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RESEARCH PAPER

Balloon catheter injury abolishes phenylephrine-induced relaxation in the rat contralateral carotid

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BACKGROUND AND PURPOSE
The consequences of compensatory responses to balloon catheter injury in rat carotid artery, on phenylephrine-induced relaxation and contraction in the contralateral carotid artery were studied.

EXPERIMENTAL APPROACH
Relaxation and contraction concentration–response curves for phenylephrine were obtained for contralateral carotid arteries in the presence of indomethacin (COX inhibitor), SC560 (COX-1 inhibitor), SC236 (COX-2 inhibitor) or 4-hydroxytetramethyl-L-piperidine-1-oxyl (tempol; superoxide dismutase mimetic). Reactive oxygen species were measured in carotid artery endothelial cells fluorimetrically with dihydroethidium.

KEY RESULTS
Phenylephrine-induced relaxation was abolished in contralateral carotid arteries from operated rats (E_{max} = 0.01 ± 0.004 g) in relation to control (E_{max} = 0.18 ± 0.005 g). Phenylephrine-induced contractions were increased in contralateral arteries (E_{max} = 0.54 ± 0.009 g) in relation to control (E_{max} = 0.38 ± 0.014 g). SC236 restored phenylephrine-induced relaxation (E_{max} = 0.17 ± 0.004 g) and contraction (E_{max} = 0.34 ± 0.018 g) in contralateral arteries. Tempol restored phenylephrine-induced relaxation (E_{max} = 0.19 ± 0.012 g) and contraction (E_{max} = 0.42 ± 0.014 g) in contralateral arteries, while apocynin did not alter either relaxation (E_{max} = 0.01 ± 0.004 g) or contraction (E_{max} = 0.54 ± 0.009 g). Dihydroethidium fluorescence was increased in contralateral samples (18 882 ± 435 U) in relation to control (10 455 ± 303 U). SC236 reduced the fluorescence in contralateral samples (8250 ± 365 U).

CONCLUSIONS AND IMPLICATIONS
Balloon catheter injury abolished phenylephrine-induced relaxation and increased phenylephrine-induced contraction in contralateral carotid arteries, through O_2^- derived from COX-2.

Abbreviations
CGRP, calcitonin gene-related peptide; COX, cyclooxygenases; DHE, dihydroethidium; O_2^-, superoxide anion; ROS, reactive oxygen species; SP, substance P; Tempol, 4-hydroxytetramethyl-L-piperidine-1-oxyl; Tiron, 4,5-dihydroxy-1,3-benzenedisulphonic acid disodium salt

Introduction

Since its first description, balloon angioplasty has been widely used as an effective technique in restoring blood flow during arterial obstructive disease (Dotter and Judkins, 1964), but postoperative complications, mainly restenosis, have limited the beneficial outcomes of this procedure. Restenosis results from neointima formation induced by balloon injury, which leads to intimal thickening and luminal narrowing (Majeski, 1994).

Among the experimental models, balloon catheter injury in the Wistar rat common carotid artery has been the most commonly used model to study neointimal formation. This procedure induces endothelial denudation, medial disruption and adventitial contusion in the ipsilateral artery (Clowes *et al.*, 1983), which leads to formation of neointima and changes ipsilateral artery responsiveness. Moreover, Antonaccio *et al.* (1994) showed a significant reduction in the maximum contraction induced by phenylephrine and other vasoconstrictors in the rat ipsilateral carotid artery when compared with the contralateral carotid artery, that is, that on the opposite side from the injured artery. The contralateral carotid artery had no morphological differences in relation to carotid arteries from normal rats, thus it was used as the control tissue for the ipsilateral carotid artery (Antonaccio *et al.*, 1994).

In 1997, Milner *et al.* observed a reduction in the perivascular nerve density close to the medial wall of the ipsilateral carotid artery, one day after balloon catheter injury in rats. The authors also observed a transitory increase in substance P (SP)- and calcitonin gene-related peptide (CGRP)-containing-nerves in the rat contralateral carotid artery, suggesting a neurocompensatory response to the vascular injury by balloon catheter (Milner *et al.*, 1997). These alterations could impair the use of the contralateral carotid artery as the control tissue for the ipsilateral artery. Surprisingly, Accorsi-Mendonça *et al.* (2004) showed that the maximum contraction induced by phenylephrine was increased in the contralateral carotid artery, but reduced in ipsilateral carotid artery, 4 days after balloon catheter injury, when compared with the carotid arteries from normal control rats. These findings also suggested a compensatory mechanism elicited by balloon injury in the contralateral artery. Moreover, the authors demonstrated that the increased production of metabolites by COX-2 may underpin the hyperreactivity to phenylephrine in the contralateral carotid artery, suggesting a local expression of COX-2. In fact, COX-2 expression can be induced by both SP and CGRP (Yamada *et al.*, 2006), and the density of SP and CGRP is increased in the perivascular nerves from the contralateral carotid artery (Milner *et al.*, 1997).

Vascular tone results from a balance between contraction and relaxation mechanisms, which negatively modulate each other. In the rat carotid artery, contraction elicited by phenylephrine is mediated by muscular α_{1A}-and α_{1D}-adrenoceptors (Villalobos-Molina and Ibarra, 1996; de Oliveira *et al.*, 1998; receptor nomenclature follows Alexander *et al.*, 2009). Moreover, α_1-adrenoceptors activation mediates an endothelium-dependent relaxation in different arteries. The activation of α_1-adrenoceptors induces relaxation in the rabbit bronchial artery (Zschauer *et al.*, 1997) and rat pulmonary arteries (Boer *et al.*, 1999), in a process involving the endothelial production and release of nitric oxide (NO). In the rat mesenteric vascular bed, picomolar to nanomolar concentrations of phenylephrine produce an endothelium-dependent relaxant effect mediated by α_{1D}-adrenoceptors (Filippi *et al.*, 2001). In the rat carotid artery, picomolar concentrations of phenylephrine produce an endothelium-dependent relaxation mediated by α_{1D}-adrenoceptors and involving the endothelial NO synthase (eNOS) (de Andrade *et al.*, 2006). These data suggest that vasorelaxation mediated by α_{1D}-adrenoceptors may represent a local control mechanism that is involved, at least in part, in the modulation of adrenergic contraction in some vessels (Filippi *et al.*, 2001). In fact, de Andrade *et al.* (2006) observed an increase in phenylephrine-induced contraction and a decrease in phenylephrine-induced relaxation in carotid arteries from hyperhomocysteinemic rats, which suggested that the enhanced contraction mediated by α_1-adrenoceptors resulted directly from an impaired relaxation evoked by α_{1D}-adrenoceptors in these arteries.

Based on these observations, we tested the hypothesis that the neurocompensatory response to balloon injury could impair the relaxation induced by phenylephrine in the rat contralateral carotid artery 4 days after surgery, thus leading to an augmented maximum phenylephrine-induced contraction in this vessel. Considering that COX-2 is able to generate superoxide anion (O_2^-) (Kulkarni and Armstead, 2002), which inactivates NO (Beckman *et al.*, 1990) and impairs NO-dependent vasorelaxation (Tawfik *et al.*, 2008) such as that induced by phenylephrine (de Andrade *et al.*, 2006), we further hypothesized that O_2^- derived from COX-2 modulated the adrenergic functional alterations in the rat contralateral carotid artery.

In order to test these hypotheses, we first studied the consequences of the compensatory response to balloon catheter injury in the rat carotid artery, on phenylephrine-induced relaxation and contraction in the rat contralateral carotid artery, comparing them with carotid arteries from normal control rats. We then characterized the potential biological mediators involved in the alterations of these responses. The study of the effects of balloon injury in regions distant from the site of injury may contribute to the understanding of the potential systemic adverse effects of angioplasty and their pathophysiological significance.

Methods

Experimental groups

All animal care and experimental procedures were approved by the Animal Ethics Committee of the School of Medicine from Ribeirão Preto, University of São Paulo, Brazil (protocol 121/2007). Individual adult male Wistar rats (350–400 g) were separated into the control, operated and sham-operated groups. Animals from the control group did not receive any surgical manipulation, while the animals from the operated group received balloon catheter injury. Sham rats underwent to the same surgical manipulation as operated rats, but without the introduction of the balloon catheter into the left carotid artery.

Our main objective was to compare the responses induced by phenylephrine (relaxation and contraction) in the contralateral carotid artery, that is, the right carotid artery which had not been injured by the balloon catheter, from operated rats with the responses observed in the control carotid arteries from normal rats (control group). Thus, all the experimental protocols were carried out with control and contralateral carotid arteries.

Balloon catheter injury

Animals in the operated group were given a standardized unilateral balloon catheter injury (Clowes *et al.*, 1983; Accorsi-Mendonça *et al.*, 2004). In brief, the rats were anaesthetized with the short-lasting anaesthetics ketamine (50 mg·kg^{-1}, i.p.) and xylazine (50 mg·kg^{-1}, i.p.). The left common carotid artery (ipsilateral) was exposed to allow access for a 2F Fogarty balloon catheter, which was distended and passed along the carotid artery three times. The catheter was removed, the external carotid was ligated and the wound was closed. Sham-operated rats were similarly anaesthetized, the left common carotid artery was exposed and the external carotid artery was ligated without catheterization.

Vascular reactivity studies

Vessel ring preparation. Operated rats were used 4 days after surgery, and control animals were from the same age group as the operated rats. Rats were anaesthetized with isoflurane and were killed by aortic exsanguination. The common carotid artery was removed and placed immediately in Krebs solution (composition in mmol·L^{-1}: NaCl 118.4; KCl 4.7; $CaCl_2$ 1.9; KH_2PO_4 1.2; $MgSO_4.7H_2O$ 1.2; $NaHCO_3$ 25; $C_6H_{12}O_6$ 11.6) at pH 7.4. Rings (4 mm) from control, contralateral (right) or ipsilateral (left) carotid arteries were placed in 5.0 mL of Krebs solution in organ bath chambers, gassed with 95% O_2 and 5% CO_2, and maintained at 37°C, pH 7.4, with periodic checking. The arterial rings were connected to an isometric force transducer (Letica Scientific Instruments, Barcelona, Spain) to measure changes in the isometric tension of the arterial rings. After 60 min of stabilization at a resting tension of 1 g, the viability of the carotid rings was tested using potassium chloride (KCl, 90 mmol·L^{-1}) and phenylephrine (0.1 μmol·L^{-1}). The integrity of the endothelium was verified using acetylcholine (ACh, 1 μmol·L^{-1}) following phenylephrine (0.1 μmol·L^{-1}) pre-contraction. When necessary, the endothelium was mechanically removed from the arterial rings by gentle rubbing. Viable control carotid arteries presented a maximum contraction of 0.41 ± 0.026 g in response to KCl (90 mmol·L^{-1}) and 0.20 ± 0.017 g in response to phenylephrine (0.1 μmol·L^{-1}), as previously determined by cumulative concentration–response curves for KCl (10–120 mmol·L^{-1}) or phenylephrine (10^{-10}–10^{-5} mol·L^{-1}). Viable ipsilateral carotid arteries presented a maximum contraction of 0.16 ± 0.012 g in response to KCl (90 mmol·L^{-1}) and 0.11 ± 0.009 g in response to phenylephrine (0.1 μmol·L^{-1}), as previously determined by cumulative concentration–response curves for KCl (10–120 mmol·L^{-1}) or phenylephrine (10^{-10}–10^{-5} mol·L^{-1}). Viable contralateral carotid arteries presented a maximum contraction of 0.43 ± 0.031 g in response to KCl (90 mmol·L^{-1}) and 0.28 ± 0.021 g in response to phenylephrine (0.1 μmol·L^{-1}), as previously determined by cumulative concentration–response curves for KCl (10–120 mmol·L^{-1}) or phenylephrine (10^{-10}–10^{-5} mol·L^{-1}). Endothelium-intact control and contralateral carotid arteries presented 100% of maximum relaxation induced by ACh over the pre-contraction induced by phenylephrine (0.1 μmol·L^{-1}). Endothelium-denuded control, ipsilateral and contralateral carotid arteries presented 0% of maximum relaxation induced by ACh over the pre-contraction induced by phenylephrine (0.1 μmol·L^{-1}).

Experimental protocols

Consequences of balloon catheter injury on phenylephrine-induced relaxation and contraction in carotid arteries. To study the consequences of balloon catheter injury on phenylephrine-induced relaxation in rat carotid arteries, cumulative concentration–response curves for phenylephrine (10^{-15}–10^{-10} mol·L^{-1}) were obtained in rings from control, contralateral and ipsilateral carotid arteries, after pre-contraction with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 30 μmol·L^{-1}). To exclude additional effects induced by phenylephrine on α_{1A}-adrenoceptors (inducing contractions) (de Andrade *et al.*, 2006), which impairs the relaxant response induced by phenylephrine, all the protocols for phenylephrine-induced relaxation were studied in the presence of WB 4101, a α_{1A}-adrenoceptor antagonist (0.1 nmol·L^{-1}) (de Oliveira *et al.*, 1998), which was added to the organ bath 30 min before the addition of $PGF_{2\alpha}$.

To study the consequences of balloon catheter injury on phenylephrine-induced contraction in rat carotid arteries, cumulative concentration–response curves for phenylephrine (10^{-10}–10^{-5} mol·L^{-1}) were obtained for the rings from control, contralateral and ipsilateral carotid arteries.

Participation of the endothelium in the modulation of phenylephrine-induced relaxation and contraction in the contralateral carotid artery. To study the participation of the endothelium in the modulation of phenylephrine-induced relaxation in the rat carotid artery, cumulative concentration–response curves for phenylephrine (10^{-15}–10^{-10} mol·L^{-1}) were obtained using endothelium-denuded rings from control and contralateral carotid arteries after pre-contraction with $PGF_{2\alpha}$.

To study the participation of the endothelium in the modulation of phenylephrine-induced contraction in the rat carotid artery, cumulative concentration–response curves for phenylephrine (10^{-10}–10^{-5} mol·L^{-1}) were obtained using endothelium-denuded rings from control and contralateral carotid arteries.

Participation of COX metabolites in the modulation of phenylephrine-induced relaxation and contraction in the contralateral carotid artery. To study the participation of COX metabolites in the modulation of phenylephrine-induced relaxation in the rat carotid artery, cumulative concentration–response curves for phenylephrine (10^{-15}–10^{-10} mol·L^{-1}) were obtained using endothelium-intact rings from control and contralateral carotid arteries after pre-contraction with $PGF_{2\alpha}$ in the presence of the non-selective COX inhibitor indomethacin (10 μmol·L^{-1}) (Tirapelli *et al.*, 2006), the selective COX 1 inhibitor SC560 (9 nmol·L^{-1}) (Smith *et al.*, 1998) or the selective COX-2 inhibitor 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzenesulfonamide (SC236, 10 nmol·L^{-1}) (Penning *et al.*, 1997),

which were added to the sample 30 min prior to the pre-contraction.

To study the participation of COX metabolites in the modulation of phenylephrine-induced contraction in the rat carotid artery, cumulative concentration–response curves for phenylephrine (10^{-10}–10^{-5} mol·L^{-1}) were obtained using endothelium-intact rings from control and contralateral carotid arteries in the presence of indomethacin (10 μmol·L^{-1}), SC560 (9 nmol·L^{-1}) or SC236 (10 nmol·L^{-1}), which were added to the sample 30 min before phenylephrine.

Participation of superoxide anion (O_2^-) in the modulation of phenylephrine-induced relaxation and contraction in the contralateral carotid artery. To study the participation of O_2^- on the modulation of phenylephrine-induced relaxation in the rat carotid artery, cumulative concentration–response curves for phenylephrine (10^{-15}–10^{-10} mol·L^{-1}) were obtained in endothelium-intact rings from control and contralateral carotid arteries after pre-contraction with $PGF_{2\alpha}$ in the presence of the superoxide dismutase (SOD) mimetic, 4-hydroxytetramethyl-L-piperidine-1-oxyl (tempol; 1 mmol·L^{-1}, 20 min) (Zheng *et al.*, 2003). Because phenylephrine-induced relaxation and contraction are calcium (Ca^{2+})-dependent mechanisms (Filippi *et al.*, 2001; Pereira *et al.*, 2010a, respectively), in order to avoid Ca^{2+} chelating by the non-enzymatic O_2^- scavenger, 4,5-dihydroxy-1,3-benzenedisulphonic acid disodium salt (tiron; Ghosh *et al.*, 2002), tempol was used in the functional studies. The dismutation of O_2^- by tempol generates hydrogen peroxide (H_2O_2) (Chen *et al.*, 2003; 2007). Thus, these protocols were performed in the presence of tempol combined or not with the selective H_2O_2 scavenger, polyethylene glycol (PEG)-catalase (250 U·mL^{-1}, 30 min).

To study the participation of O_2^- in the modulation of phenylephrine-induced contraction in the rat carotid artery, cumulative concentration–response curves for phenylephrine (10^{-10}–10^{-5} mol·L^{-1}) were obtained using endothelium-intact rings from control and contralateral carotid arteries in the presence of tempol (1 mmol·L^{-1}, 20 min) (Zheng *et al.*, 2003), with or without PEG-catalase (250 U·mL^{-1}, 30 min).

Participation of NADPH oxidase metabolites in the modulation of phenylephrine-induced relaxation and contraction in the contralateral carotid artery. To study the participation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase metabolites in the modulation of phenylephrine-induced relaxation in the rat carotid artery, cumulative concentration–response curves for phenylephrine (10^{-15}–10^{-10} mol·L^{-1}) were obtained using endothelium-intact rings from control and contralateral carotid arteries in the presence of the selective NADPH oxidase inhibitor, apocynin (0.1 mmol·L^{-1}, 30 min) (Zheng *et al.*, 2003).

To study the participation of NADPH oxidase metabolites in the modulation of phenylephrine-induced contraction in the rat carotid artery, cumulative concentration–response curves for phenylephrine (10^{-10}–10^{-5} mol·L^{-1}) were obtained using endothelium-intact rings from control and contralateral carotid arteries in the presence of apocynin (0.1 mmol·L^{-1}, 30 min) (Zheng *et al.*, 2003).

Measurement of reactive oxygen species (ROS)

Isolation of endothelial cells. Control and contralateral carotid arteries were isolated and sectioned longitudinally. Endothelial cells were mechanically isolated from the vessels via gentle friction with a plastic stem in plates containing Hanks' solution (composition in mmol·L^{-1}: $CaCl_2$ 1.6; $MgSO_4$ 1.0; NaCl 145.0; KCl 5.0; NaH_2PO_4 0.5; dextrose 10.0; HEPES 10.0) at pH 7.4. The cell suspensions were centrifuged at 137.5× *g* for 5 min, and the pellets were resuspended in 0.5 mL of Hanks' solution in a humidified incubator at 37°C until use (Bonaventura *et al.*, 2008). Each sample comprised the pooled cells from six carotid arteries. Cell viability was determined previously by Trypan blue staining (2%) and counting in a Neubauer chamber (Weber Scientific International, Lauda-Königshofen, Germany).

ROS measurement in endothelial cells by flow cytometry

To study the effects of balloon catheter injury on ROS production in endothelial cells from contralateral carotid arteries, flow cytometry analysis using a non-selective fluorescent dye for ROS, dihydroethidium (DHE) (de Iuliis *et al.*, 2006) was performed in endothelial cells isolated from control and contralateral carotid arteries. Cells are permeable to DHE, which is oxidized to 2-hydroxyethidium (2-OHEt$^+$) by O_2^-, or to ethidium (Et$^+$) by other ROS (Zhao *et al.*, 2005). Both 2-OHEt$^+$ or Et$^+$ are trapped by intercalation into the DNA, emitting a red fluorescence (Zhao *et al.*, 2005). The number of fluorescent nuclei represents the DHE fluorescence intensity, which indicates the relative level of ROS in the cells.

A cytofluorographic analysis was performed using a Becton–Dickinson FACScan (San Jose, CA, USA) with an argon ion laser set at 488 nm with an output of 15 mW. The first flow cytometric analysis of the cell suspension was performed in the absence of DHE to verify the basal fluorescence of blank samples. The cells were then incubated with DHE (2.5 μmol·L^{-1}) for 20 min before the analysis. For each experimental protocol, 10 000 cellular events were acquired by the flow cytometer.

Experimental protocols

Effects of balloon catheter injury on O_2^- bioavailability in endothelial cells from the contralateral carotid artery. To determine the presence and the levels of O_2^- in endothelial cells from contralateral carotid arteries, flow cytometry analysis was performed in the presence of the selective membrane-permeable O_2^- scavenger, tiron (1 mmol·L^{-1}), which was added to the samples 30 min before the analysis (Shi *et al.*, 2007). Tiron acts as a spin trap that stoichiometrically scavenges O_2^-, but does not dismutate O_2^- (Ledenev *et al.*, 1986). Thus, tiron replaced tempol in DHE experiments to avoid H_2O_2 generation after dismutation by the SOD mimetic tempol (Chen *et al.*, 2003; 2007) and consequent DHE oxidation (Zhao *et al.*, 2005).

Participation of COX-2 in ROS production. To study the participation of COX-2 in the production of ROS in endothelial cells from contralateral carotid arteries, flow cytometry analysis was performed in the presence of SC236 (10 nmol·L^{-1}), which was added to the samples 30 min before the analysis.

Statistical analysis

In the functional studies, relaxant responses were recorded as reductions in the muscular tone evoked by the pre-constrictor agent and expressed as grams (g) of tension (absolute relaxation values, not normalized as a percentage). Contraction responses were recorded as increases in the muscular tension from baseline and expressed as g of tension (absolute contraction values). In the graphs, relaxant responses are expressed on a negative scale, whereas contraction is expressed on a positive scale. Phenylephrine concentration–response curves were fitted using a nonlinear interactive fitting programme (GraphPad Prism 3.00; GraphPad Software Inc., San Diego, CA, USA). The potencies and maximum responses to phenylephrine are expressed as pD_2 (negative logarithm of the molar concentration of the agonist that produces 50% of the maximum response) and E_{max} (maximum effect elicited by the agonist), respectively. pD_2 values were obtained from the nonlinear regression of the phenylephrine-induced responses. E_{max} values were obtained from the concentration–response curves for phenylephrine-induced relaxation or from the nonlinear regression for phenylephrine-induced contraction. In the flow cytometry analysis, the median values of the fluorescence intensity (FI) were determined using DIVA software and expressed in fluorescence units (U). Data are expressed as the mean ± SEM., and the differences between the mean values were assessed using the one-way analysis of variance (ANOVA) followed by Newman–Keuls *post hoc* test. The significance level considered in all of the tests was 0.05.

Materials

The following drugs were used: phenylephrine hydrochloride, acetylcholine hydrochloride, $PGF_{2\alpha}$ Tris salt, tempol, PEG-catalase, apocynin, tiron (Sigma, St. Louis, MO, USA); WB 4101 hydrochloride (Tocris, Avonmouth, UK); indomethacin, SC560, SC236 (Calbiochem, Darmstadt, Germany); KCl, $CaCl_2$ and other salts (Synth, São Paulo, Brazil); isoflurane (Forane, Abbott, Sao Paulo, SP, Brazil); ketamine (União Química, Jabaquara, SP, Brazil); xylazine (Calier Laboratories, Juatuba, MG, Brazil); DHE (Invitrogen, Carlsbad, CA, USA). Indomethacin was dissolved in Tris buffer (*p*H 8.4). SC560, SC236 and DHE were prepared as stock solutions in dimethyl sulphoxide (DMSO). The other drugs were dissolved in distilled water. The bath concentration of DMSO did not exceed 0.5% and was shown to have no effects on the basal tonus of the preparations or on the agonist-mediated contraction or relaxation.

Results

Consequences of balloon catheter injury on phenylephrine-induced relaxation in carotid arteries

$PGF_{2\alpha}$ (30 μmol·L^{-1}) evoked a stable contraction response (plateau) of 0.46 ± 0.014 g (n = 5) in control carotid arteries (Figure 1A). Picomolar to nanomolar concentrations of phenylephrine produced a time-dependent relaxant response during the pre-contraction induced by $PGF_{2\alpha}$ in endothelium-intact control carotid arteries, which reduced the muscular

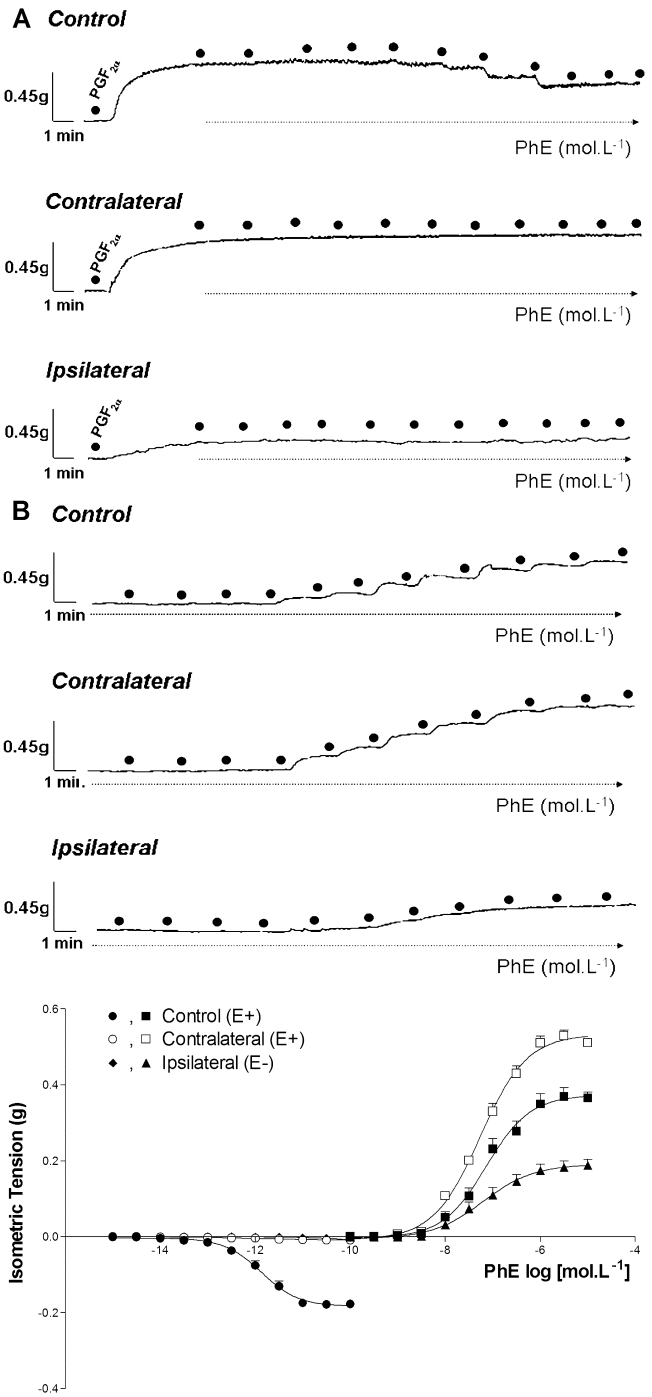

Figure 1

Concentration-dependent responses to phenylephrine in endothelium-intact (E+) control or contralateral carotid arteries, and in endothelium-denuded (E–) ipsilateral carotid arteries. (A) Representative records of phenylephrine-induced relaxation (10^{-15}–10^{-10} mol·L^{-1}); (B) representative records of phenylephrine-induced contraction (10^{-10}–10^{-5} mol·L^{-1}); (C) Concentration–response curves for phenylephrine-induced relaxation and contraction.

Table 1

E_{max} values for phenylephrine-induced relaxation or contraction in rat control, contralateral and ipsilateral carotid arteries

Groups	Phenylephrine E_{max} (g) Relaxation E+	E–	Contraction E+	E–
Control	0.18 ± 0.005	0.01 ± 0.004*	0.38 ± 0.014	0.56 ± 0.023*
Contralateral	0.01 ± 0.004*	0.01 ± 0.005*	0.54 ± 0.009*	0.35 ± 0.029§
Ipsilateral	–	0.01 ± 0.004*	–	0.19 ± 0.016*,#

Data represent the mean ± SEM (n = 5) for endothelium-intact (E+) or endothelium-denuded (E–) carotid rings.
Significant difference (P < 0.001) in relation to control E+ (*), contralateral E+ (#), or contralateral E– (§) in the respective response.

tone (Figure 1A and C, Table 1). The pD_2 value for phenylephrine during the relaxant response induced in control carotid arteries (11.844 ± 0.089) was greater than the agonist potency observed during contraction (7.103 ± 0.117) (Figure 1C).

The contraction induced by $PGF_{2\alpha}$ (30 μmol·L^{-1}) in both endothelium-intact ipsilateral (0.47 ± 0.023 g, n = 5) and contralateral (0.44 ± 0.019 g, n = 5) carotid arteries from sham rats was not different from the data obtained in endothelium-intact carotid arteries from intact control rats (0.46 ± 0.014 g, n = 5). The relaxant response induced by phenylephrine in both endothelium-intact ipsilateral (E_{max} = 0.16 ± 0.010 g, pD_2 = 11.763 ± 0.072, n = 5) and contralateral (E_{max} = 0.18 ± 0.005 g, pD_2 = 11.844 ± 0.089, n = 5) carotid arteries from sham rats was not different from the data obtained in endothelium-intact carotid arteries from intact control rats (E_{max} = 0.18 ± 0.005 g, pD_2 = 11.844 ± 0.089, n = 5). Thus, carotid arteries from intact rats were used as the control group to avoid unnecessary animal suffering.

$PGF_{2\alpha}$ (30 μmol·L^{-1}) evoked a contraction response of 0.43 ± 0.011 g (n = 5) in endothelium-intact contralateral carotid arteries (Figure 1A). The relaxant response induced by phenylephrine was abolished in contralateral carotid arteries when compared with the control group (Figure 1A and C, Table 1).

In endothelium-denuded ipsilateral carotid arteries, $PGF_{2\alpha}$ (30 μmol·L^{-1}) evoked a contraction response of 0.18 ± 0.008 g (n = 5) (Figure 1A). The relaxant response induced by phenylephrine was also abolished in ipsilateral carotid arteries when compared with the control group (Figure 1A and C, Table 1).

Consequences of balloon catheter injury on phenylephrine-induced contraction in carotid arteries

Phenylephrine evoked concentration-dependent contractions in control, contralateral and ipsilateral carotid arteries at nanomolar to micromolar concentrations (Figure 1B and C). Balloon catheter injury significantly increased the maximum contraction induced by phenylephrine in endothelium-intact contralateral carotid arteries when compared with the control (Figure 1B and C, Table 1). In contrast, a significant reduction in phenylephrine-induced maximum contraction was observed in endothelium-denuded ipsilateral carotid arteries when compared with the control (Figure 1B and C, Table 1). The pD_2 values for phenylephrine during the contraction induced in contralateral (7.254 ± 0.047) and ipsilateral (7.229 ± 0.136) carotid arteries did not differ from that in the control group.

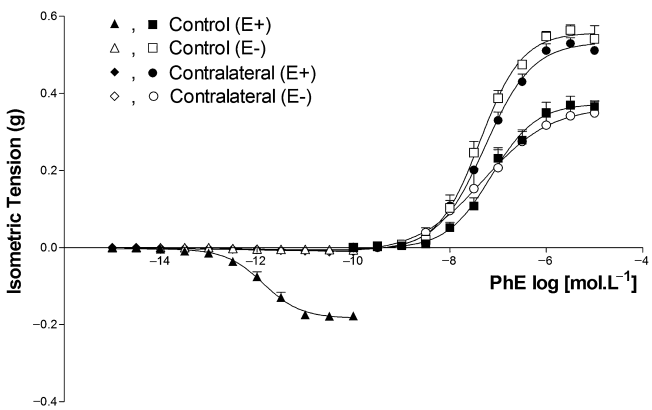

Figure 2

Concentration–response curves for phenylephrine-induced relaxation or contraction in endothelium-intact (E+) or endothelium-denuded (E–) control or contralateral carotid arteries.

Participation of the endothelium in the modulation of phenylephrine-induced relaxation in the contralateral carotid artery

Removal of the endothelium abolished phenylephrine-induced relaxation in control carotid arteries (Figure 2, Table 1). In endothelium-denuded contralateral carotid arteries, phenylephrine-induced relaxation was still absent (Figure 2, Table 1).

Participation of the endothelium in the modulation of phenylephrine-induced contraction in the contralateral carotid artery

Removal of the endothelium significantly increased the maximum phenylephrine-induced contraction in control carotid arteries in relation to endothelium-intact control ones (Figure 2, Table 1). In contralateral carotid arteries, removal of the endothelium reduced the maximum phenylephrine-induced contraction to within the control levels in the pres-

ence of endothelium (Figure 2, Table 1). The pD_2 values for phenylephrine during the contraction induced in the endothelium-denuded control (7.385 ± 0.059) and contralateral (7.407 ± 0.262) carotid arteries did not differ from those observed in the endothelium-intact control arteries.

Participation of COX metabolites in the modulation of phenylephrine-induced relaxation in the contralateral carotid artery

Phenylephrine-induced maximum relaxation in endothelium-intact control carotid arteries was not altered by indomethacin, SC560 or SC236, compared with the absence of the inhibitors (Figure 3A–C, Table 2). The pD_2 values for phenylephrine during the relaxation induced in endothelium-intact control carotid arteries in the presence of indomethacin (11.956 ± 0.276), SC560 (11.762 ± 0.079) or SC236 (11.928 ± 0.038) did not differ from those obtained in the absence of these inhibitors.

Phenylephrine-induced relaxation in endothelium-intact contralateral carotid arteries were restored by indomethacin or SC236, but not by SC560 (Figure 3A–C, Table 2). The pD_2 values for phenylephrine during the relaxation induced in endothelium-intact contralateral carotid arteries in the presence of indomethacin (11.940 ± 0.152) or SC236 (11.920 ± 0.282) did not differ from that observed in the control group in the absence of the inhibitors.

Participation of COX metabolites in the modulation of phenylephrine-induced contraction in the contralateral carotid artery

Similar to the findings obtained for COX inhibitors in phenylephrine-induced relaxation, the maximum phenylephrine-induced contraction was not altered in endothelium-intact control carotid arteries by treatment with indomethacin, SC560 or SC236 (Figure 3A–C, Table 2). The pD_2 values for phenylephrine during the contraction induced in endothelium-intact control carotid arteries in the presence of indomethacin (7.209 ± 0.166), SC560 (7.123 ± 0.061) or SC236 (7.032 ± 0.062) did not differ from that observed in the absence of these inhibitors.

In endothelium-intact contralateral carotid arteries, the maximum phenylephrine-induced contraction was reduced to within control levels by indomethacin or SC236, but not by SC560 (Figure 3A–C, Table 2). The pD_2 values for phenylephrine during the contraction induced in endothelium-intact contralateral carotid arteries in the presence of indomethacin (7.150 ± 0.076), SC560 (7.338 ± 0.031) or SC236 (7.097 ± 0.082) did not differ from that observed in the control group in the absence of the inhibitors.

Participation of O_2^- in the modulation of phenylephrine-induced relaxation in the contralateral carotid artery

In endothelium-intact control carotid arteries, tempol did not alter phenylephrine-induced relaxation (Figure 4, Table 2). Combined administration of tempol and PEG-catalase did not alter phenylephrine-induced relaxation in endothelium-intact control carotid arteries when compared with the values obtained with tempol in the absence of PEG-catalase (Figure 4, Table 2). The pD_2 values for phenylephrine during the relaxation induced in endothelium-intact control carotid arteries in the presence of tempol (11.810 ± 0.054) or tempol combined with PEG-catalase (11.766 ± 0.059) did not differ from that observed in the absence of these scavengers.

In endothelium-intact contralateral carotid arteries, tempol restored phenylephrine-induced relaxation (Figure 4, Table 2). Combined administration of tempol and PEG-

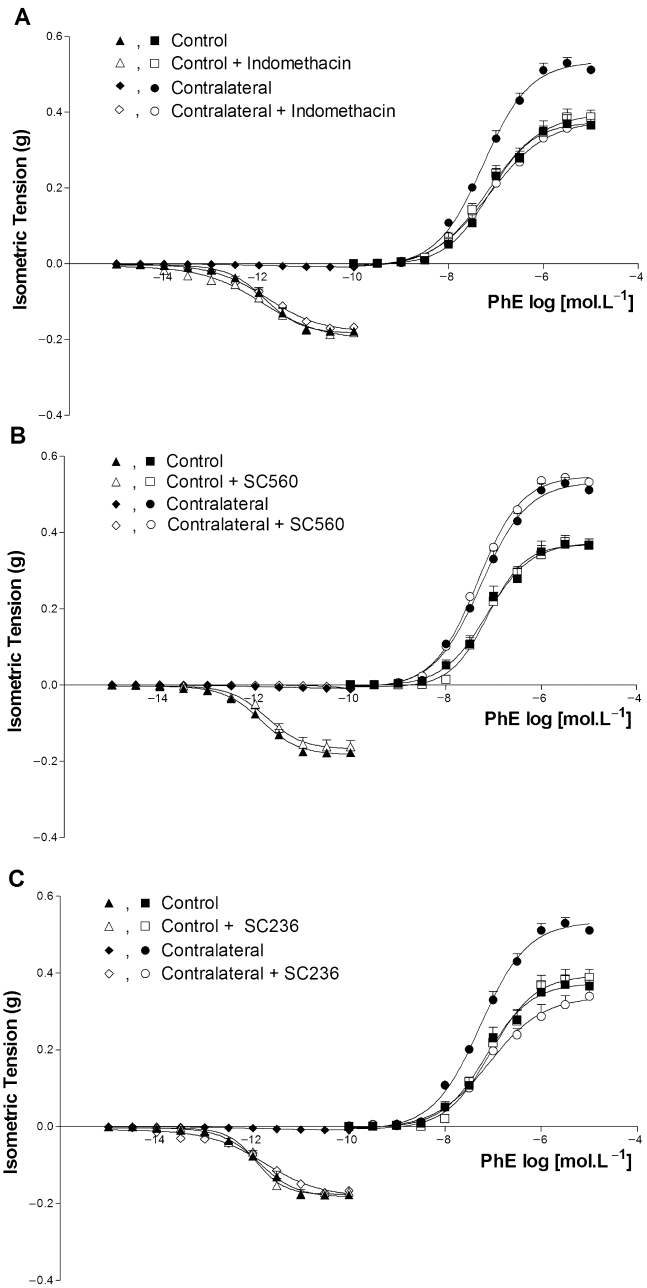

Figure 3

Concentration–response curves for phenylephrine-induced relaxation or contraction in endothelium-intact control or contralateral carotid arteries in the absence (no pretreatment) or presence of COX inhibitors. (A) Pretreatment with indomethacin. (B) Pretreatment with SC560. (C) Pretreatment with SC236.

Table 2

E_{max} values for phenylephrine-induced relaxation or contraction in rat control or contralateral carotid arteries in the absence or presence of COX inhibitors, ROS scavengers or apocynin

Groups	Phenylephrine E_{max} (g) Relaxation Control	Contralateral	Contraction Control	Contralateral
No pretreatment	0.18 ± 0.005	0.01 ± 0.004*	0.38 ± 0.014	0.54 ± 0.009*
Indomethacin	0.19 ± 0.005	0.17 ± 0.008#	0.40 ± 0.022	0.38 ± 0.022#
SC560	0.16 ± 0.016	0.01 ± 0.003*	0.37 ± 0.018	0.55 ± 0.003*
SC236	0.18 ± 0.013	0.17 ± 0.004#	0.40 ± 0.021	0.34 ± 0.018#
Tempol	0.18 ± 0.007	0.19 ± 0.012#	0.39 ± 0.015	0.42 ± 0.014#
Tempol + PEG-catalase	0.17 ± 0.014	0.18 ± 0.012#	0.40 ± 0.031	0.39 ± 0.019#
Apocynin	0.17 ± 0.008	0.01 ± 0.004*	0.40 ± 0.012	0.54 ± 0.009*

Data represent the mean ± SEM (n = 5) for endothelium-intact (E+) or endothelium-denuded (E–) carotid rings.
Significant difference ($P < 0.001$) in relation to control no pre-treatment (*) or contralateral no pre-treatment (#) in the respective response
ROS, reactive oxygen species; Tempol, 4-hydroxytetramethyl-L-piperidine-1-oxyl; tiron, 4,5-dihydroxy-1,3-benzenedisulphonic acid disodium salt.

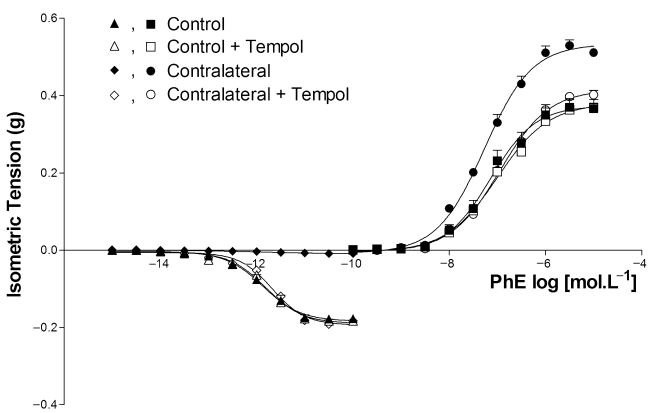

Figure 4

Concentration–response curves for phenylephrine-induced relaxation or contraction in endothelium-intact control or contralateral carotid arteries in the absence (no pretreatment) or presence of tempol.

catalase did not alter phenylephrine-induced relaxation in endothelium-intact contralateral carotid arteries when compared with the values obtained with tempol in the absence of PEG-catalase (Table 2). The pD_2 values for phenylephrine during the relaxation induced in endothelium-intact contralateral carotid arteries in the presence of tempol (11.688 ± 0.051) or tempol combined with PEG-catalase (11.714 ± 0.051) did not differ from that observed in the control group in the absence of the scavengers.

Participation of O_2^- in the modulation of phenylephrine-induced contraction in the contralateral carotid artery

In endothelium-intact control carotid arteries, tempol did not alter the maximum phenylephrine-induced contraction (Figure 4, Table 2). Combined administration of tempol and PEG-catalase did not alter the maximum phenylephrine-induced contraction in endothelium-intact control carotid arteries when compared with the values obtained with tempol in the absence of PEG-catalase (Figure 4, Table 2). The pD_2 values for phenylephrine during the contraction induced in endothelium-intact control carotid arteries in the presence of tempol (6.906 ± 0.192) or tempol combined with PEG-catalase (7.223 ± 0.083) did not differ from that observed in the absence of these scavengers.

In endothelium-intact contralateral carotid arteries, tempol reduced the maximum phenylephrine-induced contraction to within control values (Figure 4, Table 2). Combined administration of tempol and PEG-catalase did not alter the maximum phenylephrine-induced contraction in endothelium-intact contralateral carotid arteries when compared with the values obtained with tempol in the absence of PEG-catalase (Table 2). The pD_2 values for phenylephrine during the contraction induced in endothelium-intact contralateral carotid arteries in the presence of tempol (6.951 ± 0.045) or tempol combined with PEG-catalase (7.164 ± 0.209) did not differ from that observed in the control group in the absence of the scavengers.

Participation of NADPH metabolites in the modulation of phenylephrine-induced relaxation in the contralateral carotid artery

Apocynin did not alter phenylephrine-induced relaxation in endothelium-intact control or contralateral carotid arteries when compared with the absence of the inhibitor (Figure 5, Table 2). The pD_2 values for phenylephrine during relaxation induced in endothelium-intact control carotid arteries in the presence of apocynin (11.734 ± 0.068) did not differ from those observed in the control group in the absence of the inhibitor.

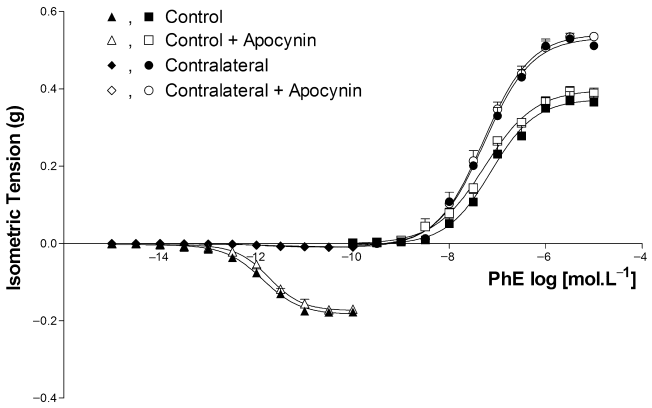

Figure 5

Concentration–response curves for phenylephrine-induced relaxation or contraction in endothelium-intact control or contralateral carotid arteries in the absence (no pretreatment) or presence of apocynin.

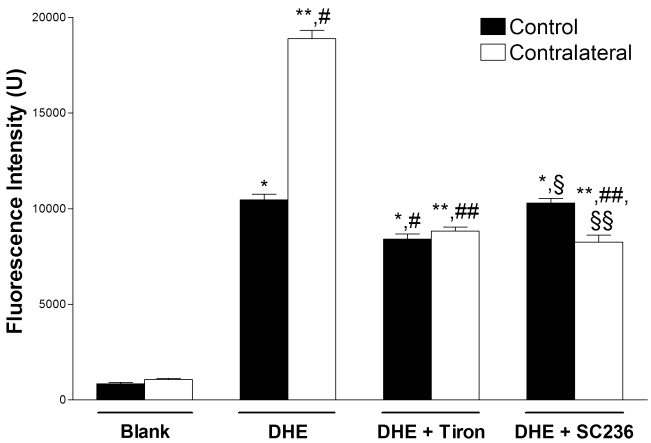

Figure 6

Cytofluorographic analysis of the fluorescence emitted by blank or dihydroethidium (DHE)-leaded samples of endothelial cells from control and contralateral carotid arteries in the absence or presence of tiron or SC236. Significant difference ($P < 0.001$) compared with the blank samples from control endothelial cells (*), blank samples from contralateral endothelial cells (**), and from DHE samples of control endothelial cells in the absence of tiron or SC236 (#), DHE samples from contralateral endothelial cells in the absence of tiron or SC236 (##), DHE samples from control endothelial cells in the presence of tiron (§) or DHE samples from control endothelial cells in the presence of SC236 (§§).

Participation of NADPH oxidase metabolites in the modulation of phenylephrine-induced contraction in the contralateral carotid artery

Apocynin did not alter the maximum phenylephrine-induced contraction in endothelium-intact control or contralateral carotid arteries when compared with the absence of the inhibitor (Figure 5, Table 2). The pD_2 values for phenylephrine during contraction induced in endothelium-intact control (7.289 ± 0.089) or contralateral (7.304 ± 0.083) carotid arteries in the presence of apocynin did not differ from those observed in the absence of the inhibitor.

Consequences of balloon catheter injury on ROS bioavailability in endothelial cells from the contralateral carotid artery

The basal fluorescence emitted by blank samples of endothelial cells from the control and contralateral carotid arteries showed a low intensity (Figure 6, Table 2). The addition of DHE significantly increased the fluorescence intensity of the samples compared with the blank ones (Figure 6). A cytofluorographic analysis revealed an increase in DHE fluorescence in contralateral carotid artery endothelial cells when compared with control samples (Figure 6).

Consequences of balloon catheter injury on O_2^- bioavailability in endothelial cells from the contralateral carotid artery

Tiron significantly reduced the fluorescence intensity of control and contralateral samples loaded with DHE, as compared with the samples in the absence of the scavenger (Figure 6).

Participation of COX 2 in ROS production in endothelial cells from the contralateral carotid artery

In the presence of DHE, SC236 did not alter the fluorescence intensity of the samples from control carotid arteries, but reduced the fluorescence emitted by endothelial cells derived from the contralateral carotid arteries to the same extent as tiron, comparedwith the samples in the absence of the inhibitor (Figure 6).

Discussion and conclusions

In the present study, we have shown that the neurocompensatory response to balloon catheter injury enhances phenylephrine-induced maximum contraction but abolishes phenylephrine-induced relaxation in the rat contralateral carotid artery 4 days after the balloon injury. Furthermore, these responses involve the participation of the same biological mediator in both functional alterations, that is, O_2^- derived from endothelial COX-2.

Our data are in accord with those of Accorsi-Mendonça *et al*. (2004), in that we have shown that the maximum phenylephrine-induced contraction is augmented in contralateral carotid arteries but reduced in ipsilateral arteries. The most significant finding showed that phenylephrine-induced relaxation was completely abolished in both ipsilateral and contralateral arteries.

In the rat carotid artery, phenylephrine induces a relaxant response mediated by α_{1D}-adrenoceptors (de Andrade *et al*., 2006), as well as a contraction response mediated by α_{1A}- and α_{1D}-adrenoceptors at higher concentrations (de Oliveira *et al*., 1998). These findings suggest that phenylephrine-induced relaxation negatively regulates phenylephrine-induced contraction. Considering these findings, the abolition of phenylephrine-induced relaxation in the contralateral carotid

artery could be responsible for the enhanced phenylephrine-induced contraction which is also observed in this vessel. In the ipsilateral carotid artery, the absent phenylephrine-induced relaxation and the reduced phenylephrine-induced contraction may be related to the disruption of the muscular integrity by balloon catheter injury.

Relaxation induced by phenylephrine is endothelium-dependent, as the removal of the endothelium abolished this response in control carotid arteries. This observation is in agreement with the findings of de Andrade *et al.* (2006). In contrast, removal of the endothelium in contralateral arteries did not alter phenylephrine-induced relaxation, which remained abolished. These findings suggest that the production and/or bioavailability of the endothelial compound involved in the phenylephrine-induced relaxation may be impaired. Endothelial removal enhanced the maximum phenylephrine-induced contraction in control arteries, confirming a negative regulatory role played by the endothelium over the adrenergic contraction (Egleme *et al.*, 1984; Carrier and White, 1985). In contrast, the maximum phenylephrine-induced contraction was restored in contralateral carotid arteries, suggesting a positive regulatory role played by the endothelium on adrenergic contraction. These findings strongly suggest an endothelial dysfunction in the contralateral carotid artery, involving an impaired production and/or bioavailability of relaxant factors and an increased production and/or bioavailability of constrictor factors.

Moreover, we found that the same biological mediator, that is, O_2^- derived from endothelial COX-2, was involved in both the augmentation of the maximum phenylephrine-induced contraction and the abolition of phenylephrine-induced relaxation in the contralateral carotid artery, thus confirming our second hypothesis.

The inhibition of COX-1 or COX-2 did not alter phenylephrine-induced relaxation or contraction in control carotid arteries, excluding the participation of COX products in the modulation of these responses, data which are in agreement with those of de Andrade *et al.* (2006) and Accorsi-Mendonça *et al.* (2004). Interestingly, indomethacin and SC236, but not SC560, restored the relaxation and contraction induced by phenylephrine in contralateral carotid arteries to within control levels, suggesting the involvement of a COX-2 metabolite in these functional alterations. These findings strongly suggest a local expression of COX-2 in the contralateral carotid artery, which may be induced by SP and CGRP during the neurocompensatory response to balloon catheter injury.

COX-2 can generate O_2^- (Kulkarni and Armstead, 2002), which inactivates NO during oxidative stress (Beckman *et al.*, 1990). NO is the cellular mediator of phenylephrine-induced relaxation (Filippi *et al.*, 2001; de Andrade *et al.*, 2006) that negatively regulates adrenergic contraction. Based on these observations, we investigated the participation of O_2^- in the adrenergic functional alterations in contralateral carotid arteries.

Tempol did not alter phenylephrine-induced relaxation and contraction in control carotid arteries, which excluded the participation of O_2^- in the modulation of these responses. Interestingly, tempol restored both phenylephrine-induced relaxation and contraction in contralateral carotid arteries, suggesting the involvement of O_2^- in the loss of phenylephrine-induced relaxation and in the enhancement of phenylephrine-induced contraction in these vessels. These findings also suggest an increase in O_2^- bioavailability in contralateral carotid arteries.

The dismutation of O_2^- by SOD or tempol evokes the generation of H_2O_2 (Chen *et al.*, 2003; Shi *et al.*, 2007), which is involved in many complex dilator and contractor signalling pathways. The endothelium-dependent contraction evoked by the Ca^{2+} ionophore A23187 in the femoral arteries from diabetic rats is mediated by hydroxyl radicals derived from H_2O_2 (Shi *et al.*, 2007). In contrast, Edwards *et al.* (2008) showed that H_2O_2 enhances the endothelium-derived hyperpolarization factor-dependent relaxation in rabbit iliac arteries by potentiating Ca^{2+} release from endothelial stores.

Based on these observations, it is likely that endogenous production of H_2O_2 could be increased in parallel with the increased O_2^- bioavailability in the contralateral carotid artery. Moreover, the H_2O_2 generated during O_2^- dismutation by tempol could be involved in the adrenergic alterations and/or in the restored phenylephrine-induced responses after tempol treatment in the contralateral carotid artery. To investigate these hypotheses, phenylephrine-induced responses were studied in the presence of tempol combined with PEG-catalase. The results showed that PEG-catalase combined with tempol did not alter phenylephrine-induced relaxation or contraction in relation to the isolated treatment with tempol. These findings exclude the participation of H_2O_2 derived from the endogenous or tempol-induced dismutation of O_2^- in these responses. Furthermore, the vascular levels of H_2O_2 could be not changed by tempol. In fact, H_2O_2 generation by tempol is a non-steady-state effect, as it lasts only for 1–4 min after tempol treatment (Chen *et al.*, 2007). Moreover, tempol produces a catalase-like effect after a few minutes (Krishna *et al.*, 1996).

Considering that the vascular cells have functionally active NADPH oxidase (Johnson *et al.*, 2002; Touyz *et al.*, 2002), critically involved in O_2^- generation and oxidative stress in the vascular wall, we investigated the participation of NADPH oxidase products in the modulation of phenylephrine-induced relaxation and contraction in contralateral carotid artery, by using apocynin to inhibit NADPH oxidase. The inhibitory effect of apocynin on NADPH oxidase depends on apocynin oxidation in dimers that block the assembly of the functional NADPH oxidase complex (Ximenes *et al.*, 2007). In fact, apocynin is converted to a symmetrical dimer in endothelial cells, that dose-dependently inhibits NADPH oxidase activity and ROS formation (Johnson *et al.*, 2002). Interestingly, apocynin did not alter phenylephrine-induced relaxation and contraction in contralateral carotid arteries, while tempol restored them, excluding NADPH oxidase as the vascular source of O_2^- involved in adrenergic functional alterations in the contralateral carotid artery.

The endothelial enzyme, eNOS, plays an important role in the generation of ROS in vascular tissues (Matoba *et al.*, 2000). However, Pereira *et al.* (2010b) showed that the ROS derived from eNOS did not change the increased phenylephrine-induced maximum contraction in the contralateral carotid artery, but negatively regulated the adrenergic Ca^{2+} influx. Thus, ROS derived from eNOS did not enhance phenylephrine-induced contraction in the contralateral carotid artery as effectively as COX-2-derived O_2^-, which

impaired the phenylephrine-induced relaxation pathways in this vessel. These findings suggest that phenylephrine-induced relaxation, which was sensitive to COX-2-derived O_2^-, was more important to counter-regulate the adrenergic contraction than the impaired Ca^{2+} influx in the contralateral carotid artery, 4 days after balloon catheter injury.

Our functional findings suggested an increase in the bioavailability of O_2^- derived from endothelial COX-2 in the contralateral carotid artery. To investigate this hypothesis, we measured ROS levels in endothelial cells from contralateral carotid arteries by flow cytometry using DHE. Our data revealed an increase in DHE fluorescence from contralateral carotid artery endothelial cells in relation to the control, suggesting an increase in the bioavailability of ROS in the endothelium of contralateral carotid arteries. Tiron significantly reduced DHE fluorescence from control samples, suggesting the presence of a basal level of O_2^- in these cells. Endogenous production of O_2^- has been postulated since the purification of SOD (McCord and Fridovich, 1969) and demonstrated in numerous studies (see Lassegue and Clempus, 2003). Tiron significantly reduced the level of DHE fluorescence from contralateral carotid artery endothelial cells to control levels, suggesting an increase in the endothelial bioavailability of O_2^- from contralateral arteries. These findings confirmed our functional data.

Considering the involvement of COX-2 metabolites in the adrenergic alterations in the contralateral carotid artery, we investigated the participation of COX-2 in the production of ROS in endothelial cells from carotid arteries. In control endothelial cells, SC236 did not alter DHE fluorescence, excluding the participation of COX-2 in ROS production in these cells. In contrast, SC236 reduced DHE fluorescence from contralateral carotid artery endothelial cells to the same extent as tiron, suggesting that COX-2 was responsible for the augmented ROS production, that is, O_2^- production, in these cells.

Our findings suggest a local expression of COX-2 in endothelial cells from contralateral carotid arteries, which generates O_2^-, which in turn is responsible for both the loss of phenylephrine-induced relaxation and the enhanced phenylephrine-induced contraction in these vessels.

In summary, our findings have shown that balloon catheter injury abolishes phenylephrine-induced relaxation, which enhances phenylephrine-induced contraction in the contralateral carotid artery. These effects involve the participation of O_2^- derived from endothelial COX-2 in both adrenergic functions. Moreover, the abolished adrenergic relaxation and augmented adrenergic contraction impair the tone of the contralateral carotid artery. The demonstration that oxidative stress is involved in the pathophysiological response of the vasculature to balloon angioplasty suggests that the effects of antioxidants or COX-2-inhibitors should be evaluated in an appropriate clinical investigation.

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Conflict of interest

None to declare.

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