Aging and total stenosis triggers differential responses of carotid and basilar arteries to endothelin-1 and phenylephrine

Claudia Roberta de ANDRADE1, Fernando Morgan de A. CORRÊA1 and Ana Maria de OLIVEIRA2

1Department of Pharmacology, School of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil
2Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

Received July 13, 2009; Accepted October 14, 2009

Abstract

Our aim was to investigate the effects of ageing on the vascular contractility of carotid and basilar arteries from guinea-pigs, in a model of total stenosis. Moreover, we attempted to identify whether total stenosis of the left common carotid (stenosed) in adult guinea-pigs, would affect the contractions of contralateral carotid (intact) and basilar arteries to different vasoconstrictors. With this purpose, the left carotid was occluded with a silk thread at a position close to its origin. Vascular reactivity experiments using standard muscle bath were performed 7, 15, 30, and 90 days after carotid occlusion. Reactivity of carotid and basilar arteries to endothelin-1, phenylephrine and KCl was reduced with ageing in naive guinea-pigs. The endothelin-1 and KCl-induced contractions were unaltered in arteries from SHAM-operated animals. Moreover, phenylephrine-induced contractions were reduced in both carotids from 7 days SHAM-operated guinea-pigs, when compared to naive group. Stenosis induced progressive reduction in the contraction induced by endothelin-1, phenylephrine and KCl in the stenosed carotid, when compared to their respective age-matched naive and SHAM groups. Interestingly, an increased contractile-response to vasoconstrictor agents in all the contralateral carotids was observed. Stenosis (30 and 90 days) also induced an increase in the contractions induced by endothelin-1 in the basilar artery. Conversely, phenylephrine and KCl-induced contractions were reduced in basilar arteries 7, 15, 30 and 90 days after stenosis. These results showed that stenosis in adult guinea-pigs induce alterations of vascular reactivity in arteries distant from the site of injury. Thus, in spite of the common use of contralateral carotid as control, it must be aware of the potential alteration induced by stenosis in the vascular motility of such vessels. Additionally, it was verified a relationship between the period of stenosis and the alterations in the vascular reactivity to these vasoconstrictors.

Key words: total stenosis, contralateral carotid artery, basilar artery, vascular reactivity, endothelin-1
Introduction

Vascular diseases remain the most common cause of death in the world (Ross, 1999), and these chronic diseases progress slowly with age and can affect the reactivity of blood vessels (Bashour et al., 1990; Mizuno et al., 1992). The new effective experimental models of vascular injury have enhanced the understanding on the pathophysiological processes leading to vascular occlusion.

Age-related changes in the cardiovascular system and other organ systems are associated with the increasing prevalence of cardiovascular diseases at older age (Thomas and Rich, 2007). A possible mechanism involved in this process is the ageing-associated mechanical alterations in blood vessels that lead to altered responsiveness to vasoactive agents. In this line, the potency of noradrenaline increased in the isolated aorta from rat and mouse with age from 3 to 10 weeks, but decreased thereafter from 10 to 40 weeks (Satoh et al., 1995; Tanaka et al., 2006).

Animal models of vascular occlusive diseases (Kennedy et al., 2006), as well as models of vascular injury (Milner et al., 1997; Accorsi-Mendonça et al., 2004; Fukada et al., 2004a; 2004b), are associated with reduced contractility of the injured artery to catecholamines and angiotensin II. Interestingly, the effects of stenosis on the vascular responses to KCl were reported to be tissue and age-dependent (Asano et al., 1993). The ageing effects on the vasculature may differ depending on agonist, vessel type, and the tissue studied. Alternatively, the varying effects of ageing could reflect differences in the animal strains and the variety of techniques for evaluation of contractility used in these studies (For review please refer Ferrari et al., 2003). In fact, we have recently reported that unilateral stenosis of guinea-pig carotids induces alterations of the vascular reactivity on arteries distant from the site of injury, such as contralateral carotid and basilar arteries (de Andrade et al., 2008). However, it is important to note that our data were obtained in carotid and basilar arteries from guinea-pigs, which had not reached their adulthood, when the injury was established. Thus, the consequences of unilateral total stenosis of carotid on reactivity of contralateral carotid and basilar arteries, in adult guinea-pigs, and the effect of ageing in this response remain to be investigated.

The contralateral carotid is widely used as control in animal models of vascular injury or occlusive diseases (Manderson et al., 1989; Lippolis et al., 2003; Popolo et al., 2005). However, Milner et al. (1997) reported that the carotid injury triggers compensatory mechanisms in arteries located distant from the site of injury. Such effects were also observed by Accorsi-Mendonça et al. (2004), who have showed that these compensatory mechanisms alter the vascular reactivity of rat carotids to phenylephrine and angiotensin II, after balloon injury. Similarly, an increased response to phenylephrine and angiotensin II was observed in the contralateral carotid artery after perivascular injury induced by a silicone collar (Fukada et al., 2004a; 2004b). Moreover, it was previously described that partial stenosis of carotids, due to the atherosclerosis process, increases the vascular reactivity of the carotid and cerebral arteries to contractile agonists (Muller et al., 1995; van Everdingen et al., 2000; de Nie et al., 2001). These results indicate that vascular injury or occlusion evokes alterations in the reactivity of the contralateral carotid.

Based on the aforementioned observations, the aim of this study was to investigate the...
effects of ageing in the vascular contractility in a model of total stenosis. Moreover, we attempted to verify whether total stenosis of the left common carotid artery (stenosed) would affect the vascular responsiveness of the contralateral carotid (intact) as well as the basilar artery from adult guinea-pigs. With this purpose, concentration-response curves for endothelin-1, phenylephrine and KCl were performed on the left (stenosed) or right (contralateral) carotids and basilar arteries 7, 15, 30 and 90 days after stenosis.

**Material and Methods**

*Experimental design*

Male guinea-pigs were housed under standard laboratory conditions with free access to food and water. Housing conditions and experimental protocols are in accordance with the Ethical Animal Committee from the University of São Paulo, *Campus* of Ribeirão Preto.

Adult male guinea pigs (6-month old) were randomly divided into three groups:

A. Naive group: Intact animals: without any surgical proceeding. Both left and right carotid and basilar arteries were intact.

B. SHAM-operated group: animals were anesthetized with dihydrotiazine chloride (0.2 ml/Kg, *i.m.*, Bayer, Germany). The left common carotid was exposed, separated from the vagus nerve and manipulated. The right carotid and basilar arteries were intact.

C. Stenosis: animals were anesthetized with dihydrotiazine chloride (0.2 ml/Kg, *i.m.*, Bayer, Germany). The left carotid was exposed, isolated from the vagus nerve and completely occluded with a silk thread at a position close to its origin. The right carotid and basilar arteries were intact.

The schemes outlined in Fig. 1 illustrate the surgical proceedings in the three experimental groups, and Fig. 2 represents the design of the experimental protocol.

Carotid occlusion was performed in 6-month old guinea-pigs, when it is assumed that they reach adulthood (Charles River Institutes). Vascular reactivity experiments were performed 7, 15, 30 and 90 days after carotid occlusion. Vascular reactivity of the left carotid was performed in the distal portion to manipulation (SHAM-operated group) or occlusion (stenosis group).

*Artery preparation and measurements*

The guinea-pigs were anaesthetized and sacrificed by aortic exsanguination. The carotids were quickly removed, cleaned of adherent connective tissues and cut into rings. After isolation of the carotid rings, the skull was opened and the brain was carefully removed. The brain stem was isolated to allow dissection of the basilar artery. The basilar artery was quickly removed, cleaned of adherent connective tissues and one ring was dissected. Two stainless-steel stirrups were passed through the lumen of each ring (carotid or basilar). One stirrup was connected to an isometric force transducer (Letica Scientific Instruments, Barcelona, Spain) to measure vessel tension. The rings were placed in a organ chamber (volume 5 ml) containing Krebs solution (pH 7.4). The composition of the Krebs solution was as follows (in mM): NaCl, 118.4; KCl, 4.7; KH₂PO₄, 1.2; MgSO₄, 1.2; NaHCO₃, 25.0; Glucose, 11.6; and CaCl₂, 1.9. The solution was continuously gassed with carbogen (95% O₂ + 5% CO₂) at 37°C. The rings were initially
stretched until a basal tension of 0.8 g (carotids) and 0.3 g (basilar), which were determined by length-tension relationship experiments. The rings were allowed to equilibrate for 60 min with the bath fluid being changed every 15–20 min. 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).

After equilibration for 60 min in Krebs solution, each ring was exposed twice to phenylephrine ($10^{-7}$ M) to assess 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 ($10^{-11}$ to $10^{-5}$ M), endothelin-1 ($10^{-15}$ to $10^{-8}$ M) or KCl (10 to 120 mM) were determined on endothelium-intact rings. The vascular responsiveness to the agonists was studied in carotid and basilar arteries from total stenosis groups (7, 15, 30 and 90 days) and their respective age-matched naive and SHAM groups.

At the end of the assays the rings were dehydrated and weighed. Contractions were recorded as changes in the displacement (grams per mg of dry tissue) from baseline.

**Reagents**

The following drugs were used: dihydrotiazine chloride (Bayer®, Germany), phenylephrine hydrochloride, acetylcholine hydrochloride and endothelin-1 (Sigma, St. Louis, Mo., USA), and KCl (Synth, São Paulo, Brazil). The drugs were dissolved in distilled water. Stock solutions of endothelin-1 were prepared in 0.02 mM acetic acid and frozen. Suitable dilutions were made on the day of the experiment.

**Data analysis**

Results are expressed as mean ± standard error of the mean (S.E.M.). Agonist potencies and maximal responses were expressed as $pD_2$ (negative logarithm of the molar concentration of agonist producing 50% of the maximal response) and $E_{max}$ (maximum effect elicited by the agonist), respectively. The $E_{max}$ and $pD_2$ values were calculated for each individual concentration-response curve by nonlinear regression analysis using GraphPad Prism version 3.0 for Windows (GraphPad Software Inc., San Diego, USA). Statistical analysis of the $E_{max}$ and $pD_2$ values was performed using one-way analysis of variance (ANOVA). *Post-hoc* comparisons were performed using Bonferroni’s multiple comparison test. Values of $P\leq0.05$ were taken to be statistically significant.
Fig. 3. Effect of ageing on the responsiveness of carotid and basilar arteri es to endothelin-1, KCl and phenylephrine. Concentration-response curves for (A) endothelin-1 ($10^{-15}$–$10^{-8}$ mol/l); (B) KCl (10 to 120 mmol/l) and (C) phenylephrine ($10^{-12}$–$10^{-5}$ mol/l) were performed in the carotid and basilar artery segments from adult (6-month old) naive guinea pigs, 7, 15, 30 and 90 days. Values are means ± S.E.M. of n=7 preparations for each group. *, compared to 6-month old ($P<0.05$, ANOVA followed by Bonferroni’s multiple comparison test).
Results

Effect of ageing on vascular reactivity

Vascular reactivity evoked by endothelin-1, phenylephrine and KCl was reduced with ageing (Fig. 3). The carotid and basilar responses to endothelin-1 and phenylephrine were reduced in 7 and 9-month old animals (Fig. 3, A and B).

Similarly, the Emax and potency of endothelin-1 were reduced in basilar arteries from 7 and 9-month old animals (Fig. 3A). While endothelin-1 potency was reduced in both, carotid and basilar arteries, no differences were found for potency for phenylephrine in these arteries. In addition, KCl-induced contraction was significantly reduced in carotid rings from 9-month old guinea pigs (Fig. 3C). On the other hand, a decrease on Emax values for KCl in basilar arteries was observed in animals 7 and 9-month old. No differences were observed in the pHD2 values (potency) for KCl in carotid or basilar arteries.

Effect of SHAM surgery on vascular reactivity

The responsiveness of carotid and basilar arteries from naive animals was compared to those observed in SHAM-operated guinea-pigs 7, 15, 30 and 90 days after SHAM-surgery. Endothelin-1 and KCl evoked similar concentration-dependent responses in carotid arteries from intact and SHAM-operated guinea-pigs (Figs. 4 and 5). However, there was a reduction in
the Emax values for phenylephrine in the left (manipulated) and right (intact) carotids from SHAM-operated animals, when compared to their respective age-matched naive animals. However, no differences in the Emax values for phenylephrine were detected in basilar arteries from SHAM-operated animals, when compared to the respective age-matched naive group (Fig. 6). SHAM-surgery did not alter the $\rho D_2$ values for endothelin-1, KCl and phenylephrine in neither carotid nor basilar arteries.

**Effect of unilateral total stenosis on the vascular contraction induced by endothelin-1, KCl and phenylephrine**

Total stenosis for 30 and 90 days induced a reduction on endothelin-1-induced contraction in the stenosed carotids. Conversely, total stenosis increased the contraction induced by endothelin-1 in intact carotids 30 and 90 days after stenosis, when compared to the arteries from the respective age-matched SHAM groups (Fig. 4). The Emax for endothelin-1 was reduced in basilar arteries 7 days after stenosis when compared to the respective naive and SHAM-operated groups. However, endothelin-1-induced contraction was significantly higher in basilar arteries isolated 30 and 90 days after the stenosis procedure, when compared to their age matched naive and SHAM groups (Fig. 4).

Stenosis induced a progressive reduction on KCl-induced contraction in the stenosed
Vascular reactivity and injury

carotids that were removed 15, 30 and 90 days after surgery (Fig. 5). The Emax values for KCl were higher in the intact carotids from animals in the total stenosis group that were sacrificed 30 or 90 days after the procedure (Fig. 5). In basilar arteries, total stenosis for 7, 15, 30 and 90 days induced a progressive reduction on KCl-induced contraction, when compared to the respective age-matched naive and SHAM groups (Fig. 5).

The phenylephrine-induced contractions in the stenosed carotids were also reduced, despite the increase observed in contralateral carotids 15, 30 and 90 days after stenosis (Fig. 6). This stenosis process for 7, 15, 30 and 90 days induced a decrease on phenylephrine-induced contraction (Fig. 6).

Stenosis did not alter the potency of endothelin-1, phenylephrine and KCl in either carotid or basilar arteries (data not shown).

**Discussion**

The present findings show that ageing reduces the reactivity of the guinea-pig carotid and basilar arteries for endothelin-1, KCl and phenylephrine, which suggest that ageing triggered a non-specific decrease in the contraction responses in these arteries. This observation is in accordance with previous findings from the literature describing that ageing reduces urotensin II-induced contraction in the rat aorta (Ishihata et al., 2005) and carotid responses for
phenylephrine and KCl (De Oliveira et al., 1998). Ageing is described to induce mechanical and morphological alterations as well as endothelial dysfunction in blood vessels. The morphological bases for these mechanical alterations are the well known vascular transformations that occur during senescence: arteries become tortuous and elongated with intimal and medial thickening together with an increase in collagen accumulation in the media, and elastin fragmentation (Ferrara et al., 2003).

The findings from animal studies, which have examined the effects of ageing on endothelial function and vascular contractility, have been contradictory. As an example, endothelial-dependent vasodilatation has been reported to remain unchanged (Ishihata et al., 2005), decreased (Ibarra et al., 2006) and possibly enhanced with ageing (Shipley et al., 2005). Similarly, contractile response was also unaltered (Ririe et al., 2001) or decreased (Matz et al., 2001) with ageing. Taken together, these results suggest that the effects of ageing in the vasculature are complex and may differ depending on agonist, vessel type, and the tissue studied. Alternatively, the varying effects of ageing could reflect differences in the animal strains, the age of study, and the variety of techniques for evaluation of contractility used in these studies. Our data support the notion that ageing simultaneously affects the reactivity of two different vascular tissues to three vasoactive substances. The importance of this observation is that ageing became an important factor that alters the vascular reactivity, in a same experimental model.

Another point observed in the present investigation is the absence of alterations in the contractile response induced by endothelin-1 and KCl in arteries from SHAM-operated guinea-pigs when compared to tissues from naive animals. This observation indicates that the surgical procedure, which consists in the dissection of the vagus nerve in left carotid, was not able to affect per se the reactivity of the carotid and basilar arteries. Interestingly, there was a reduction in the responsiveness evoked by phenylephrine in carotids from SHAM-operated animals. However, further investigation is needed to verify the mechanisms underlying this response.

Vascular compensation and remodelling are poorly understood complex processes. Few studies have investigated the altered reactivity seen in the contralateral and collateral arteries and, therefore, further studies are required to examine which of the above mechanisms are responsible for this phenomenon. Our results indicate that important information concerning possible compensatory mechanisms may be derived from the study of the consequences of total stenosis on the responsiveness of contralateral and ipsilateral carotid arteries. Our data, obtained using a model of total stenosis in guinea pigs (Cava porcellus) (Majewska-Michalska et al., 1998) confirm the importance of adaptation to total stenosis on the vascular reactivity to different vasoconstrictor agents. In these animals, the basilar artery is responsible by one third of cerebral circulation, whereas the carotid arteries supply the other two third (Majewska-Michalska et al., 1998). Unilateral carotid occlusion induced significant changes in the vascular reactivity of the ipsilateral and contralateral carotids and the collateral basilar arteries to endothelin-1, phenylephrine and KCl.

The present investigation shows that unilateral total stenosis of the common carotid induced a reduction on endothelin-1-, phenylephrine- and KCl-induced contraction in the stenosed carotid, further evidencing a relation between the period after stenosis and the magnitude in the
decrease of the contraction induced by these vasoactive agents. Our findings are in agreement with previous studies describing reduced contraction for several agonists such as endothelin-1, phenylephrine and angiotensin II in injured arteries (Milner et al., 1997; Accorsi-Mendonça et al., 2004; Fukada et al., 2004a; 2004b).

Endothelin-1 is a 21-amino-acid peptide produced by the endothelium, belonging to a family of potent vasoconstrictors (Yanagisawa et al., 1988). The vascular actions of endothelins are mediated by two receptors named ET_A and ET_B. The former is restricted to vascular smooth muscle and mediates vasoconstriction (Haynes et al. 1993). Endothelial ET_B receptors are described to mediate relaxation via production of nitric oxide (NO) (Hirata et al. 1993) and (or) prostacyclin (PGI_2) (Filep et al. 1991), while the ET_B receptors located on vascular smooth muscle induce contraction (Ihara et al., 1992; Tirapelli et al., 2005). Endothelin-1-induced contraction of guinea-pig carotid is mediated by ET_A receptors (Rubany et al. 1994), which induces contraction, via influx of extracellular Ca^{2+} (Rubany et al. 1994). Accordingly, the reduced reactivity to endothelin-1 could be related to a lesser participation of ET_A receptors located on the vascular smooth muscle. Interestingly, the reduced reactivity to endothelin-1 is not due to a reduction of contractile machinery of the stenosed guinea pig carotid, since the contractile response of these arteries to phenylephrine, a selective α_1-adrenoreceptor agonist, and KCl, a depolarizing agent, was also reduced by stenosis.

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 (Wesselman et al. 1996; Lee et al., 2001). Likewise, endothelin-1-induced contraction involves the influx of extracellular Ca^{2+} through receptor-operated and voltage-sensitive Ca^{2+} channels (Rubany et al. 1994). Since stenosis reduced the contraction induced by these vasoactive agents, it could be suggested that stenosis alters Ca^{2+} influx through voltage- and receptor-operated channels.

Another point to be considered is the age of animals, since Sandoval et al. (2007) reported that 5-HT-induced myofilament Ca^{2+} sensitization, is attributed to agonist enhancement of thick-filament reactivity in ovine fetal cerebral arteries mediated by G protein receptor activation of RhoA-dependent and PKC-independent mechanisms. However, further investigation is needed to verify whether such mechanisms take place in the model of stenosis described in the present work.

We also reported a significant increase in the contraction induced by endothelin-1, phenylephrine and KCl in the right (intact) carotid from stenosed animals. The increased response of the right carotid artery is in accordance with previous observations from our laboratory showing that vascular injury caused by balloon catheter increased phenylephrine-induced contraction in the contralateral arteries (Accorsi-Mendonça et al., 2004). These results indicate that there may be compensatory mechanisms in the contralateral artery that are activated after vascular injury.

Compensatory mechanisms of the vascular reactivity are complex processes, which are poorly understood. More recently we investigate the participation of vasoconstrictor endothelial prostanoids derived from the arachidonic acid-cyclooxygenase pathway in the compensatory
mechanism observed in the carotid artery (de Andrade et al. 2008). By using the same model to induce stenosis, we found that indomethacin prevented the increase in tension induced by endothelin-1 in the contralateral artery. However, this response was not prevented by endothelial denudation. Taken together, our results suggest a role for endothelium-derived prostanoids in the increased reactivity of contralateral carotids to endothelin-1.

Total stenosis of carotid artery triggered alterations on basilar artery responsiveness. However, we observed that these alterations varied according to the agonist studied. While stenosis increased endothelin-1-induced contraction, it reduced the contractions induced by phenylephrine and KCl. The agonist used may explain the heterogeneous and apparently contradictory observations. The co-activation of ET\(_A\) receptors located on the smooth muscle and endothelial ET\(_B\) receptors results in opposing vasomotor effects, but the contraction covers relaxation under normal conditions (Tirapelli et al., 2005). Loss of ET\(_B\)-receptor-mediated relaxation (due to down-regulation of these receptors or alterations in the receptor-coupled intracellular transduction signaling system that mediates the vasorelaxant response) in basilar arteries after stenosis could be the cause of the increased contraction induced by endothelin-1. This observation is strengthened by previous findings from our laboratory describing that endothelial denudation, as well as incubation of endothelium-intact tissues with L-NAME, did not alter the contraction induced by endothelin-1 when compared to the endothelium-intact tissues from the stenosis group in the absence of the inhibitor (de Andrade et al., 2008). Since ET\(_B\)-mediated relaxation involves the release of NO (Tirapelli et al., 2005), these results suggest that activation of the NO pathway was attenuated by stenosis.

The most interesting observation from the present study is that vascular occlusion can induce physiological changes in arteries distant from the site of injury. This mechanism might be especially critical in some conditions, such as those with underlying progressive arterial occlusion or stenosis, since these conditions would compromise arterial blood flow in different vascular territories. This observation is in accordance with previous data from the literature (Accorsi-Mendonça et al., 2004; Fukada et al., 2004a; 2004b). Milner et al. (1997) reported that balloon catheter-induced stenosis includes damage to perivascular nerves and induces a transient increase in the density of sensory neuropeptide-containing nerves innervating the contralateral carotid. Thus, after injury, changes in the vasculature innervation may be more widespread than previously thought. Plasticity of the autonomic nervous system is well documented (Burnstock, 1991; Crowe and Iannone, 1993), often involving changes contralateral to the site of injury (Joly et al. 1995). The contralateral carotid artery is often used as a control in studies regarding the effect of stenosis or vascular injury (Kennedy et al., 2006). Based on the present findings we can assume that caution is required when using vessels contralateral to the site of injury as controls since vascular injury can induce changes in the reactivity of arteries distant from the site of injury.

In conclusion, our results show that stenosis in adult guinea pigs induces alterations of the vascular reactivity in arteries distant from the site of injury. Thus, in spite of the common use of contralateral carotid artery as control, we must be aware of the potential alteration induced by stenosis in the vascular motility of these vessels. Additionally, it was verified a relationship between the period of stenosis and the alterations in the vascular reactivity to endothelin-1, KCl and phenylephrine.
Acknowledgements

The authors wish to thank CNPq for founds and grants. We also thank Juliana A. Vercesi, Miriam C. C. de Melo, Ivanilda A. C. Fortunato and Idália Aguiar for their technical assistance.

References


Hudgins, A. and Weiss, G. (1968) Effects of Ca2+ removal upon vascular smooth muscle contraction...
induced by noripenephrine, histamine and potassium. *J. Pharm. Exp. Ther.* **159**: 91–97.


