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The role of reactive oxygen species in the modulation of the contraction induced by angiotensin II in carotid artery from diabetic rat

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ABSTRACT

The modulation played by reactive oxygen species on the angiotensin II-induced contraction in type I-diabetic rat carotid was investigated. Concentration–response curves for angiotensin II were obtained in endothelium-intact or endothelium-denuded carotid from control or streptozotocin-induced diabetic rats, pre-treated with tiron (superoxide scavenger), PEG-catalase (hydrogen peroxide scavenger), dimethylthiourea (hydroxyl scavenger), apocynin [NAD(P)H oxidase inhibitor], SC560 (cyclooxygenase-1 inhibitor), SC236 (cyclooxygenase-2 inhibitor) or Y-27632 (Rho-kinase inhibitor). Reactive oxygen species were measured by flow cytometry in dihydroethidium (DHE)-loaded endothelial cells. Cyclooxygenase and AT_1-receptor expression was assessed by immunohistochemistry. Diabetes increased the angiotensin II-induced contraction but reduced the agonist potency in rat carotid. Endothelium removal, tiron or apocynin restored the angiotensin II-induced contraction in diabetic rat carotid to control levels. PEG-catalase, DMTU or SC560 reduced the angiotensin II-induced contraction in diabetic rat carotid at the same extent. SC236 restored the angiotensin II potency in diabetic rat carotid. Y-27632 reduced the angiotensin II-induced contraction in endothelium-intact or -denuded diabetic rat carotid. Diabetes increased the DHE-fluorescence of carotid endothelial cells. Apocynin reduced the DHE-fluorescence of endothelial cells from diabetic rat carotid to control levels. Diabetes increased the muscular cyclooxygenase-2 expression but reduced the muscular AT_1-receptor expression in rat carotid. In summary, hydroxyl radical, hydrogen peroxide and superoxide anion-derived from endothelial NAD(P)H oxidase mediate the hyperreactivity to angiotensin II in type I-diabetic rat carotid, involving the participation of cyclooxygenase-1 and Rho-kinase. Moreover, increased muscular cyclooxygenase-2 expression in type I-diabetic rat carotid seems to be related to the local reduced AT_1-receptor expression and the reduced angiotensin II potency.

1. Introduction

Type I Diabetes Mellitus is an important risk factor for the development of atherosclerosis in common carotid (Järvisalo et al., 2002; Gül et al., 2010). Recently, Margeirsdottir et al. (2010) showed that the carotid intima-media thickness, which is correlated with carotid atherosclerosis, is significantly increased in 13.1% of type I-diabetic children. Moreover, Dalla Pozza et al. (2011) found a significant progression of subclinical carotid atherosclerosis in children during four years of type I Diabetes.

One of the early events involved in the pathogenesis of the atherosclerosis triggered by Diabetes is the endothelial dysfunction (Dandona, 2009), characterized by an impairment of the endothelium-dependent vasorelaxation mechanisms (De Vriese et al., 2000). In addition to the impairment of vasorelaxant responses, the diabetic endothelial dysfunction also affects the agonist-induced vasoconstrictor responses. For instance, few studies have shown that type I Diabetes enhances the contractile responses induced by angiotensin II, endothelin 1 and norepinephrine through tyrosine-kinases and phosphatidylinositol-3-phosphate kinase (PI_3K)-mediated mechanisms in rat carotid (Yousif et al., 2005; 2006).

Oxidative stress contributes to the diabetic endothelial dysfunction by impairing the endothelium-dependent vasorelaxation mechanisms through the inactivation of nitric oxide (NO) or the uncoupling of endothelial NO synthase (*e*NOS) triggered by superoxide (O_2^-)

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(Szabó, 2009). In addition, some reactive oxygen species, like O_2^-, hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH), also act by intrinsic mechanisms producing vasoconstrictor and/or vasorelaxant effects (Liu and Gutterman, 2002; Yang et al., 2002; Gao et al., 2003; Du et al., 2005; Ding et al., 2007; Shi et al., 2007). Indeed, reactive oxygen species enhance the agonist-induced contractile responses in type I-diabetic rat aorta (Saini et al., 2006) or femoral artery (Shi et al., 2007). In vascular cells, reactive oxygen species generation has been attributed to angiotensin II, which generates O_2^- and H_2O_2 (Dikalov et al., 2008) by activating reduced nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase (Szabó, 2009) through AT_1-receptors (Zhang et al., 2007). Although the effects produced by reactive oxygen species on agonist-induced contractile responses in aorta and femoral arteries from diabetic animals have already been described (Saini et al., 2006; Shi et al., 2007), there are no studies concerning the modulation played by these species over the contraction induced by angiotensin II in carotid artery during type I Diabetes. Thus, the present study was aimed to investigate the role of the main reactive oxygen species in the modulation of the contraction induced by angiotensin II in carotid from type I-diabetic rat. Moreover, since reactive oxygen species are able to activate cyclooxygenases (COX) (Wong and Vanhoutte, 2010) and Rho-kinase (Kajimoto et al., 2007), which are involved in the vascular smooth muscle contraction (Wong and Vanhoutte, 2010; Somlyo and Somlyo, 2003), we also investigated the participation of COX metabolites and Rho kinase in the modulation of angiotensin II-induced contraction in diabetic rat carotid artery.

The study of the redox signaling underlying the abnormalities of the carotid tone during Diabetes Mellitus may contribute to further investigate the therapeutic approaches to attenuate the diabetic complications correlated with these alterations, mainly atherosclerosis.

2. Material and methods

2.1. Experimental groups

The experimental procedures were approved by the Animal Ethics Committee of the School of Medicine from Ribeirão Preto, University of São Paulo, Brazil (protocol 007/2009). The animals were kept under a 12 h light:12 h darkness cycle (light from 06:00 to 18:00 h), at room temperature, fed with regular chow and given free access to water. Basal fasting glucose levels were determined from rat tail blood samples prior to Diabetes induction, using a one-touch glucometer (LifeScan Inc., Milpitas, CA, USA). Diabetes was induced in 8-weeks-old male Wistar rats (350–400 g) by a single intraperitoneal injection of streptozotocin (STZ, 55 mg/kg) dissolved in citrate buffer (0.09 mol/L, *p*H 4.5) (day 0), as previously described by Yousif et al. (2006). Fasting blood glucose levels were measured 48 h after STZ-injection, and diabetic rats presented a glycaemia higher than 300 mg/dl (Yousif et al., 2006). A positive control group was composed by age-matched normoglycaemic rats that underwent to citrate buffer injection. The control group was composed by intact (not treated) age-matched normoglycaemic rats, without citrate buffer injection. Normoglycaemic rats presented fasting glucose levels lesser than 90 mg/dl. Six weeks after STZ- or vehicle-injection (day 42), the fasting glucose was measured, the animals were anesthetized with isoflurane and sacrificed by aortic exsanguination. Intact control animals were sacrificed at the same age as the diabetic ones.

2.2. Functional studies

2.2.1. Carotid ring preparation

All the experiments were performed in isolated carotid arteries from male Wistar rats, as previously described by Accorsi-Mendonça et al. (2004). After animal sacrifice, the common carotid artery was removed and placed immediately in Krebs solution (composition in mmol/l: 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 *p*H 7.4. Carotid rings (4 mm in length) from control or diabetic rats were placed in organ bath chambers containing 5.0 ml of Krebs solution, gassed with 95% O_2 and 5% CO_2 and maintained at 37 °C, *p*H 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 the appropriated molar concentration of phenylephrine that produces 50% of the maximum contraction response (EC_{50}) in each experimental group ($EC_{50\ Phenylephrine} = 0.1$ μmol/l for control group, or $EC_{50\ Phenylephrine} = 10$ nmol/l for diabetic group), as previously determined by cumulative concentration–response curves for phenylephrine. The endothelial integrity was verified using the appropriated molar concentration of acetylcholine that produces 100% of the maximum relaxation response (EC_{100}) in each experimental group ($EC_{100\ Acetylcholine} = 1.0$ μmol/l for control group, or $EC_{100\ Acetylcholine} = 100$ μmol/l for diabetic group), over phenylephrine-induced pre-contraction. When necessary, the endothelium was mechanically removed by gentle rubbing the vessel in a thin wire. For studies with endothelium-intact vessels, the ring was discarded if the maximum relaxation induced by acetylcholine did not reach 100%. For studies with endothelium-denuded vessels, rings were discarded if there was any measurable degree of relaxation.

2.2.2. Experimental protocols

Cumulative concentration–response curves for angiotensin II (10^{-11}–10^{-6} mol/l) were obtained in endothelium-intact or -denuded carotid rings (Accorsi-Mendonça et al., 2004), in the absence or presence of the selective O_2^- scavenger 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate (tiron, 0.1 mmol/l, 30 min) (Pernomian et al., 2011), the selective H_2O_2 scavenger polyethylene glycol (PEG)-catalase (250 U/ml, 30 min) (Pereira et al., 2010), the selective hydroxyl radicals (·OH) scavenger, N,N′-dimethyltiourea (DMTU, 100 μmol/l, 40 min) (Yang et al., 2002), the selective NAD(P)H oxidase inhibitor apocynin (0.1 mmol/l, 30 min) (Zheng et al., 2003), the selective COX-1 inhibitor SC560 (9 nmol/l, 30 min), the selective COX-2 inhibitor 4-[5-(4-chlorophenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl]benzene-sulfonamide (SC236, 10 nmol/l, 30 min) (Pernomian et al., 2011) or the selective Rho-kinase inhibitor Y-27632 (1.0 μmol/l, 15 min) (Jin et al., 2006).

Tiron was used instead of SOD mimetics since it acts as a spin trap that does not dismutate O_2^- (Ledenev et al., 1986), which avoids the generation of H_2O_2 by O_2^- dismutation (Mori and Iwahashi, 2007), and the interference of H_2O_2 in the modulation of the contractile responses. Moreover, tiron does not chelate calcium in the concentration used (0.1 mmol/l) (Ghosh et al., 2002), allowing the study of calcium-dependent phenomena, like angiotensin II-induced contraction.

Apocynin was used as NADPH oxidase inhibitor since the oxidation of apocynin in dimers that block the assembly of the functional NADPH oxidase complex occurs in vascular cells (Johnson et al., 2002). Moreover, apocynin seemly does not act as a reactive oxygen species scavenger in rat carotid (Pernomian et al., 2011), but as a NAD(P)H oxidase inhibitor.

2.2.3. Normalization of the angiotensin II-induced contraction by the arterial dry mass

At the end of the reactivity studies, the carotid rings (4 mm) were dried in a dry incubator for 24 h. The arterial dry mass (mg) was used to normalize the contractile response induced by angiotensin II.

2.3. Reactive oxygen species measurements

2.3.1. Isolation of endothelial cells

Control and diabetic rat 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: $CaCl_2$ 1.6; $MgSO_4$ 1.0; NaCl 145.0; KCl 5.0; NaH_2PO_4 0.5; dextrose 10.0; HEPES 10.0) at *p*H 7.4. The cell suspensions were centrifuged at 1.375 × *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 (Bonventura et al., 2008). Each *n* comprised a pool of six carotid arteries. The cell viability was determined previously by trypan blue staining (2%) and counting in a Neubauer chamber (Weber Scientific International, Germany).

2.3.2. Flow cytometry

Flow cytometry analysis using a non-selective fluorescent dye for reactive oxygen species, dihydroethidium (DHE) (de Iuliis et al., 2006) was performed in endothelial cells isolated from control and diabetic rat carotid arteries. Cells are permeable to DHE, which is oxidized to 2-hydroxyethidium (2-OHEt$^+$) by O_2^-, or to ethidium (Et$^+$) by other reactive oxygen species (Zhao et al., 2005). Both 2-OHEt$^+$ or Et$^+$ are trapped by intercalation into the DNA, emitting red fluorescence (Zhao et al., 2005). The number of fluorescent nuclei represents the DHE fluorescence intensity, which indicates the relative level of reactive oxygen species 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) for 20 min before the analysis. For each experimental protocol, 10,000 cellular events were acquired by the flow cytometer.

2.3.3. Experimental protocols

The basal levels of O_2^- from endothelial cells of rat carotid was measured in the absence or presence of tiron (0.1 mmol/l, 30 min) or apocynin (0.1 mmol/l, 30 min), added to the cellular samples during DHE loading.

In DHE studies, the spin-trap tiron was used instead of SOD mimetics to avoid the oxidation of DHE by H_2O_2 generated after O_2^- dismutation induced by SOD mimetics (Chen et al., 2003; Chen et al., 2007).

2.4. COX or AT_1-receptor expression by immunohistochemistry

Staining for COX-1, COX-2 or AT_1-receptors was performed in ion paraffin included carotid rings, cut into 3 μm sections and mounted on poly-L-lysine-coated slides. The slides were rinsed with phosphate-buffered saline (PBS) and immersed in 3% hydrogen peroxide for 20 min to block endogenous peroxidase. Non-specific protein binding was blocked with normal serum (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc., Burlingame, CA, USA) for 30 min. The sections were then incubated with rabbit monoclonal primary antibody specific for COX-1 (dilution 1:250), rabbit polyclonal primary antibody specific for COX-2 (dilution 1:200) (ABCAM, Cambridge, MA, USA) or rabbit polyclonal primary antibody specific for AT_1-receptors (dilution 1:500) (ABCAM, Cambridge, MA, USA) for 2 h at 25 °C in a humid chamber. Following washes in PBS, biotinylated pan-specific universal secondary antibody (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc., dilution 1:300) was applied for 30 min. Thus, the slides were incubated with the avidin–biotin peroxidase complex (Vectastain Elite ABC Kit, Universal, Vector Laboratories Inc.) for 30 min and developed with Vector NovaRED Kit (Vector Laboratories Inc.) for 5 min. The slides were counterstained by Harris haematoxylin, dehydrated and mounted with Permount (Biomeda, Foster City, CA, USA). As negative controls, all specimens were incubated with an isotope-matched control antibody under identical conditions. The immunolabeling was considered positive when distinct red nuclear or cytoplasmic staining was homogenously present.

2.5. Drugs

The following drugs were used: STZ, citrate buffer solution, phenylephrine hydrochloride, acetylcholine hydrochloride, (ASN1,VAL5)-angiotensin II acetate, tiron, PEG-catalase, DMTU, apocynin, Y-27632 (Sigma, St. Louis, Mo., USA); SC560, SC236 (Calbiochem, Darmstadt, Germany); KCl, $CaCl_2$ and other salts (Synth, São Paulo, SP, Brazil); isoflurane (Forane, ABBOTT, São Paulo, SP, Brazil); DHE (Invitrogen, Carlsbad, CA, USA); rabbit monoclonal anti-COX-1 antibody, rabbit polyclonal anti-COX-2 antibody, rabbit polyclonal anti-AT_1 antibody (ABCAM, Cambridge, MA, USA). SC560, SC236 and DHE were prepared as stock solutions in dimethyl sulfoxide (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-induced contraction.

2.6. Statistical analysis

In the functional studies, contraction responses were recorded as increases in the muscular tension from baseline and expressed as grams of tension normalized per milligrams of dry tissue. The concentration–response curves were fitted using a nonlinear interactive fitting program (GraphPad Prism 3.00; GraphPad Software Inc., San Diego, CA). The potencies and maximum responses to angiotensin II were 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 non-linear regression of the angiotensin II-evoked contraction. E_{max} values were obtained from the concentration–response curves for angiotensin II.

In the flow cytometry analysis, the median values of the fluorescence intensity (FI) were determined using DIVA software and expressed in fluorescence units (U).

In immunohistochemical assays, the percentage (%) of the stained area was analyzed by Image J software.

Data were expressed as the mean ± S.E.M. The differences between the mean values from the functional and fluorescent studies were assessed by the one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test, since multiple comparisons were performed between experimental groups that differ by only one factor (same kind of animal with different treatments/times, or different kinds of animal with same treatment/time). In the immunohistochemical assays, the differences between the mean values were assessed by the Student *t* test, since the comparisons were made between two experimental groups. The significance level considered in all of the tests was 0.05.

3. Results

3.1. Blood glucose levels and body weight from STZ-treated rats

The fasting glucose levels from vehicle- or STZ-treated rats were not different from the control group at the beginning of the studies (day 0). Six weeks after vehicle or STZ injection (day 42), STZ-treated rats presented a fasting blood glucose levels higher than control or vehicle-treated rats. The body weight from vehicle- or STZ-treated rats were not different control ones at day 0. Six weeks after vehicle or STZ injection (day 42), control and vehicle-treated rats gained weight, while STZ-treated rats lost weigh (Table 1). These data validate the diabetogenic efficacy of STZ treatment in rats.

As vehicle injection did not alter the rat fasting blood glucose levels or body weight, only normoglycaemic intact (not treated) rats were used as the control group.

Table 1
Fasting blood glucose levels and body weight from control, vehicle- or STZ-treated rats.

Groups	Biological parameters			
	Glucose level (mg/dl)		Body weight (g)	
	Day 0	Day 42	Day 0	Day 42
Control	73.58 ± 2.89	80.28 ± 3.94	362.12 ± 8.27	729.85 ± 10.09[b]
Vehicle-treated	81.04 ± 4.15	76.72 ± 2.57	376.74 ± 6.93	741.03 ± 9.87[b]
STZ-treated	79.22 ± 3.61	587.31 ± 9.05[a,b]	355.26 ± 7.34	291.47 ± 8.59[a,b]

Data represent the mean ± S.E.M. (n = 11). Significant difference ($P<0.001$) from control group at the same day (a) or the same group at day 0 (b).

3.2. Contraction induced by angiotensin II in STZ-treated rat carotid artery

STZ treatment increased the absolute maximum contraction value for angiotensin II in endothelium-intact carotid rings (0.61 ± 0.057 g) when compared to endothelium-intact carotid rings from control rats (0.28 ± 0.019 g) ($P<0.05$, $n=6$) (Fig. 1). Since the mean dry mass of the carotid rings from STZ-treated rats (0.40 ± 0.007 mg) was reduced when compared to the carotid rings from control rats (0.54 ± 0.013 mg) ($P<0.05$, $n=6$), the contractile response induced by angiotensin II was normalized by the arterial dry mass.

After the normalization, the E_{max} value for angiotensin II in endothelium-intact carotid arteries from STZ-treated rats remained higher than the value obtained in endothelium-intact carotid arteries from control rats (Fig. 2, Table 2). Endothelium removal increased the E_{max} value for angiotensin II in control carotid arteries but reduced it in carotid arteries from STZ-treated rats (Fig. 2, Table 2). These data suggest the generation of endothelial contractile factors in carotid artery from type I-diabetic rat.

The pD_2 value for angiotensin II in endothelium-intact carotid arteries from STZ-treated rats (7.40 ± 0.141) was significantly lesser than the control value (8.97 ± 0.059) ($P<0.05$, $n=6$) (Fig. 2). Endothelium removal did not alter the pD_2 value for angiotensin II in control carotid arteries (8.82 ± 0.427) but significantly increased it in carotid arteries from STZ-treated rats (8.96 ± 0.457) when compared to the presence of endothelium ($P<0.05$, $n=6$) (Fig. 2), suggesting the generation of endothelial factors that impair the receptor occupation by angiotensin II.

3.3. Modulation played by reactive oxygen species on the contraction induced by angiotensin II in STZ-treated rat carotid artery

The O_2^- scavenger tiron or the ·OH scavenger DMTU did not alter the E_{max} values for angiotensin II in endothelium-intact or -denuded

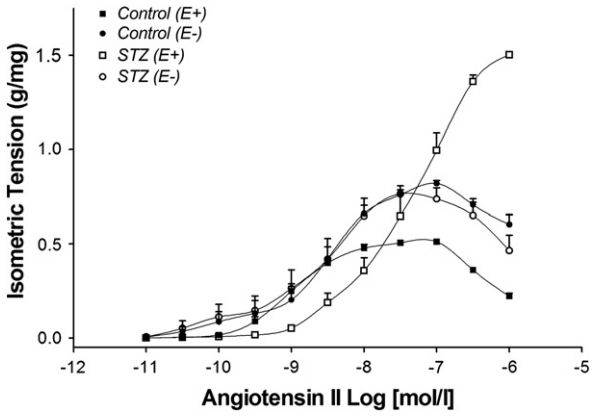

Fig. 2. Concentration–response curves for angiotensin II in endothelium-intact (E+) or endothelium-denuded (E−) carotid arteries from control or diabetic (STZ-treated) rats. Data represent the mean ± S.E.M. ($n=6$).

control carotid arteries, while the H_2O_2 scavenger PEG-catalase reduced them (Fig. 3A and C, Table 2). The inhibitory effect of PEG-catalase was also observed in endothelium-intact control carotid arteries pre-treated with tiron or DMTU (Fig. 4A). Taken together, these data suggest that muscular H_2O_2 acts as a local contractile factor.

Tiron, PEG-catalase or DMTU reduced the E_{max} value for angiotensin II in endothelium-intact carotid arteries from STZ-treated rats (Fig. 3B, Table 2). The inhibitory effect of tiron, PEG-catalase or DMTU was abolished in endothelium-denuded carotid arteries from STZ-treated rats (Fig. 3D, Table 2), suggesting that endothelial O_2^-, H_2O_2 and ·OH plays as contractile factors in these vessels. When combined with tiron, PEG-catalase did not reduce the E_{max} value for angiotensin II in endothelium-intact carotid arteries from STZ-treated rats (Fig. 4B), suggesting that the local generation of H_2O_2 is derived from O_2^-. In the presence of DMTU, the inhibitory effect of PEG-catalase in endothelium-intact carotid rings from STZ-treated rats was abolished (Fig. 4B), suggesting that the local generation of ·OH depends on H_2O_2.

3.4. Modulation played by NAD(P)H oxidase metabolites on the contraction induced by angiotensin II in STZ-treated rat carotid artery

At the same extent as PEG-catalase, apocynin reduced the E_{max} value for angiotensin II in endothelium-intact or -denuded control

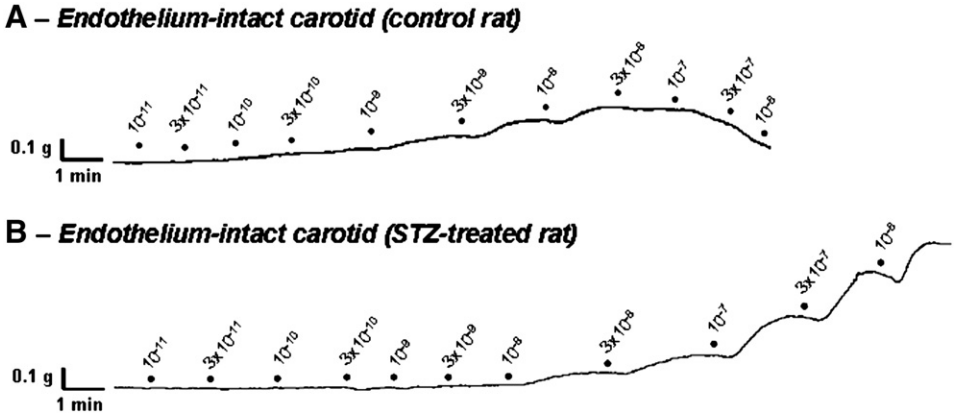

Fig. 1. Representative traces of the contractile response (g) induced by cumulative concentrations (10^{-11}–10^{-6} mol/l) of angiotensin II in endothelium-intact carotid rings from control or diabetic (STZ-treated) rats.

Table 2
E_{max} values for angiotensin II-induced contraction in endothelium-intact (E+) or -denuded (E−) carotid arteries from control or STZ-treated rats.

Groups	Angiotensin II Emax (g/mg)	
	Control	STZ
No pre-treated (E+)	0.52 ± 0.010	1.50 ± 0.014[a]
No pre-treated (E-)	0.82 ± 0.013[a]	0.80 ± 0.033[b]
Tiron (E+)	0.51 ± 0.014	0.74 ± 0.023[b,e]
Tiron (E-)	0.79 ± 0.014[e]	0.82 ± 0.060
PEG-catalase (E+)	0.39 ± 0.018[a]	1.06 ± 0.013[b,f]
PEG-catalase (E-)	0.69 ± 0.018[c,f]	0.79 ± 0.062[l,q]
DMTU (E+)	0.54 ± 0.061	1.05 ± 0.026[b,g]
DMTU (E-)	0.85 ± 0.029[g]	0.84 ± 0.071[r]
Apocynin (E+)	0.43 ± 0.006[a]	0.76 ± 0.011[b,h]
Apocynin (E-)	0.69 ± 0.015[c,h]	0.79 ± 0.031[m]
SC560 (E+)	0.36 ± 0.021[a]	1.04 ± 0.049[b,i]
SC560 (E-)	0.63 ± 0.038[c,i]	0.77 ± 0.026[s,n]
SC236 (E+)	0.55 ± 0.031	1.56 ± 0.068[j]
SC236 (E-)	0.83 ± 0.042[j]	0.78 ± 0.051[t]
Y-27632 (E+)	0.54 ± 0.041	0.91 ± 0.023[b,k]
Y-27632 (E-)	0.81 ± 0.063[k]	0.50 ± 0.026[d,o,u]
Tiron (E+)	0.51 ± 0.014	0.74 ± 0.023[b,e]
Y-27632 + Tiron (E+)	0.52 ± 0.036	0.52 ± 0.043[p]

Data represent the mean ± S.E.M. (n = 6). Significant difference ($P<0.05$) in relation to control (E+) (a), STZ (E+) (b), control (E−) (c), STZ (E−) (d), control (E+) pre-treated with tiron (e), PEG-catalase (f), DMTU (g), apocynin (h), SC560 (i), SC236 (j) or, Y-27632 (k), control (E−) pre-treated with PEG-catalase (l) apocynin (m), SC560 (n) or Y-27632 (o), STZ (E+) pre-treated with tiron (p), PEG-catalase (q), DMTU (r), SC560 (s) or SC236 (t), or STZ (E−) pre-treated with Y-27632 (u).

carotid arteries (Fig. 5A and B, Table 2), suggesting that, in these vessels, the contractile factor derived from muscular NAD(P)H oxidase is H_2O_2.

At the same extent as the endothelium removal or the pre-treatment with tiron in the presence of endothelium, apocynin reduced the E_{max} value for angiotensin II in endothelium-intact carotid arteries from STZ-treated (Fig. 5A, Table 2). Moreover, the inhibitory effect of apocynin was abolished in endothelium-denuded carotid arteries from STZ-treated (Fig. 5B, Table 2). These data suggest that, in these vessels, the contractile factor derived from endothelial NAD(P)H oxidase is O_2^-.

3.5. Basal levels of O_2^- in endothelial cells from STZ-treated rat carotid artery

The cytofluorographic analysis showed an increase in the fluorescence from DHE-loaded endothelial cells of STZ-treated rat carotid arteries when compared to DHE-loaded control samples (Fig. 6). Tiron or apocynin reduced the fluorescence intensity of DHE-loaded samples from STZ-treated rat carotid arteries to the control levels obtained in the presence of these chemicals (Fig. 6), confirming the previous functional data suggesting that O_2^- is generated by endothelial NAD(P)H oxidase in these vessels.

3.6. Expression of AT_1-receptors in STZ-treated rat carotid artery

The endothelial staining for AT_1-receptors in STZ-treated rat carotid artery was increased when compared to control one (Fig. 7A, B and C), which may be related to the endothelial generation of O_2^- derived from NAD(P)H oxidase during type I Diabetes.

The muscular staining for AT_1-receptors was reduced in STZ-treated rat carotid artery when compared to control one (Fig. 7A, B and C), which may be related to the impaired potency for angiotensin II observed in the diabetic rat artery.

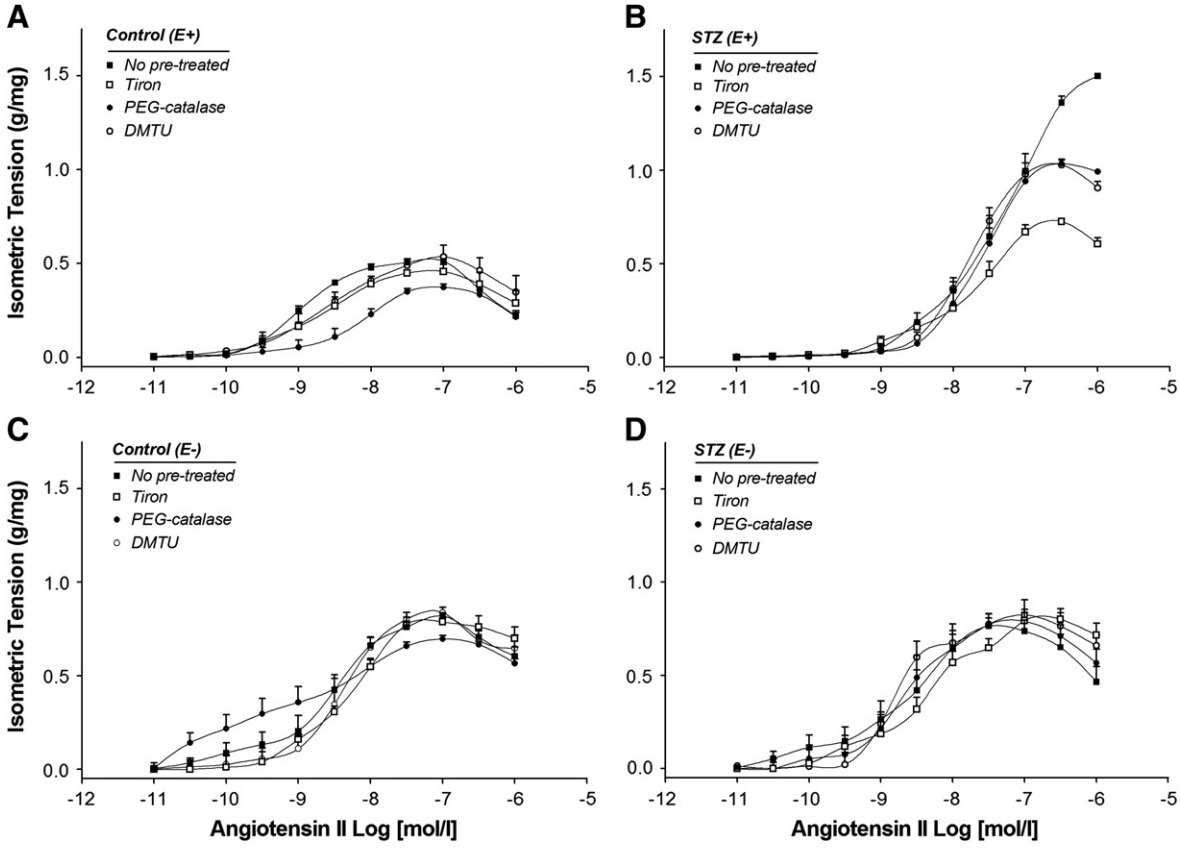

Fig. 3. Concentration–response curves for angiotensin II in endothelium-intact (E+) or endothelium-denuded (E−) carotid arteries from control or diabetic (STZ-treated) rats pre-treated or not with tiron (0.1 mmol/l, 30 min), PEG-catalase (250 U/ml, 30 min) or DMTU (100 μmol/l, 40 min). (A) Effect of reactive oxygen species scavengers in carotid arteries (E+) from control rats. (B) Effect of reactive oxygen species scavengers in carotid arteries (E+) from diabetic rats. (C) Effect of reactive oxygen species scavengers in carotid arteries (E−) from control rats. (D) Effect of reactive oxygen species scavengers in carotid arteries (E−) from diabetic rats. Data represent the mean ± S.E.M. ($n = 6$).

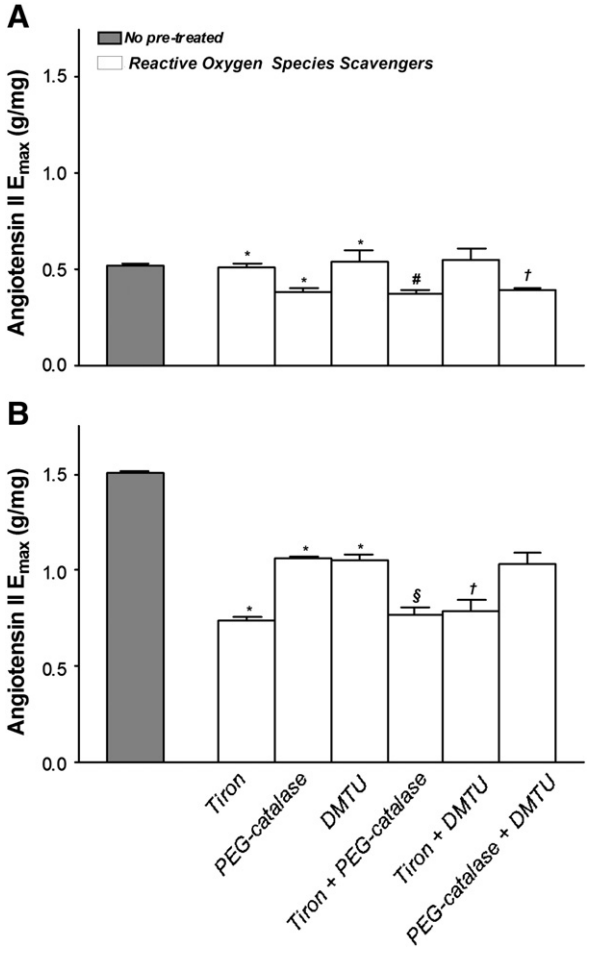

Fig. 4. E_{max} values for angiotensin II in endothelium-intact carotid arteries from control or diabetic (STZ-treated) rats pre-treated or not with tiron (0.1 mmol/l, 30 min) combined or not with PEG-catalase (250 U/ml, 30 min) or DMTU (100 μmol/l, 40 min), or pre-treated or not with PEG-catalase combined with DMTU. (A) Effect of reactive oxygen species scavengers in control carotid arteries. (B) Effect of reactive oxygen species scavengers in diabetic rat carotid arteries. Data represent the mean ± S.E.M. ($n = 6$). Significant difference ($P < 0.05$) compared to the respective group not pre-treated (*), pre-treated with tiron ($^{\#}$), PEG-catalase (§), or DMTU (†). One-way ANOVA (multiple comparisons made between experimental groups that differ by one factor); Bonferroni post-hoc test.

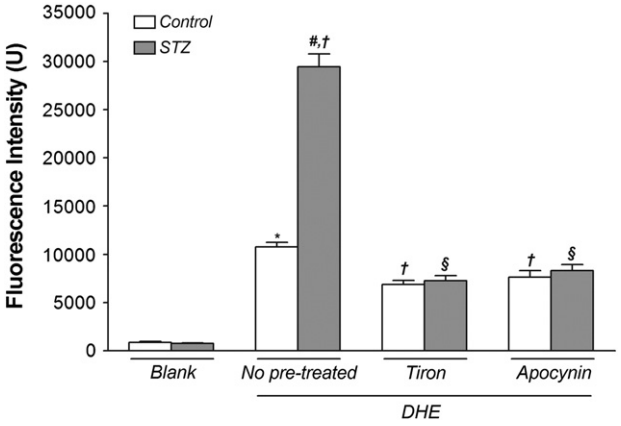

Fig. 6. Cytofluorographic analysis of the basal fluorescence emitted by blank or DHE-loaded samples of endothelial cells from control or diabetic rat carotid arteries in the absence or presence of tiron (0.1 mmol/l, 30 min) or apocynin (0.1 mmol/l, 30 min). Data represent the mean ± S.E.M. ($n = 5$). Significant difference ($P < 0.01$) compared to the blank samples from control (*) or diabetic ($^{\#}$) rat carotid endothelial cells, or DHE-loaded samples from control (†) or diabetic (§) rat carotid endothelial cells in the absence of tiron or apocynin. One-way ANOVA (multiple comparisons made between experimental groups that differ by one factor); Bonferroni post-hoc test.

3.7. Modulation played by COX-1 metabolites on the contraction induced by angiotensin II in STZ-treated rat carotid artery

SC560 reduced the E_{max} value for angiotensin II in endothelium-intact or –denuded control carotid arteries (Fig. 8A, Table 2). The inhibitory effect of SC560 in these vessels was abolished when combined with PEG-catalase (Fig. 8A), suggesting that the local contractile tone induced by H_2O_2 is mediated by muscular COX-1 metabolites.

SC560 reduced the E_{max} value for angiotensin II in endothelium-intact, but not in endothelium-denuded carotid arteries from STZ-treated rats (Fig. 8B, Table 2). In the presence of tiron, PEG-catalase or DMTU, the inhibitory effect of SC560 was abolished in endothelium-intact carotid arteries from STZ-treated rats (Fig. 8B). Taken together, these data suggest that endothelial COX-1 metabolites mediate the local contractile tone induced by ·OH seemly derived from H_2O_2, previously generated from O_2^{-}.

3.8. Modulation played by COX-2 metabolites on the contraction induced by angiotensin II in STZ-treated rat carotid artery

SC236 did not alter the contraction induced by angiotensin II in endothelium-intact or -denuded control carotid arteries (Table 2).

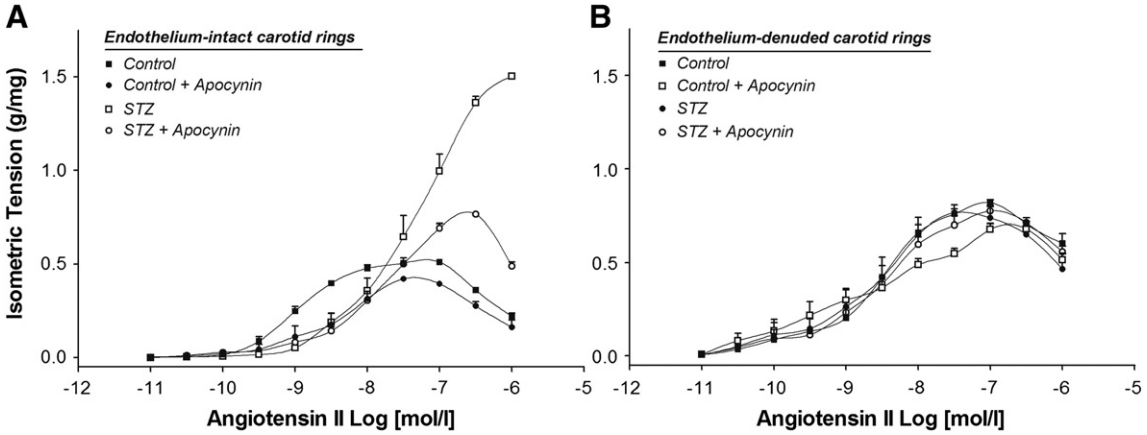

Fig. 5. Concentration–response curves for angiotensin II in endothelium-intact or endothelium-denuded carotid arteries from control or diabetic (STZ-treated) rats pre-treated or not with apocynin (0.1 mmol/l, 30 min). (A) Effect of apocynin in endothelium-intact carotid arteries. (B) Effect of apocynin in endothelium-denuded carotid arteries. Data represent the mean ± S.E.M. ($n = 6$).

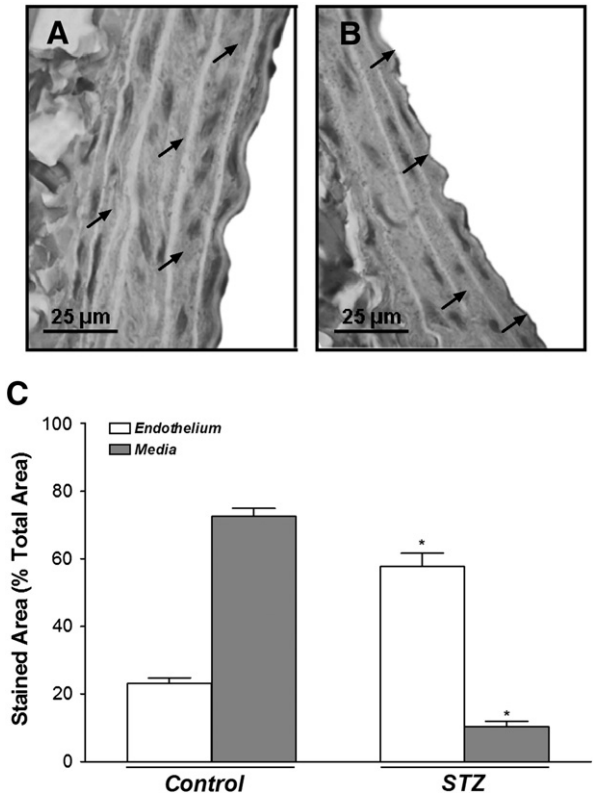

Fig. 7. Expression of AT_1-receptors in control or diabetic (STZ-treated) rat carotid arteries by immunohistochemistry. (A) Representative image from control rat carotid artery stained with anti-AT_1. (B) Representative image from diabetic rat carotid artery stained with anti-AT_1. (C) Endothelial and muscular expression of AT_1-receptors in control or diabetic rat carotid arteries. The staining is denoted by the arrows (magnification: 100×). Significant difference ($P<0.05$) compared to the respective tissue from control artery (*). Student *t* test (comparisons made between two experimental groups).

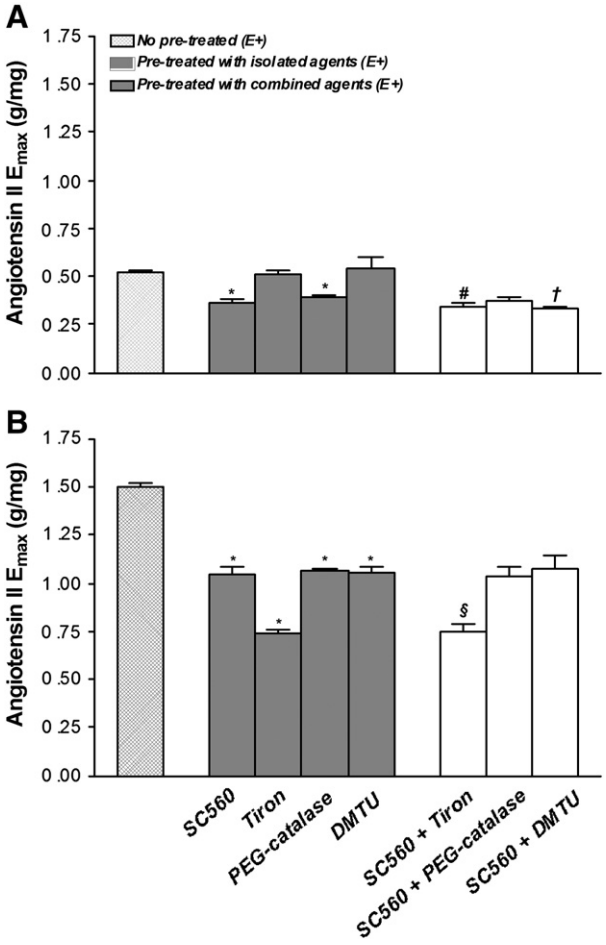

Fig. 8. E_{max} values for angiotensin II in endothelium-intact carotid arteries from control or diabetic (STZ-treated) rats pre-treated or not with SC560 (9 nmol/l, 30 min), combined or not with tiron (0.1 mmol/l, 30 min), PEG-catalase (250 U/ml, 30 min) or DMTU (100 µmol/l, 40 min). (A) Effect of SC560 combined with reactive oxygen species scavengers in control carotid arteries. (B) Effect of SC560 combined with reactive oxygen species scavengers in diabetic rat carotid arteries. Data represent the mean ± S.E.M. ($n=6$). Significant difference ($P<0.05$) compared to the respective group not pre-treated (*), or pre-treated with SC560 (§), tiron ($^{\#}$) or DMTU (†). One-way ANOVA (multiple comparisons made between experimental groups that differ by one factor); Bonferroni post-hoc test.

Although SC236 did not alter the E_{max} value for angiotensin II in endothelium-intact carotid arteries from STZ-treated rats (Table 2), this inhibitor restored the pD_2 value in these vessels (9.04 ± 0.336, $n=6$), suggesting that COX-2 metabolites impair the receptor occupation by angiotensin II in type I-diabetic rat carotid.

3.9. Expression of COX-1 and COX-2 in STZ-treated rat carotid artery

The muscular staining for COX-1 in STZ-treated rat carotid artery was not different from the muscular staining for COX-1 in control carotid artery (Fig. 9A, B and C). The muscular staining for COX-2 was increased in STZ-treated rat carotid artery when compared to control one (Fig. 10A, B and C), which may be related to the COX-2 metabolite-mediated impairment of the angiotensin II potency on the contraction receptors.

3.10. Modulation played by Rho-kinase on the contraction induced by angiotensin II in STZ-treated rat carotid artery

Y-27632 did not alter the E_{max} value for angiotensin II in control carotid arteries when compared to the absence of the inhibitor (Fig. 11A, Table 2). However, Y-27632 reduced the E_{max} values for angiotensin II in endothelium-intact or -denuded carotid arteries from STZ-treated rats (Fig. 11B, Table 2), suggesting that the endothelium-dependent and -independent activation of Rho-kinase contributes to the contraction evoked by angiotensin II in this vessel. Tiron reduced the inhibitory effect of Y-27632 in endothelium-intact carotid arteries from STZ-treated rats (Fig. 11C, Table 2), suggesting that the local endothelium-dependent activation of Rho-kinase depends on O_2^-.

4. Discussion

Our major new findings showed that the contractile hyperreactivity to angiotensin II in rat carotid triggered by type I Diabetes is mediated by ·OH, H_2O_2 and O_2^--derived from endothelial AT_1-NAD(P)H oxidase and involves the participation of COX-1 and Rho-kinase.

The contractile hyperreactivity to angiotensin II in type I-diabetic rat carotid had already been described by Yousif et al. (2005; 2006). In agreement with these findings, we also showed that type I Diabetes increased the maximum contraction induced by angiotensin II in rat carotid, but we demonstrated for the first time that this is an endothelium-dependent mechanism, suggesting the release of endothelial contracting factors during this condition. Shi et al. (2007) found that reactive oxygen species acts as endothelium-derived contracting factors in diabetic rat femoral artery. These data prompted us to investigate the participation of reactive oxygen species in the modulation of the angiotensin II-induced contraction in diabetic rat carotid. Our results suggest that O_2^- acts as a contracting factor in diabetic rat carotid, since tiron reduced the angiotensin II-induced contraction

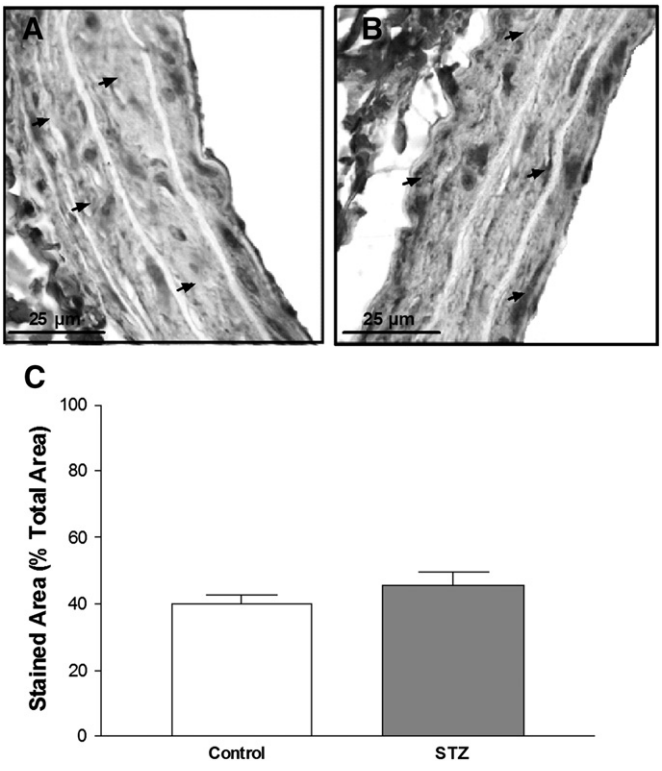

Fig. 9. Expression of COX-1 in control or diabetic (STZ-treated) rat carotid arteries by immunohistochemistry. (A) Representative image from control rat carotid artery stained with anti-COX-1. (B) Representative image from diabetic rat carotid artery stained with anti-COX-1. (C) Muscular expression of COX-1 in control or diabetic rat carotid arteries. The staining is denoted by the arrows (magnification: 100×). Student *t* test (comparisons made between two experimental groups).

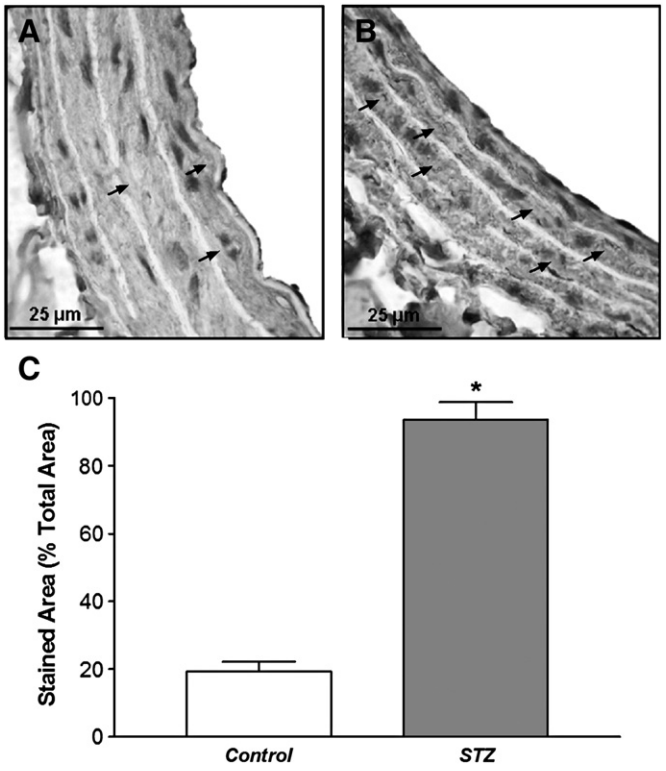

Fig. 10. Expression of COX-2 in control or diabetic (STZ-treated) rat carotid arteries by immunohistochemistry. (A) Representative image from control rat carotid artery stained with anti-COX-2. (B) Representative image from diabetic rat carotid artery stained with anti-COX-2. (C) Muscular expression of COX-2 in control or diabetic rat carotid arteries. The staining is denoted by the arrows (magnification: 100×). Significant difference ($P<0.05$) compared to the control artery (*).Student *t* test (comparisons made between two experimental groups).

in this vessel. The inhibitory effect of tiron on angiotensin II-induced contraction in diabetic rat carotid was similar to the effect produced by endothelium removal, suggesting that O_2^- is derived from the endothelium. Indeed, endothelium removal abolished the inhibitory effect of tiron on angiotensin II-induced contraction in diabetic rat carotid, confirming the endothelial nature of O_2^- in this vessel. Furthermore, Diabetes increased the basal DHE-fluorescence of carotid endothelial cells, which was reduced by tiron to control levels, indicating that the endothelial O_2^- generation is increased during this condition.

Interestingly, despite tiron reduced the angiotensin II-induced contraction in diabetic rat carotid, this value was higher than that one observed in control artery pre-treated with the scavenger, but similar to the value obtained in endothelium-denuded control carotid. These data suggest that the bioavailability of endothelial-derived relaxant factors in diabetic rat carotid is impaired by O_2^--independent pathways, and that the redox signaling underlying the local hyperreactivity to angiotensin II involves intrinsic contractile mechanisms triggered by O_2^-. Indeed, O_2^- induces a contractile tone by closing ATP sensitive potassium channels or calcium activated potassium channels (Liu and Gutterman, 2002), or by activating extracellular signaling-regulated kinase 1/2 (Ding et al., 2007), ryanodine receptors (Du et al., 2005) or Rho-kinase (Jin et al., 2004; 2006).

In addition to O_2^-, we showed that H_2O_2 partially mediates the angiotensin II-induced contraction in carotid arteries from control and type I-diabetic rats, since PEG-catalase reduced this response in these vessels at a lesser extent as tiron. These results corroborate with Gao et al. (2003), who showed that H_2O_2 induces a contractile in rat mesenteric artery. Moreover, we showed that endothelium removal or pre-treatment with tiron abolished the inhibitory effect of PEG-catalase on the angiotensin II-induced contraction in diabetic rat carotid, but not in control one. These data suggest that Diabetes impairs the O_2^--independent muscular generation of H_2O_2 in rat carotid but induces the O_2^--dependent endothelial generation of H_2O_2, which partially mediates the contractile tone induced by O_2^-. Indeed, SOD-induced or spontaneously dismutation of O_2^- generates H_2O_2 (Mori and Iwahashi, 2007).

Our findings also suggest that ·OH partially mediates the contractile hyperreactivity to angiotensin II in type I-diabetic rat carotid, since DMTU reduced the angiotensin II-induced contraction in this vessel at a lesser extent as tiron. These results corroborate with Shi et al. (2007), who showed that ·OH mediates the endothelium-dependent contraction in diabetic rat femoral artery. Indeed, ·OH induces a contractile tone in rat aorta (Yang et al., 2002).

The inhibitory effect of DMTU in diabetic rat carotid had the same extent as that one produced by PEG-catalase. Also, endothelial removal, tiron or PEG-catalase abolished this effect of DMTU. These findings suggest that Diabetes induces the endothelial generation of ·OH in an O_2^-- and H_2O_2-dependent pathway. Indeed, ferrous ions (Fe^{2+}) catalyze the conversion of H_2O_2 in OH by the Fenton reaction (Mori and Iwahashi, 2007). Thus, our data suggest that type I Diabetes induces the generation of ·OH from H_2O_2 derived from endothelial O_2^- in rat carotid.

Both O_2^- and H_2O_2 can be generated by NAD(P)H oxidase: while Nox 4 generates H_2O_2 in vascular smooth muscle cells, Nox 1 generates O_2^-, which is latter converted to H_2O_2 (Dikalov et al., 2008). Moreover, Nox1 generates O_2^- in endothelial cells (Honjo et al., 2008). In agreement with these findings, we showed that apocynin reduced angiotensin II-induced contraction in control carotid at the same extent as PEG-catalase, suggesting that H_2O_2 is the NAD(P)H oxidase metabolite that partially mediates this response in this vessel. Furthermore, apocynin reduced the angiotensin II-induced contraction in diabetic rat carotid at the same extent as tiron or endothelium

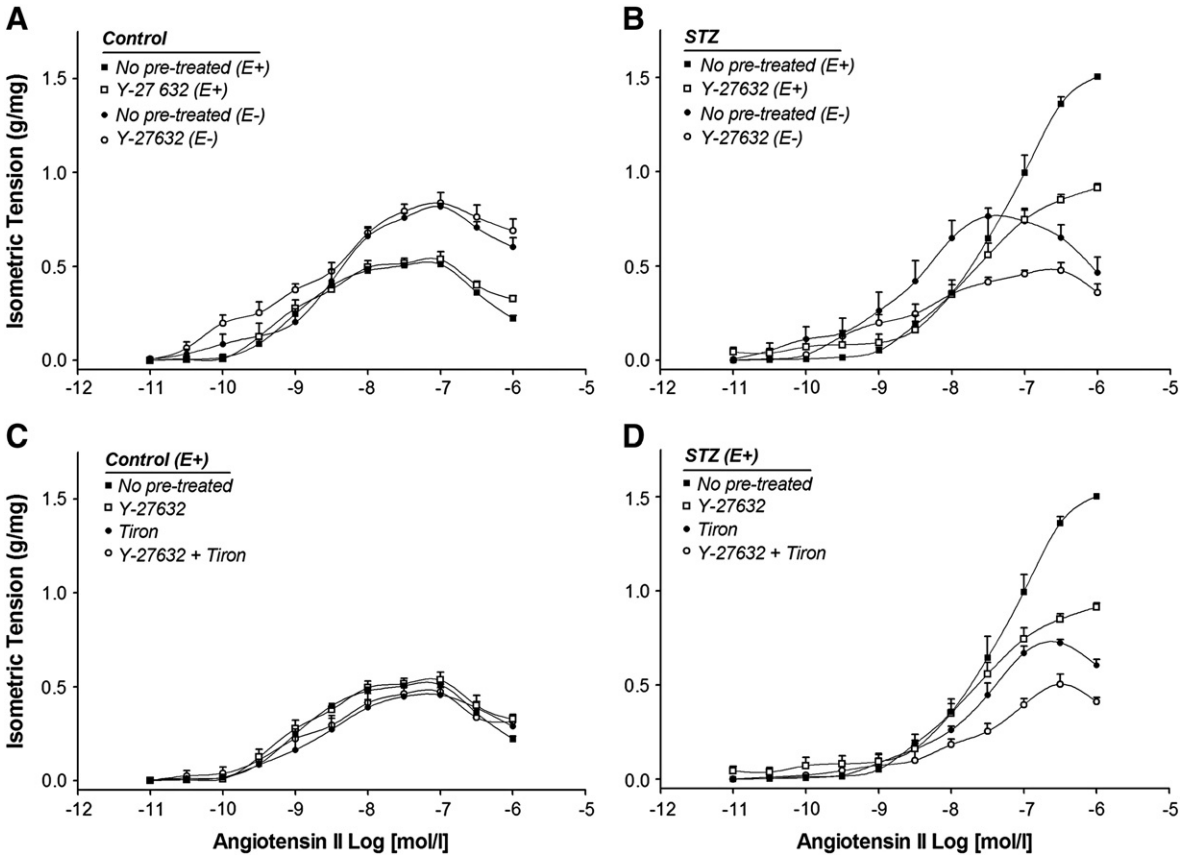

Fig. 11. Concentration–response curves for angiotensin II in carotid arteries from control or diabetic (STZ-treated) rats pre-treated or not with Y-27632 (1.0 μmol/l, 15 min), combined or not with tiron (0.1 mmol/l, 30 min). (A) Effect of Y-27632 in endothelium-intact carotid arteries from control or diabetic rats. (B) Effect of Y-27632 in endothelium-denuded carotid arteries from control or diabetic rats. (C) Effect of Y-27632 combined with tiron in endothelium-intact carotid arteries from control or diabetic rats. (D) Effect of Y-27632 combined with tiron in endothelium-intact carotid arteries from control or diabetic rats. Data represent the mean ± S.E.M. ($n = 6$).

removal, which in turn abolished the inhibitory effect of apocynin on this vessel. These data suggest that endothelial NAD(P)H oxidase generates O_2^- in type I-diabetic rat carotid. Indeed, apocynin reduced the basal DHE-fluorescence of endothelial cells from diabetic rat carotid, confirming the participation of NAD(P)H oxidase in the local generation of O_2^-, which seems to be converted to H_2O_2. In addition to these data, we showed that endothelial AT_1-receptor expression is increased in diabetic rat carotid, which may be related to the reactive oxygen species generation derived from endothelial NAD(P)H oxidase in this vessel, since the activation of AT_1-receptors by angiotensin II induces to the NAD(P)H oxidase-dependent reactive oxygen species generation (Zhang et al., 2007).

The contractile tone induced by ·OH or H_2O_2 in vascular beds seems to be mediated by COX-1 metabolites (Yang et al., 2002; Gao et al., 2003). In fact, our results showed that COX-1 metabolites contribute to the angiotensin II-induced contraction in diabetic rat carotid. Moreover, the inhibitory effect of SC560 was abolished by PEG-catalase, DMTU or endothelium removal in diabetic rat carotid, suggesting that COX-1 metabolites mediate the local contractile tone induced by endothelial ·OH and H_2O_2.

Interestingly, SC236 restored the reduced angiotensin II potency in diabetic rat carotid, suggesting that COX-2 metabolites impair the occupation of AT_1-receptors by angiotensin II. Furthermore, muscular expression of COX-2 is increased but muscular expression of AT_1-receptors is reduced in diabetic rat carotid. These data suggest that COX-2 metabolites impair angiotensin II potency by reducing the muscular expression of AT_1-receptors in diabetic rat carotid. Indeed, Morinelli et al. (2008) showed that COX-2 expression evokes the internalization of AT_1-receptors in vascular smooth muscle cells.

As the inhibitory effect of PEG-catalase or DMTU on the angiotensin II-induced contraction in diabetic rat carotid was lesser than the effect of tiron, which abolished the inhibitory effect of PEG-catalase or DMTU, we suggest that only a part of the contractile tone induced by O_2^- is mediated by H_2O_2 and ·OH in this vessel. This observation prompted us to investigate the participation of Rho-kinase in the modulation of the contraction induced by angiotensin II in diabetic rat carotid, since reactive oxygen species enhance the Rho-kinase activity (Jin et al., 2006), which is involved in the vascular smooth muscle contraction (Somlyo and Somlyo, 2003). Y-27632 attenuated the angiotensin II-induced contraction in both endothelium-intact and -denuded carotid arteries from diabetic rat, but not in control ones. These results suggest that Rho-kinase contributes to the angiotensin II-induced contraction in diabetic rat carotid by endothelium-dependent and independent mechanisms. Our findings corroborate with Bagi et al. (2011), who showed that the sustained contraction induced by angiotensin II in resistance arteries from diabetic rat skeletal muscle involves the activation of Rho-kinase by high glucose.

As our previous results suggest that the reactive oxygen species generated in diabetic rat carotid are derived from the endothelium, we can assume that the endothelial removal withdraws the influence of these species on the Rho-kinase activation in the endothelium-independent inhibitory effect of Y-27632 in this vessel. Moreover, in the presence of tiron, Y-27632 remains producing an inhibitory effect on the angiotensin II-induced contraction in endothelium-intact diabetic rat carotid at the same extent as in endothelium-denuded artery. These findings suggest that the endothelium-independent activation of Rho-kinase in carotid from diabetic rat does not depend on reactive oxygen species. Indeed, Xie et al. (2010) showed that high

glucose activates Rho-kinase by a protein-kinase-C-dependent pathway in the vasculature of diabetic rats.

Interestingly, the inhibitory effect of Y-27632 in endothelium-intact diabetic rat carotid was reduced by tiron, suggesting that the endothelium-dependent activation of Rho-kinase depends on O_2^- in this vessel. Moreover, the contractile tone mediated by the endothelial O_2^--activated Rho-kinase in diabetic rat carotid had the same extent as the H_2O_2/·OH/COX-1-independent contractile tone mediated by O_2^-. These findings suggest that O_2^- partially mediates the contractile hyperreactivity to angiotensin II in carotid from diabetic rat by the activation of Rho-kinase in a H_2O_2/·OH/COX-1-independent pathway.

In summary, our findings suggest that the contractile hyperreactivity to angiotensin II in type I-diabetic rat carotid is mediated by endothelial AT_1-NAD(P)H oxidase derived-O_2^-, that triggers the generation of H_2O_2 and ·OH and the activation of COX-1 and Rho-kinase. In contrast to the increased angiotensin II-induced contraction, the reduced angiotensin II potency in type I-diabetic rat carotid seems to be related to the reduced muscular AT_1-receptor expression in a COX-2-dependent pathway.

5. Conclusions

Although type I Diabetes accounts for only 5–10% of all cases of Diabetes (Daneman, 2006), this disease still represents a healthy problem for children and adolescents, in which the diabetic vascular complications, like carotid atherosclerosis (Margeirsdottir et al., 2010; Dalla Pozza et al., 2011), impair their quality of life. Thus, studies aimed to investigate the mechanisms underlying the vascular dysfunction during type I Diabetes may contribute to the development of strategic therapies to attenuate the diabetic vascular complications in type I-diabetic patients.

To the best of our knowledge, this study is the first one in describing the participation of reactive oxygen species in the contractile hyperreactivity to angiotensin II in carotid artery from type I-diabetic rat. Our results suggest that endothelial AT_1-NAD(P)H oxidase generates O_2^-, that triggers the generation of H_2O_2 and ·OH and the activation of COX-1 and Rho-kinase in carotid artery from diabetic rat. Both the O_2^--dependent H_2O_2/·OH, COX-1 and Rho-kinase contribute to enhance the contraction induced by angiotensin II in carotid artery from diabetic rat. Furthermore, we also show that Diabetes induces the activation of Rho-kinase through an O_2^--independent pathway, which sustains the contraction induced by angiotensin II in rat carotid artery when the endothelial reactive oxygen species are removed from this vessel. Interestingly, our findings also show that COX-2 impairs the angiotensin II potency in rat carotid, which seems to be related to the reduced muscular expression of AT_1-receptors in diabetic rat carotid.

Taken together, these findings clearly show that type I Diabetes triggers an endothelial and muscular dysfunction mediated by pro-oxidant and pro-inflammatory factors in rat carotid, which can be correlated to the pathogenesis of atherosclerosis. According to our findings, the diabetic endothelial dysfunction in rat carotid artery, which is mediated by reactive oxygen species, leads to the muscular hyperresponsiveness to angiotensin II in diabetic rat carotid artery, while the O_2^--independent activation of Rho-kinase compensates the muscular hyporresponsiveness to angiotensin II in the absence of the endothelial reactive oxygen species. This observation is important to guide the choice of an appropriated therapeutic approach to attenuate the diabetic complications in carotid artery, placing the antioxidants as agents of first choice for this purpose.

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