range from 1–10 μM, with a significantly higher level in men than women (Ueland and Refsum, 1989). A significant risk is observed at only slightly higher levels (15–20 μM or more). Thromboembolism is a major cause of death in patients which presents hyperhomocysteinemia (Ueland and Refsum, 1989). Several mechanisms have been proposed to explain a causative link between hyperhomocystinemia and atherosclerosis (Welch et al., 1997). One of the more well accepted is that the autoxidation of homocysteine leads to the formation of superoxide and hydrogen peroxide, that consequently alters endothelial cell function and predisposes the affected vessel to atherosclerosis (Welch et al., 1997; Starkebaum and Harlan, 1986). It is now well established that an elevation of vascular superoxide generation is a viable mechanism of endothelial dysfunction and can be proatherogenic by virtue of the rapid reaction between nitric oxide and superoxide (Harrison, 1997).

The lack of effect induced by DL-homocysteine thiolactone treatment on KCl-induced contraction described in the present investigation was also observed by Bonaventura et al. (2004). Contractions of vascular tissues induced by KCl rely almost exclusively on $Ca^{2+}$ influx through activation of voltage-sensitive channels (Hudgins and Weiss, 1968), whereas contractions induced by phenylephrine are mediated by an increase in $Ca^{2+}$ influx through both receptor-operated channels (Hirata et al., 1998) and voltage-sensitive channels (Lee et al., 2001). Since HHcy did not alter the contraction induced by KCl, it could be suggested that HHcy does not affect $Ca^{2+}$ influx through voltage-operated channels. However, it has been previously suggested that alteration of phosphoinositide turnover or transmembranous $Ca^{2+}$ influx in vascular smooth muscle cells is a possible mechanism for the effect of HHcy on blood vessels (Okatani et al., 2001). We found that the contractile response to phenylephrine in $Ca^{2+}$-free medium, which is used to verify the contribution of intracellular $Ca^{2+}$, was significantly increased in arteries from adult but not young rats, suggesting that $IP_3$-induced intracellular $Ca^{2+}$ release after stimulation with phenylephrine (Horowitz et al., 1996) contribute to the enhanced contractile response of DL-homocysteine thiolactone-treated carotid arteries. Moreover, we observed that the enhanced responsiveness to phenylephrine was associated with an enhanced contractility to increasing extracellular $Ca^{2+}$ in carotid arteries from adult, but not young rats. Based on these results, we can suggest that HHcy mediate the enhanced reactivity to phenylephrine by mechanisms that alter the mobilization or sensitivity to intra and extracellular $Ca^{2+}$ in adult rats.

Rho/Rho-kinase signaling, as well as $Ca^{2+}$ mobilization, is thought to play important roles in vasoconstriction and may contribute to the etiology of cardiovascular diseases both in humans and experimental animals (Jin et al., 2004; Nakakuki et al., 2005). Moreover, basal peripheral and systemic vascular tone depends on Rho-associated kinase (Büssemaker et al., 2007). In this study, the effect of Rho-kinase inhibitor (Y-27632) on arteries from HHcy treated rats markedly indicates that the contraction induced by phenylephrine requires the activation of Rho-kinase pathway, being this activation more important in young than in adult rats. Rho-kinase pathway negatively regulates vascular NO bioavailability by increasing superoxide concentrations. Moreover, increased superoxide levels can modify the contraction of vascular muscle smooth through activation of the Rho-kinase pathway (Jin et al., 2004). The present findings show that carotid arteries from young and adult rats treated with DL-homocysteine thiolactone possess significantly higher levels of superoxide anion when compared to control tissues. This observation corroborates previous findings from our laboratory showing that HHcy increases reactive oxygen species generation, as indicated by the overproduction of nitrotyrosine (de Andrade et al., 2006). Our results also show that maximum contraction induced by phenylephrine was not altered by indomethacin in endothelium-intact carotid rings from control or DL-homocysteine thiolactone-treated rats. This result shows that endothelial prostanoids derived from the arachidonic acid-cyclooxygenase pathway do not modulates the contraction induced by phenylephrine in the rat carotid artery. Moreover, we can conclude that HHcy does not alter the generation of prostanoids.

The mechanisms by which HHcy 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; Weiss, 2005) and reduction of NO bioavailability (Stamler et al., 1993; Powers et al., 2003). Blood vessels maintain a balanced state between vasodilatation and vasoconstriction through biochemical signaling by endothelial cells to smooth muscle cells (Rubanyi, 1993). The disturbance of these cell functions is known to generally cause decreased relaxation and increased vasoconstriction (Luscher et al., 1993; Alexander, 1995; Sellke et al., 1996). Our findings show that although HHcy induced increased responses to phenylephrine in young rats, no differences were found for the endothelium-dependent relaxation induced by acetylcholine, which is mediated by NO in this tissue (Tirapelli et al., 2006). Interestingly, HHcy did not modify nitrite or nitrate, the levels of which are used as an indicator of NO production. Taken together, these observations suggest that in young rats the enhanced responsiveness of DL-homocysteine thiolactone-treated carotid arteries to phenylephrine was not due to an impaired release/generation of NO. Thus, it seems that in young rats, the hyperreactivity to phenylephrine induced by HHcy is related to an increased generation of superoxide levels, which in turn would modify the contraction of vascular muscle smooth through activation of the Rho-kinase pathway. On the other hand, our findings in carotid arteries from old rats suggests that the enhanced responsiveness of DL-homocysteine thiolactone-treated carotid arteries to phenylephrine was due to an impaired release/generation of NO since reduced levels of nitrite and impaired relaxation to acetylcholine was observed in these arteries. A possible explanation to this response is that increased generation of reactive oxygen species in the arteries obtained from HHcy rats may lead to NO inactivation, thus reducing NO bioavailability, and increasing the formation of peroxynitrite and secondary nitro-oxidative species (de Andrade et al., 2006).

Aging is associated with a generalized endothelial dysfunction (Celermajer et al., 1994; Eskurza et al., 2006) and an increased incidence of cardiovascular disease (Seals et al., 2006). Vascular endothelial dysfunction is mainly characterized by a progressive

reduction of the bioavailability of NO (Brandes et al., 2005). Limited availability of tetrahydrobiopterin (a cofactor for eNOS) observed in old rats may contribute to eNOS uncoupling, and the subsequent decrease in NO signaling and increased eNOS-derived superoxide anion formation (Delp et al., 2008; Sindler et al., 2009). The decrease in NO bioavailability due to excess amount of superoxide anion formation is a major cause of endothelial dysfunction in aging (Brandes et al., 2005). Thus, the age-related endothelial dysfunction could be suggested as a source for the different responses induced by HHcy on carotid artery responsiveness to phenylephrine in young and adult rats. Based on our findings we can conclude that old rats are more susceptible to the vascular dysfunction induced by HHcy.

## 5. Conclusions

The major new finding of the present work is that arteries from young rats are more resistant to vascular changes evoked by HHcy than arteries from adult. This information provides important aspects for elucidating the mechanisms involved in the development and progression of arterial diseases and the importance of temporal consequences of this process. Also, we verified that the enhanced vascular response to phenylephrine observed in carotid arteries of DL-homocysteine thiolactone-treated rats is mediated by different mechanisms in young and adult rats.

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