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Impaired calcium influx despite hyper-reactivity in contralateral carotid following balloon injury: eNOS involvement

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ABSTRACT

Balloon catheter injury results in hyper-reactivity to phenylephrine in contralateral carotids. Decreased nitric oxide (NO) modulation and/or increased intracellular calcium concentration triggers vascular smooth muscle contraction. Therefore, this study explores the participation of NO signaling pathway and calcium mobilization on hyper-reactivity to phenylephrine in contralateral carotids. Concentration–response curves for calcium ($CaCl_2$) and phenylephrine were obtained in control and contralateral carotids four days after balloon injury, in the presence and absence of the inhibitors (L-NAME, L-NNA, 1400W, 7-NI, Oxyhemoglobin, ODQ or Tiron). Confocal microscopy using Fluo-3AM or DHE was performed to detect the intracellular levels of calcium and reactive oxygen species, respectively. The modulation of NO on phenylephrine-induced contraction was absent in the contralateral carotid. Phenylephrine-induced intracellular calcium mobilization was not altered in contralateral carotids. However, extracellular calcium mobilization by phenylephrine was reduced in the contralateral carotid compared to control arteries, and this result was confirmed by confocal microscopy. L-NAME increased phenylephrine-induced extracellular calcium mobilization in the contralateral carotid to the control levels. Results obtained with L-NNA, 1400W, 7-NI, OxyHb, ODQ or Tiron showed that this response was mediated by products from endothelial NOS (eNOS) different from NO and without soluble guanylate cyclase activation, but it involved superoxide anions. Furthermore, Tiron or L-NNA reduced the levels of reactive oxygen species in contralateral carotids. Data suggest that balloon catheter injury promoted eNOS uncoupling in contralateral carotids, which generates superoxide rather than NO, and reduces phenylephrine-induced extracellular calcium mobilization, despite the hyper-reactivity to phenylephrine in contralateral carotids.

1. Introduction

The most common intervention for improving blood flow in the treatment of blood vessel stenoses is the balloon catheter angioplasty (Schwartz and De Blois, 1995; Schwartz and Henry, 2002; Grech 2003). However, despite its widespread application, this technique may cause extensive endothelial injury (Schwartz and De Blois, 1995).

Balloon catheter injury induces specific changes in the contralateral carotid artery (Milner et al., 1997; Bruijns et al., 1998; Accorsi-Mendonça et al., 2004). Our lab previously observed an endothelium dependent hyper-reactivity to phenylephrine in the contralateral rat carotid following balloon injury, occurring between 4 and 7 days, thereafter returning to control levels (Accorsi-Mendonça et al., 2004). This process could be due to an increase in the release of an endothelium-derived constricting (Wilson 2004), but further studies are necessary to understand this process in the contralateral carotid.

Alpha$_1$-adrenoceptors play critical roles in many physiological processes, including vascular smooth muscle contraction and relaxation (de Oliveira et al., 1998; Hague et al., 2002; Jahnichen et al., 2004; Boer et al., 1999). In rat carotid arteries, both contract and relaxant responses are mediated by the alpha$_{1D}$ subtype (de Oliveira et al., 1998; Filippi et al., 2001; de Andrade et al., 2006), present in two different populations on smooth muscle and endothelium. These receptors initiate their physiological effect by activation of G protein-coupled pathways, mainly phospholipase C (Zhong and Minneman 1999). Phospholipase C catalyzes the formation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (Minneman 1988; Zhong and Minneman 1999). IP_3 mobilizes calcium from intracellular store (Minneman 1988; Berridge 1993), and DAG stimulates protein kinase C (PKC), which activates extracellular calcium influx (Berridge 1993). Alpha$_{1D}$ adrenoceptor-induced relaxation involves nitric oxide (NO) synthase activation and NO production (Filippi et al., 2001; de Andrade et al., 2006) in endothelium. The physiological role of this endothelial vasorelaxant adrenoceptors may represent a local control mechanism, which is, at least in part, involved in the modulation of vasoconstrictor responses to sympathomimetic amines (Filippi et al., 2001).

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Nitric oxide is well recognized as an important modulator of vascular tone (Marin and Rodriguez-Martinez 1997). Production of NO in vascular beds occurs mainly by endothelial NO synthase (eNOS) activation (Moncada et al., 1991) in presence of L-arginine, O_2 and tetrahydrobiopterin (Forstermann et al., 1994). In vascular smooth muscle cells, nitric oxide activates soluble guanylate cyclase (sGC) to produce cyclic GMP (cGMP), which activates cyclic GMP-dependent protein kinase (PKG) (Moncada et al., 1991). NO can enhance the activity of large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) either directly (Bolotina et al., 1994) or indirectly via PKG (Moncada et al., 1991; Robertson et al., 1993). Together, these actions reduce $[Ca]_i$ through calcium desensitization mechanisms (Moncada et al., 1991, Hanafy et al., 2001).

Intracellular calcium homeostasis is regulated by nitric oxide signaling pathway (Hanafy et al., 2001). A rise in $[Ca]_i$ is a classic trigger for force development and smooth muscle contraction (Berridge 1993). In the present study we tested the hypothesis that phenylephrine-induced hyper-reactivity in the contralateral carotid results from increased calcium mobilization and reduced participation of NO regulatory mechanisms.

2. Material and methods

2.1. Surgery

All procedures employed in this study conformed to International Guidelines and Ethical Animal Committee of the Ribeirão Preto Campus, University of São Paulo, Brazil (process 06.1.1063.53.4). Adult male Wistar rats (400–450 g) underwent unilateral balloon catheter injury. The surgery was carried out as previously described (Clowes et al., 1983). In brief, general anesthesia was installed using the short-lasting anesthetics Ketamine/Xylazine (100 mg/kg, intraperitoneal). The left common carotid artery (ipsilateral) was exposed for access with a 2F Fogarty balloon catheter, which was distended and passed tree times along the carotid artery. The catheter was removed, the external carotid was ligated and the wound was closed. Intact animals were used as control in all experimental protocols.

2.2. Vascular studies

Four days after surgery, rats were anaesthetized with Isoflurane and killed by aortic exsanguination. Control and contralateral carotids were removed and immediately placed in Krebs solution (NaCl 118.4; KCl 4.7; $CaCl_2$ 1.9; KH_2PO_4 1.2; $MgSO_4 \cdot 7H_2O$ 1.2; $NaHCO_3$ 25; $C_6H_{12}O_6$ 11.6, in mmol/l), pH 7.4, at 37 °C, bubbled with 95% O_2 and 5% CO_2. Carotid rings were connected to an isometric force transducer (Letica Scientific Instruments, Barcelona, Spain) to measure tension in the vessels. After 60 min of stabilization (tension of 1 g), viability was tested using KCl (90 mmol/l) and the $alpha_1$-adrenergic agonist, phenylephrine (10^{-7} mol/l). Endothelium integrity was verified using acethylcholine (10^{-6} mol/l) after phenylephrine (10^{-7} mol/l) induced pre-contraction. Cumulative concentration–response curves for phenylephrine (10^{-10}–10^{-5} mol/l) were obtained in preparations with intact endothelium, in the absence and in presence of L-NAME (10^{-4} mol/l, a non-selective nitric oxide synthase—NOS, inhibitor) (Tirapelli et al., 2005).

To study calcium mobilization, normal Krebs solution was changed to a solution without calcium, and phenylephrine (10^{-7} mol/l) was added. The observed contractile response corresponded to intracellular calcium mobilization. Successive phenylephrine (10^{-7} mol/l) stimulations were performed in the presence of EGTA (1 mmol/l) to deplete intracellular Ca^{2+} stores. Preparations were rinsed in Ca^{2+}-free solution (without EGTA) containing phenylephrine (10^{-7} mol/l). Cumulative concentration–response curves for $CaCl_2$ (0.05–2.5 mmol/l) (Tostes et al., 1995, 1996) were obtained in the absence and presence of L-NAME (10^{-4} mol/l), Oxyhemoglobin (an nitric oxide scavenger, 10^{-5} mol/l, OxyHb), ODQ (soluble guanylate cyclase inhibitor, 10^{-6} mol/l), L-NNA (eNOS inhibitor, 10^{-4} mol/l), 7-NI (nNOS inhibitor, 10^{-4} mol/l) or 1400 W (iNOS inhibitor, 10^{-7} mol/l) in order to evaluate the participation of NO signaling pathway on this response (Tirapelli et al., 2005, 2007; de Andrade et al., 2006). Tiron (10^{-3} mol/l), a superoxide scavenger, was also used. The incubation period was 30 min.

2.3. Detection of intracellular calcium levels in carotid rings

Calcium levels were detected as previously described (Lunardi et al., 2006; Oliveira et al., 2009). Carotid rings were placed in a glass coverslip covered with poly-L-lysine (50% Hanks solution—NaCl 145.0; KCl 5.0; $MgSO_4$; $7H_2O$ 1.0; $CaCl_2$ 1.6; NaH_2PO_4 0.5; Glucose 10.0; Hepes 10.0 in mmol/l, pH 7.4, at 37 °C). Then, carotid rings were loaded with Fluo-3 AM (10^{-5} mol/l, Sigma Probes) for 30 min at room temperature. After washing with Hanks solution, coverslips were placed on a chamber (1.0 ml in volume) and put on a confocal microscope (Leica TCS SP2). Ca^{2+}-images of the carotid rings were taken sequentially in Hanks buffer and assessed through a water immersion objective (63×). Fluo-3 AM was excited with the 488 nm line of an argon ion laser, and the emitted fluorescence was measured at 510 nm. Rings were stimulated by phenylephrine (10^{-7} mol/l) in presence of the sarcoendoplasmic reticulum Ca^{2+}-ATPase inhibitor, thapsigargin (10^{-6} mol/l) (Tostes et al., 1996), after 30 min of incubation. Time course software was used to capture images of the rings two minutes after phenylephrine addition, at intervals of 1.635 s (xyt mode), 512×512 pixel at 400 Hz. The computer software Leica Mycrosystem LAS AF Lite, was used to measure the intensities of the intracellular maximum or minimum fluorescence in regions of interest in the endothelium and media slice. From these data, the initial fluorescence value (F0) was used and the final fluorescence value was denoted as F. The difference in fluorescence intensity (ΔFI) was obtained for each protocol: %ΔFI (=(F–F0/F)×100.

2.4. Detection of reactive oxygen species in carotid rings

Dihydroethydine (DHE) was used to detect superoxide production in isolated carotid arteries as described earlier (Miller et al., 1998). Cells are permeable to DHE, which in the presence of superoxide is oxidized to fluorescent ethidium bromide (Bagi et al., 2008). Ethidium bromide is trapped by intercalation into DNA, and the number of fluorescent nuclei indicates the relative level of superoxide production. Carotid arteries were previously incubated with Tiron (10^{-3} mol/l) or L-NNA (10^{-4} mol/l) for 30 min in Krebs solution. Then, it was cryosectioned and put on glass slides covered with poly-L-lysine. DHE (10^{-6} mol/l) was then added to the PSS for 20 min followed by washing in normal PBS. Frozen sections of arteries were then visualized through a water immersion objective (63×) and photographed using a confocal scanning laser microscope (Leica TCS SP2). Images were analyzed using the Image J software. The intensities of ethidium bromide fluorescence were measured in the endothelium and media slice regions.

2.5. Nitrite and nitrate measurement

Nitrite and nitrate levels were measured from total carotid arteries homogenates as previously described (Lizarte et al., 2009). Arteries were prepared in Tris buffer (*p*H 7.4). Following a centrifugation at 4000 g for 10 min, supernatants were assayed for nitrite and nitrate, using a Nitric Oxide Analyser (Sievers, Model 280i). Data were collected for endothelium-intact rat carotids from control and contralateral arteries.

2.6. Drugs

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich. KCl, $CaCl_2$ and others salts were purchased from Synth (São Paulo, Brazil), Ketamine (União Quimica, Brazil), Xylazine (Calier