Doxycycline ameliorates 2K-1C hypertension-induced vascular dysfunction in rats by attenuating oxidative stress and improving nitric oxide bioavailability

Michele M. Castro a, Elen Rizzi a, Carla S. Ceron a, Danielle A. Guimaraes a, Gerson J. Rodrigues a, Luisane M. Bendhack b, Raquel F. Gerlach c, Jose Eduardo Tanus-Santos a,*

a Department of Pharmacology, Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, Av. Bandeirantes, 3900, 14049-900, Ribeirao Preto, SP, Brazil
b Department of Pharmacology, Faculty of Pharmaceutical Sciences of Ribeirao Preto, University of Sao Paulo, Av. Café, s/n, 14040-904 Ribeirao Preto, SP, Brazil
c Department of Morphology, Estomatology and Physiology, Dental School of Ribeirao Preto, University of Sao Paulo, Av. Café, s/n, 14040-904 Ribeirao Preto, SP, Brazil

Keywords:
Doxycycline
Matrix metalloproteinases
Oxidative stress
Arterial endothelial dysfunction
2K-1C hypertension
Reactive oxygen species

A R T I C L E  I N F O
Article history:
Received 11 October 2011
Revised 18 January 2012
Available online 3 February 2012

ABSTRACT
Vascular dysfunction associated with two-kidney, one-clip (2K-1C) hypertension may result from both altered matrix metalloproteinase (MMP) activity and higher concentrations of reactive oxygen species (ROS). Doxycycline is considering the most potent MMP inhibitor of tetracyclines and attenuates 2K-1C hypertension-induced high blood pressure and chronic vascular remodeling. Doxycycline might also act as a ROS scavenger and this may contribute to the amelioration of some cardiovascular diseases associated with increased concentrations of ROS. We hypothesized that in addition to its MMP inhibitory effect, doxycycline attenuates oxidative stress and improves nitric oxide (NO) bioavailability in 2K-1C hypertension, thus improving hypertension-induced arterial endothelial dysfunction. Sham operated or 2K-1C hypertensive rats were treated with doxycycline 30 mg/kg/day (or vehicle). After 8 weeks of treatment, aortic rings were isolated to assess endothelium dependent vasorelaxation to A23187. Arterial and systemic levels of ROS were respectively measured using dihydroethidine (DHE) and thiobarbituric acid reactive substances (TBARS). Neutrophils-derived ROS were tested in vitro using the fluoroprobe Carboxy-H2DCFDA and human neutrophils stimulated with phorbol 12-myristate 13-acetate (PMA). NO levels were assessed in rat aortic endothelial cells by confocal microscopy. Aortic MMP activity was determined by in situ zymography. Doxycycline attenuated 2K-1C hypertension (169 ± 17.3 versus 209 ± 10.9 mm Hg in hypertensive controls, p < 0.05) and protected against hypertension-induced reduction in endothelium-dependent vasorelaxation to A23187 (p < 0.05). Doxycycline also decreased hypertension-induced oxidative stress (p < 0.05), higher MMP activity (p < 0.01) and improved NO levels in aortic endothelial cells (p < 0.01). Therefore, doxycycline ameliorates 2K-1C hypertension-induced endothelial dysfunction in aortas by inhibiting oxidative stress generation and improving NO bioavailability, in addition to its inhibitory effects on MMP activity.

© 2012 Elsevier Inc. Open access under the Elsevier OA license.

Introduction

Hypertension is a worldwide health issue that is highly related to vascular dysfunction and chronic vascular remodeling [1–3]. Since the endothelium plays an essential role in the arterial tone regulation, its damage notably contributes to hypertension-induced vascular alterations [4]. Increased levels of reactive oxygen species (ROS) are implicated as a major cause of endothelial dysfunction associated with both clinical and experimental hypertension [5,6]. In this respect, lower nitric oxide (NO) bioavailability and increased matrix metalloproteinases (MMPs) activation have usually been described as important downstream effects of ROS-induced vascular endothelial damage in hypertension [7,8].

MMPs are a family of zinc-dependent endopeptidases widely known as their ability to degrade extracellular matrix proteins. Higher MMP-2 and MMP-9 activities are found in plasma from hypertensive patients [9,10] and in the arteries from hypertensive animals [11–17]. Increased MMPs activities lead to excessive vascular smooth muscle cells migration and proliferation as well as monocyte infiltration into the arterial intima [18,19]. These effects may contribute to hypertension-induced endothelial damage and maladaptive vascular remodeling.

The activity of MMPs can be regulated at multiple levels including gene transcription, post-translational modification and by interaction with their endogenous tissue inhibitors (TIMPs). Peroxynitrite (ONOO−), an important ROS generated by the reaction between superoxide and NO, induces post-translational modifications of MMP activity [20,21]. In vitro, peroxynitrite at very high
concentrations (0.3–10 μM) disrupts the binding between the critical cysteine residue in MMP-2 propeptide domain and the zinc ion in the catalytic site. This results in the active 72 kDa MMP-2 [21]. It was also shown that increased arterial ROS generation may be a downstream effect of increased MMP activity [22]. This interplay between ROS and MMPs may promote a constant cause-effect cycle. Some ROS per se also have oxidizing properties that are directly responsible for hypertension-induced vascular dysfunction [7]. Furthermore, since NO and superoxide react to produce ONOO⁻, the latter notably reduces vascular NO bioavailability and then contributes to hypertension-induced endothelial damage.

MMP inhibitors may be key pharmacological tools to prevent the cardiovascular alterations associated with hypertension. Indeed, doxycycline is considered the most potent MMP inhibitor of tetracyclines and inhibits MMP activity independently of its antimicrobial effect [23,24]. Studies using animal models of hypertension have shown that doxycycline attenuates both the high blood pressure and the chronic vascular remodeling found in hypertension, probably as a result of MMPs inhibition [11,15–17,25]. We have also shown that doxycycline ameliorates 2K-1C-hypertension-induced impaired endothelial-dependent vasorelaxation, arterial wall hypertrophy and excessive collagen/elastin deposition [15,26]. However, doxycycline may also act as a ROS scavenger drug [27–29]. This drug reduced doxorubicin-induced ROS production in mice heart and also myocardial cell apoptosis, thus attenuating ventricular remodeling and systolic dysfunction [27]. Similar effects on ROS levels were also observed in testes of mice treated with doxorubicin [28]. Moreover, oral treatment of spontaneous hypertensive rats with doxycycline reduced the microvascular oxidative stress and lessened the proteolytic degradation of insulin receptor in leukocytes [30]. Doxycycline may also inhibit ROS derived from neutrophils in vitro [31] and hypochloride-induced collagenase activation in osteoblast-like cells [32]. However, there are no studies showing whether treatment with doxycycline decreases 2K-1C hypertension-induced vascular ROS production (in addition to its MMP inhibitory effect) and if this effect contributes to improve the impaired endothelial-dependent vasorelaxation and arterial wall hypertrophy.

In the present study, we addressed the hypothesis that although doxycycline inhibits MMP activity, it may also ameliorate 2K-1C hypertension-induced endothelial dysfunction by attenuating oxidative stress. We used the 2K-1C hypertension model as it is associated with enhanced MMP activity and oxidative stress as well as significant vascular dysfunction.

Methods

Animals and treatments

This study complied with guidelines of Faculty of Medicine of Ribeirão Preto, University of São Paulo. Animals were handled according to guiding principles published by the National Institutes of Health (NIH). Male Wistar rats (180–200 g) obtained from the colony at University of São Paulo were maintained on 12-h light/dark cycle at room temperature (22–25 °C) with free access to standard chow and water.

The 2K-1C hypertension model was induced by clipping the left renal artery with a silver clip (0.2 mm). Sham-operated rats underwent the same surgical procedure (anesthesia with ketamine 100 mg/kg and xylazine 10 mg/kg i.p.) except for the clip placement. Animals were randomly assigned to one of four groups: 2K-1C and Sham that received tap water and 2K-1C and Sham that received doxycycline at 30 mg/kg/day by gavage. It was previously showed that this dose inhibits MMP activity in vivo [11,15,17,26]. Treatment was started two weeks after surgery and maintained for eight weeks. Tail systolic blood pressure (SBP) was assessed weekly by tail-cuff plethysmography. Plasma and aortas were removed from rats previously treated with doxycycline at 30 mg/kg/day to perform all the ex vivo experiments presented here.

Vascular reactivity

After 8 weeks of treatment, thoracic aortas were isolated and cleaned of connective tissue and fat. Aortic rings were cut at 4 mm in length and placed in organ chambers containing modified Krebs salt solution of the following composition (mM): NaCl 130, CaCl₂ 1.6, MgSO₄ 1.2, KH₂PO₄ 1.2, KCl 4.7, NaHCO₃ 14.9, glucose 5.5, which was maintained at 37 °C, pH 7.4, and bubbled with 95% O₂ and 5% CO₂. The system was connected to an isometric force displacement transducer (Leticia Scientific Instruments; Barcelona, Spain) and aortic responses were recorded on a computer system using Chart V4.04, PowerLab AD Instruments (2000) Program. Aortic rings were submitted to a tension of 1.5 g during 60 min equilibration period and were considered to have an intact functional endothelium when acetylcholine (1 μM) produced a relaxation of more than 80%. Relaxation was calculated as a percentage of contraction induced by phenylephrine (100 nM). To assess endothelium-dependent vasorelaxation, aortic rings pre-contracted with phenylephrine (100 nM) were used to construct a cumulative concentration–response curve to the calcium ionophore A23187 (1 nM–1 μM) [33].

Measurement of aortic ROS and plasma lipid peroxide levels

Dihydroethidium (DHE), a sensitive superoxide probe, was used to evaluate in situ production of ROS in aortas. Briefly, aortic tissues were vertically embedded in an OCT compound and were frozen and cut in serial 4 μm sections. Unfixed cryosections were incubated in dark, at room temperature, with DHE at 10 μM for 30 min. Sections were examined with a fluorescence microscope (Leica Imaging Systems Ltd., Cambridge, England) and images were captured at X40. Red fluorescence represents superoxide production. The intensity of fluorescent signal was evaluated by using ImageJ Program (NIH). We measured it from 20 fields selected around the vessel circumference, and the arithmetical mean of the fluorescence from 20 fields was calculated for each slide [8].

Plasma lipid peroxide levels were determined by measuring thiobarbituric acid reactive substances (TBARS) using a fluorimetric method. Lipid peroxide levels were expressed in nmol/ml and in terms of malondialdehyde (MDA). This method requires excitation at 515 nm and emission at 553 nm and uses 1,1,3,3-tetramethoxy-propane as standard [8].

Measurement of neutrophils-derived ROS in vitro

We also tested whether doxycycline inhibits neutrophil-derived ROS in vitro. Briefly, human neutrophils (1 × 10⁶ cells/ml) were isolated and plated into a sterile 96-well opaque microtiter plate (200 μl/well). Cells were loaded with 5 μM of the fluorophore Carboxy-H₂DCFDA (Molecular Probes, Oregon, USA) during 15 min at 37 °C and, then, washed three times with Hanks modified buffer. These cells were equilibrated during 5–10 min, treated or not with different concentrations of doxycycline (0.8–200 μM) and then incubated at 37 °C with 5% CO₂ for 30 min. Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) at 100 nM and plates were immediately placed into a spectrofluorometer at 37 °C (Gemini EM, Molecular Devices, Sunnyvale, CA). Readings were recorded every 1–120 min. Carboxy-H₂DCFDA requires excitation at 485 nm and emission at 529 nm. Results were expressed as arbitrary units of fluorescence.
Assessment of NO levels in aortic endothelial cells using confocal microscopy

The selective NO fluorescent dye 4,5-diaminofluorescein diacetate (DAF-2/DA) was used to detect NO levels in aortic endothelial cells [34]. Briefly, rat thoracic aortas were quickly removed, longitudinally opened and then were maintained in Hanks solution with the following composition (mM): NaCl 145.0, CaCl₂ 1.6, KCl 5.0, MgCl₂ 1.0, NaH₂PO₄ 0.5, dextrose 10.0 and HEPES 10.0. Endothelial cells were isolated from the aorta inner surface by gentle rake friction in Hanks solution. Cell suspension was centrifuged at 1000 rpm for 5 min and then, cell pellet was suspended in 0.5 ml of Hanks buffer. The suspension was placed on 10% poly-L-lysine coated slide for 30 min in a humidified 37 °C incubator with 5% CO₂. Then, cells were loaded with 5 μM DAF-2/DA during 20 min and were examined in Hanks buffer with a confocal scanning laser microscope (Leica TCS SP5). DAF-2/DA fluorescence was excited with 488 nm line of an argon ion laser and the emitted fluorescence was measured at 515 nm. Time-course software was used to capture images of cells at one second intervals (x,y,t) in the Live Data Mode acquisition at 700 Hz and 1024 × 1024 pixel. The intensity of fluorescent signal in the endothelial preparation was evaluated by the LSCM computer software. The initial fluorescence intensity (FI) value was obtained at t = 0 and it was designated F₀. Final FI value obtained after stimulation with A23187 (1 μM) was designated F. The percentage of difference in FI (%ΔFI), which reflects increase of NO concentration in endothelial cells, was obtained in relation to F₀ (100%). It was calculated by the following formula: %ΔFI = (F – F₀)/F₀ × 100.

Measurement of aortic MMP activity by in situ zymography

MMP activity in frozen thoracic aortas was measured using DQ Gelatin as a fluorescent substrate (E12055, Molecular Probes, Oregon, USA). Aortic tissues were vertically embedded in OCT compound and then frozen and cut in serial 4 μm sections. Vessels sections were incubated in dark humidified chambers for 1 h with 1 μM DQ gelatin in Tris–CaCl₂ buffer (Tris 50 mM, CaCl₂ 10 mM, ZnCl₂ 1 μM). Sections were examined by a fluorescent microscope (Leica Imaging Systems Ltd., Cambridge, England) and images were captured at 40×. Proteolytic activity was detected as a bright green fluorescence, which indicates substrate degradation by MMPs, and was evaluated using the ImageJ Program. The assessment of gelatinolytic activity was made by quantifying the intensity of fluorescence from 20 fields selected around vessel circumference. The arithmetic mean of the fluorescence from 20 fields was calculated for each slide [17]. Phenanthroline and PMSF, both at 1 mM, were used to confirm MMP activity. While phenanthroline inhibited MMP activity, PMSF produced no major effects. Doxycycline in situ at 0.1 mM produced no auto-fluorescence interference (data not shown).

Statistical analysis

Results are expressed as mean ± S.E.M. Between groups, comparisons were assessed by two-way analysis of variance (ANOVA) using Bonferroni correction or t test as appropriate. Pearson correlations and linear regression were calculated for Fig. 4 comparing arbitrary units of fluorescence resulted from PMA-stimulated ROS production in neutrophils with increasing concentrations of doxycycline (μM) (GraphPad Prism 5.01 software). A probability value of 5% was considered significant.

Results

Doxycycline attenuates hypertension in 2K-1C rats

While no significant changes in SBP were observed in Sham and Sham + doxy groups, it was significantly increased in 2K-1C rats (p < 0.01). Doxycycline attenuated higher SBP in hypertensive rats throughout the treatment (p < 0.05 effects for doxycycline in hypertensive rats, but not in Sham groups, Fig. 1A) which confirms previous results of our group [15,17,26]. The following values show SBP at 8 weeks of treatment (n = 8/group, in mmHg): Sham 103 ± 2.5, Sham + doxy 107 ± 5.2, 2K-1C 209 ± 10.9 and 2K-1C + doxy 169 ± 17.3 (Fig. 1B).

Doxycycline ameliorates hypertension-induced endothelial dysfunction in aortas

To evaluate the effect of doxycycline on 2K-1C hypertension-induced arterial endothelial dysfunction, aortic rings were isolated and their functional performance was assessed in organ chamber experiments. Fig. 2 shows endothelial cell-dependent vasorelaxation induced by A23187 (1 nM–1 μM). 2K-1C hypertension showed a significant impairment of the maximum A23187-induced aorta relaxation, thus reflecting endothelial dysfunction (p < 0.01 versus both Sham groups, Fig. 2). Although no apparent changes were seen in Sham + doxy group, treatment with doxycycline significantly attenuated 2K-1C-induced impaired endothelial-dependent vasorelaxation to A23187 which is related to its maximal relaxation response (**p < 0.05 for doxycycline effects, Fig. 2).

Doxycycline reduces hypertension-induced oxidative stress

ROS production was first evaluated in situ in isolated aortic segments by fluorescence microscopy. We found increased ROS levels distributed throughout the entire aorta of 2K-1C rats when compared with Sham groups (p < 0.05, Fig. 3A and B). Treatment with doxycycline diminished 2K-1C-induced vascular oxidative stress. Lipid peroxide levels were also determined by measuring TBARS in plasma. We found increased plasma levels of MDA in 2K-1C rats compared with Sham groups (p < 0.05), an effect which was reversed by doxycycline treatment (p < 0.05, Fig. 3C).

Doxycycline attenuates neutrophils-derived ROS in vitro

To verify whether doxycycline inhibits neutrophils-derived ROS, human neutrophils were treated or not with different concentrations of doxycycline (0.8–200 μM) and then stimulated with PMA. The arbitrary units of fluorescence resulted from PMA-stimulated ROS production in neutrophils correlated inversely with increasing concentrations of doxycycline (p < 0.05, r = –0.9234, Fig. 4). Cells that were treated either with PMA alone or PMA plus lower concentrations of doxycycline showed higher levels of ROS, as represented by the increased fluorescence intensity. However, neutrophils that were previously treated with doxycycline at higher concentrations showed significant reduction in PMA-stimulated ROS production. The estimate half maximal effective concentration (EC50) of doxycycline that inhibits neutrophils-derived ROS in vitro is approximately 181.3 μM.

Doxycycline protects against hypertension-induced loss of NO bioavailability in aortic endothelial cells

NO levels in aortic endothelial cells were assessed at each one second intervals by confocal microscopy using the selective NO fluorescent dye DAF-2/DA. As shown in Fig. 5, NO production...
Sham Sham + Doxy 2K-1C 2K-1C + Doxy

Discussion

Hypertension is associated with well known functional and structural vascular alterations that may result from the interplay of several mechanisms. This study is the first to show that doxycycline decreases the oxidative stress in 2K-1C hypertension. This effect may contribute to the improvement of the vascular dysfunction associated with hypertension, not only by decreasing vascular MMP activity, but also by increasing NO bioavailability.

Similar to what we found in previous reports using rat aortas [15,17,26], we showed here that treatment with doxycycline decreased higher MMP activity in the aortas from 2K-1C rats and significantly improved hypertension-induced impaired endothelial-dependent vasorelaxation. Using in situ zymography, we observed an important link between the localization of MMPs in hypertensive aortic wall with the functional vascular alteration. The increased MMP activity in the aortic endothelium of hypertensive rats may be related to hypertension-induced vascular dysfunction, and this association would not be detected if we assessed MMP levels by conventional gelatin zymography using the whole aorta. However, the mechanisms by which MMPs contribute to hypertension-induced vascular dysfunction still remain to be elucidated. It is possible that MMP-induced excessive degradation of extracellular matrix and vascular smooth muscle cells migration from media to intima layer contributes to such endothelial damage [18,35]. Moreover, it is possible that MMP-2-mediated vascular contractility by cleaving some peptides, such as big-endothelin-1 [36], calcitonin gene-related peptide [37] and adrenomedullin [38] also contributes to endothelial dysfunction and increased vascular resistance in hypertension.

Although non-antimicrobial effects of doxycycline are related to MMP inhibition, both in vivo and in vitro studies have also shown that this drug may have significant ROS scavenging properties [27–29,31,32]. In line with these findings, our results show for the first time that treatment with doxycycline reduced 2K-1C hypertension-induced systemic and vascular oxidative stress. We have also found that PMA-stimulated ROS production in neutrophils in vitro correlated inversely with increasing concentrations of doxycycline. In addition, high concentrations of doxycycline may also directly react with superoxide [39] and peroxynitrite in vitro and these findings corroborate the idea that doxycycline may be a potential ROS scavenger in hypertensive rats. In fact, Fig. 3A and B shows that treatment of hypertensive rats with doxycycline at 30 mg/kg/day decreased higher levels of superoxide in aortas which was represented as DHE levels. Supporting our findings, a recent study in mice showed that doxycycline prevented doxorubicin-induced myocardial ROS production, thus ameliorating the systolic dysfunction and detrimental ventricular remodeling [27]. Similar effects on ROS production were also found in testes of mice treated with doxorubicin [28].

Fig. 1. Systolic blood pressure (SBP) after 8 weeks of hypertension. SBP (mmHg) was weekly measured by tail-cuff plethysmography in all experimental groups during the study period (A). Bar graph represents the final SBP in mmHg (B). Data are shown as mean ± S.E.M. (n = 8). *p < 0.01 versus Sham and Sham + Doxy groups and †p < 0.05, significant effects for doxycycline in hypertensive rats, but not in Sham groups.

Fig. 2. Doxycycline ameliorates 2K-1C hypertension-induced arterial endothelial dysfunction. To assess endothelial cell-dependent vasorelaxation, aortic rings were pre-contracted with phenylephrine (100 nM) and then used to construct a cumulative concentration–response curve to A23187 (1 nM–1 μM). Data are shown as mean ± S.E.M. (n = 3–8). *p < 0.01 versus Sham groups and †p < 0.05 for doxycycline effects.

Doxycycline reduces increased MMP activity in aortas of 2K-1C hypertensive rats

In situ zymography was used to assess MMP activity in frozen cross-sectioned aortas. We found enhanced green fluorescence intensity in entire aortas (p < 0.01). Treatment with doxycycline markedly inhibited 2K-1C-induced loss of NO bioavailability in aortic endothelial cells (*p < 0.01 effects for doxycycline in hypertensive rats, but not in Sham groups, Fig. 5).

Supporting our findings, we have also found that PMA-stimulated ROS production in neutrophils in vitro correlated inversely with increasing concentrations of doxycycline. In addition, high concentrations of doxycycline may also directly react with superoxide and peroxynitrite in vitro and these findings corroborate the idea that doxycycline may be a potential ROS scavenger in hypertensive rats. In fact, Fig. 3A and B shows that treatment of hypertensive rats with doxycycline at 30 mg/kg/day decreased higher levels of superoxide in aortas which was represented as DHE levels. Supporting our findings, a recent study in mice showed that doxycycline prevented doxorubicin-induced myocardial ROS production, thus ameliorating the systolic dysfunction and detrimental ventricular remodeling [27]. Similar effects on ROS production were also found in testes of mice treated with doxorubicin [28].
Increased ROS concentrations play an important role in the vascular dysfunction found both in clinical and experimental hypertension [5–8]. High levels of superoxide react with NO in the aortic endothelium, thus reducing its bioavailability and leading to endothelial dysfunction and impaired vascular relaxation. Our results suggest that the protective effects of doxycycline against 2K-1C-induced vascular dysfunction may result of its action as a ROS scavenger. In fact, we found that doxycycline decreased 2K-1C-induced oxidative stress and protected against loss of NO bioavailability in aortic endothelial cells, thus contributing to the amelioration of hypertension-induced endothelial dysfunction. To assess NO and endothelial-dependent vasorelaxation, we used the calcium ionophore A23187. This drug has the ability to move calcium through cell membranes, thus inducing endothelial NO production by diverse mechanisms [33]. Interestingly, doxycycline improved NO levels in aortic endothelial cells from hypertensive animals. Our findings are consistent with previous results showing that doxycycline improved vascular dysfunction in mice with Marfan syndrome [40]. The beneficial effects of doxycycline were associated with increased vascular endothelial NO synthase phosphorylation and increased NO bioavailability [40]. Our findings suggest a potential additional effect of doxycycline that may explain the how doxycycline improved the vascular function in hypertension.

To reach circulating concentrations of doxycycline in rats similar to what it is observed in adult humans (approximately 4 μM after a single oral dose of 100 mg [41]), rats were treated with a relatively higher dose of doxycycline at 30 mg/kg/day as they have
more rapidly metabolism than humans. According to Prall et al. [41], treatment of mice with oral doses of doxycycline at 10, 50 and 100 mg/kg produced dose-dependent increases in drug serum concentrations of 1.4, 2.7 and 11.9 μg/ml which correspond to 2.7, 5.3 and 23.2 μM, respectively. If we consider that after oral administration of 30 mg/kg of doxycycline in rats, its serum concentration is proportional to what is observed in mice, we would expect to see doxycycline concentration at approximately 4.2 μM (which is closely related to what it is seen in humans after a single dose of 100 mg doxycycline). Furthermore, since it was suggested that diabetic rats orally treated with doxycycline at 15 mg/kg/day contained 2 μM of it in plasma [42], we would expect to see in our study a concentration of doxycycline around 4 μM (considering that the rat gender, specie and body weight in both studies were similar). In addition to treat the rats with 30 mg/kg/day of doxycycline, we also treated them with 3 and 10 mg/kg/day [26]. Using these lower doses, we did not observe any improvement in MMP-induced vascular alterations in hypertension. A very likely reason to explain this lack of effects is attributed to the insufficient concentrations of doxycycline that may reach rat aortas (probably lower than 4 μM). According to Prall et al. [41], doxycycline at circulating concentrations higher than 4 μM was significantly effective in reducing MMPs-induced aortic aneurysm growth in mice. This may explain why we observed improvement in MMP-induced vascular dysfunction in hypertension using only doxycycline at 30, but not 3 and 10 mg/kg/day. The resultant lower concentration of doxycycline may also be a reason to justify that its lower doses would not decrease the levels of either nitric oxide or ROS in hypertensive rats (although there is a recent article showing that doxycycline at very low-dose may restore diabetes-induced increase in plasma lipid peroxidation [43]). However, this latter hypothesis should be further investigated in hypertension.

Several studies have shown the important link between ROS and MMPs [20–22,44]. The reaction of superoxide and NO in the vasculature promotes ONOO− formation. This harmful ROS activates the 72 kDa MMP-2 by disrupting the binding between the critical cysteine residue in its propeptide and the zinc ion in the catalytic site [21,44]. In vitro studies have also shown that ROS can enhance both MMP-2 mRNA levels and activity via increasing vascular pro-oxidant enzymes activities [45,46]. Using antioxidants, such as tempol, we recently showed in a in vivo study that MMP-2 activity may be an important downstream mechanism of ROS-induced vascular endothelial damage in 2K-1C hypertension [8]. Conversely, it was also suggested that MMPs may be upstream mediators of ROS generation. Using isolated rat mesenteric arteries, the study showed that by inhibiting MMP activity, doxycycline and GM6001 prevented arterial oxidative stress production and decreased phenylephrine-induced enhances in vascular tone and hypertrophy [22]. Because doxycycline inhibited 2K-1C-induced oxidative stress and MMP activity in the present study, it is uncertain whether increased ROS levels are responsible to trigger MMP activation or whether MMPs mediate the generation of oxidative stress, thus leading to hypertension-induced vascular changes.

Doxycycline directly inhibits MMP activity through its ability to chelate the catalytic Zn2+ ion essential for MMP activity. In addition, doxycycline contains phenol rings in its structure, it may act as a ROS scavenger, thus decreasing several harmful effects induced by oxidative stress [27–29]. However, because doxycycline directly inhibits MMP activity and the deleterious effects of oxidative stress may be partially mediated by MMP activation, labeling doxycycline as an antioxidant would be overstated.

In conclusion our results suggest that doxycycline ameliorates 2K-1C hypertension-induced arterial endothelial dysfunction in part by inhibiting oxidative stress generation and improving NO bioavailability, and these effects may contribute to MMP inhibition to protect against the vascular alterations of hypertension. Since there is a link between ROS and MMPs, and doxycycline may inhibit both, it is unclear whether doxycycline-induced ROS reduction
potentiates its effect on MMP inhibition (or vice versa). Further studies should be carried out to clarify this issue in hypertension.

Acknowledgments

This study was funded by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP – Brazil) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq – Brazil).

References