Redox Biology 1 (2013) 578–585

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

Atorvastatin and sildenafil lower blood pressure and improve endothelial dysfunction, but only atorvastatin increases vascular stores of nitric oxide in hypertension

Danielle A. Guimarães [a], Elen Rizzi [a], Carla S. Ceron [a], Lucas C. Pinheiro [a], Raquel F. Gerlach [b], Jose E. Tanus-Santos [a,*]

[a] Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Av. Bandeirantes 3900, Ribeirao Preto 14049-900, SP, Brazil
[b] Department of Morphology, Physiology, and Basic Pathology, University of Sao Paulo, s/n Av. Café, Ribeirao Preto 14040-904, SP, Brazil

ARTICLE INFO

Article history:
Received 5 November 2013
Received in revised form
8 November 2013
Accepted 11 November 2013

Keywords:
Atorvastatin
Endothelial dysfunction
Nitric oxide
Nitrite
Oxidative stress
Sildenafil
2K1C hypertension

ABSTRACT

Nitric oxide (NO)-derived metabolites including the anion nitrite can recycle back to NO and thus complement NO formation independent of NO synthases. While nitrite is as a major vascular storage pool and source of NO, little is known about drugs that increase tissue nitrite concentrations. This study examined the effects of atorvastatin or sildenafil, or the combination, on vascular nitrite concentrations and on endothelial dysfunction in the 2 kidney-1 clip (2K1C) hypertension model. Sham-operated or 2K1C hypertensive rats were treated with vehicle, atorvastatin (50 mg/Kg), sildenafil (45 mg/Kg), or both for 8 weeks. Systolic blood pressure (SBP) was monitored weekly. Nitrite concentrations were assessed in the aortas and in plasma samples by ozone-based reductive chemiluminescence assay. Aortic rings were isolated to assess endothelium-dependent and independent relaxation. Aortic NADPH activity and ROS production were evaluated by luminescence and dihydroethidium, respectively, and plasma TBARS levels were measured. Aortic nitrotyrosine staining was evaluated to assess peroxynitrite formation. Atorvastatin and sildenafil, alone or combined, significantly lowered SBP by approximately 40 mmHg. Atorvastatin significantly increased vascular nitrite levels by 70% in hypertensive rats, whereas sildenafil had no effects. Both drugs significantly improved the vascular function, and decreased vascular NADPH activity, ROS, and nitrotyrosine levels. Lower plasma TBARS concentrations were found with both treatments. The combination of drugs showed no improved responses compared to each drug alone. These findings show evidence that atorvastatin, but not sildenafil, increases vascular NO stores, although both drugs exert antioxidant effects, improve endothelial function, and lower blood pressure in 2K1C hypertension.

Introduction

Nitric oxide (NO) is a major player in normal cardiovascular physiology [1] and its deficiency causes endothelial dysfunction, a critical mechanism in the pathogenesis of cardiovascular diseases including hypertension [2,3]. Oxidized NO forms the anions nitrite (NO_2^-) and nitrate (NO_3^-), and it is now clear that NO-derived metabolites can recycle back to NO through a nitrate–nitrite–NO pathway that complements NO formation independent of NO synthases [4,5]. Importantly, nitrite anions are now considered as a major vascular storage pool and source of NO [6,7], and therefore drugs that increase the vascular concentrations of nitrite may promote therapeutic effects, particularly in disease conditions associated with NO depletion [2]. However, very few studies have examined how drugs that improve endothelial dysfunction affect vascular nitrite levels, especially in hypertension. Examining this possibility is very important because NO production from nitrite occurs mostly in tissues [7] and therefore assessing tissue nitrite levels may improve our understanding of how the nitrite–NO pathway mediates critical functions [8].

In this study, we hypothesized that drugs activating the NO-cyclic guanosine monophosphate (cGMP) pathway could increase the vascular stores of NO (assessed as tissue nitrite concentrations) and thus improve the vascular function in hypertension. We examined how atorvastatin or sildenafil, or the combination of both drugs, affect the vascular nitrite concentrations in the

* Corresponding author. Tel.: +55 16 36023 163; fax: +55 16 3602 0220.
E-mail addresses: tanus@fmrp.usp.br, tanussantos@yahoo.com (J.E. Tanus-Santos).

2213-2317
http://dx.doi.org/10.1016/j.redox.2013.11.004

2 kidney-1 clip (2K1C) hypertension model. This model exhibits many features of clinical hypertension including increased vascular oxidative stress, impaired vascular NO activity, and endothelial dysfunction [9–12]. While statins improve endothelial dysfunction by mechanisms that reduce reactive oxygen species (ROS) formation and enhance NO activity [13,14], no previous study has examined whether atorvastatin increases the vascular NO stores in 2K1C hypertension. Moreover, while phosphodiesterase 5 inhibitors (PDE5i) activate the NO-cGMP pathway by inhibiting cGMP degradation, antioxidant mechanisms activated by these drugs may reduce tissue ROS concentrations and therefore may improve vascular NO bioavailability by preventing the reaction of NO with ROS [15–17]. However, it is unknown whether sildenafil improves vascular NO stores and improves endothelial dysfunction in 2K1C hypertension. The present study addresses the effects of both drugs and the combination therapy, which could offer additive effects on vascular nitrite levels and function [18–20].

Material and methods

Animals and treatments

This study was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, and the animals were handled according to the guiding principles published by the National Institutes of Health. Male Wistar rats (180–200 g) were maintained on 12-h light/dark cycle at 25 °C with free access to rat chow and water.

Surgical procedures and experimental protocols

2K1C hypertension was induced by clipping the left renal artery with a silver clip (0.2 mm). Sham-operated rats underwent the same surgical procedure (under general anesthesia with ketamine 100 mg/kg and xylazine 10 mg/kg i.p.), except for the placement of the renal artery clip. Body weight and systolic blood pressure (SBP) were assessed weekly. SBP were measured by tail-cuff plethysmography, and the rats were considered hypertensive when SBP > 160 mmHg two weeks after the surgery.

Animals were randomly assigned to one of eight groups: 2K1C and Sham groups that received ethanol 2% (vehicle used to dilute both drugs); 2K1C and Sham groups that received atorvastatin at 50 mg/kg per day [21]; 2K1C and Sham groups that received sildenafil at 45 mg/kg per day [22]; and 2K1C and Sham groups that received the combination of atorvastatin 45 mg/kg + sildenafil 50 mg/kg per day. The treatments were started two weeks after 2K1C hypertension was induced and maintained for additional eight weeks. All treatments were given daily by oral gavage and after 10 weeks of hypertension, the animals were killed by decapitation and their thoracic aortas were isolated and cleaned of connective tissue and fat. Arterial blood samples were centrifuged at 1000*g* for 10 min and plasma fractions were immediately stored at −70 °C until used for biochemical measurements.

Determination of plasma and vascular nitrite concentrations

The plasma and aortic extracts were analyzed in duplicate for their nitrite content using an ozone-based reductive chemiluminescence assay as previously described [23,24]. The aortas were homogenized in ice-cold phosphate buffer (pH 7.4; 500 μl) and homogenates were kept on ice in the dark and used within 30 min of preparation. Briefly, to measure nitrite concentration, 50 μl of plasma or 200 μl of aortic extracts samples were injected into a solution of acidified triiodide, purging with nitrogen in line with a gas phase chemiluminescence NO analyzer (Sievers Model 280 NO analyzer, Boulder, CO, USA). Approximately 8 ml of triiodide solution (2 g potassium iodide and 1.3 g iodine dissolved in 40 ml water with 140 ml acetic acid) was placed in the purge vessel into which the samples were injected. The triiodide solution reduces nitrites to NO gas, which is detected by the NO analyzer. The data were analyzed using the software Origin Lab 6.1.

Vascular reactivity

The thoracic aortas were isolated and rings 4 mm in length were cut and mounted for isometric tension recording. The rings were placed in bath chambers (5 ml) for isolated organs containing modified Krebs salt solution with the following composition (mmol/L): NaCl 130, $CaCl_2$ 1.6, $MgSO_4$ 1.2, KH_2PO_4 1.2, KCl 4.7, $NaHCO_3$ 14.9, glucose 5.5. This solution was maintained at 37 °C, pH 7.4, and bubbled with 95% O_2 and 5% CO_2. The system was connected to an isometric force displacement transducer (Letica Scientific Instruments, Barcelona, Spain) and the responses were recorded on a computer system using the Chart version 4.04, PowerLab ADInstruments (2000) program. The aortic rings were subjected to a tension of 1.5 g for a 60-min equilibration period and were considered to have intact functional endothelium when acetylcholine (10^{-3} mol/L) produced more than 80% relaxation, which was calculated as a percentage of the contraction induced by phenylephrine (10^{-7} mol/L). To assess endothelium-dependent and endothelium-independent relaxation, aortic rings precontracted with phenylephrine (10^{-4} mol/L) were used to construct cumulative concentration–response curves to acetylcholine (10^{-10}–10^{-5} mol/L) and to sodium nitroprusside (10^{-10}–10^{-5} mol/L) [25].

Assessment of vascular ROS formation, vascular NADPH activity, and lipid peroxide levels in plasma

Dihydroethidium (DHE) was used to evaluate vascular production of reactive oxygen species (ROS). Briefly, aortic tissues were embedded vertically in Tissue-tek® and criostetized. The aortic sections were incubated with DHE (10 μmol/L) as previously described [26] and examined with fluorescence microscopy (Leica Imaging Systems Ltd., Cambridge, England). The images were captured at x400 and the intensity of the red fluorescent signal was evaluated by using ImageJ Program (NIH – National Institute of Health).

Vascular NADPH-dependent superoxide production was measured in aortic rings from all experimental groups as previously described [26]. Aortic rings were transferred to luminescence vials containing 1 mL of Krebs-HEPES buffer, pH 7.4. After equilibration and background counts, a nonredox cycling concentration of lucigenin (5 μmol/L) and NADPH (300 μmol/L) were automatically added and the luminescence counts measured continuously for 15 min in a Berthold FB12 single tube luminometer at 37 °C. Background signals from aortic rings were subtracted from the NADPH-driven signals and the results were normalized for the dry weight and reported as RLU/mg/min.

Plasma lipid peroxide levels were determined by measuring thiobarbituric acid reactive substances (TBARS) using a fluorimetric method that requires excitation at 515 nm and emission at 553 nm as previously described [27]. The lipoperoxide levels were expressed in terms of malondialdehyde (MDA) (nmol/mL).

Immunohistochemistry assessment of vascular nytrotirosine levels

Vascular nitrotyrosine levels were measured in aortas fixed in 4% phosphate-buffered paraformaldehyde, pH 7.4, and embedded in paraffin. Tissue sections (5 μm thick) were deparaffinized, washed with phosphate buffer, and submitted to heat-induced antigen retrieval, endogenous peroxidase inhibition, and non-specific antibody

blocking. Subsequently, the sections were incubated overnight at 4 °C with the anti-nitrotyrosine primary antibody (1:100; Millipore) and with the secondary antibody (1:200; Millipore). Antigen was visualized with a labelled streptavidin biotin peroxidase technique (Vectastain ABC kit; Vector Laboratories Inc.) with 3,3′-diaminobenzidine (DAB) substrate [28]. Sections were counterstained with haematoxylin, and examined using light microscopy (Leica Imaging Systems Ltd., Cambridge, England) and the image was captured at x400. Immunoreactivity intensity was measured using the ImageJ Program.

Statistical analysis

The results are expressed as means ± S.E.M. Comparisons between groups were assessed by two-way or one way ANOVA followed by the Tukey test using GraphPad Prism software. A probability value < 0.05 was considered significant.

Results

Effects of treatments on systolic blood pressure (SBP) and body weight

The baseline SBP was similar in the eight experimental groups, and no significant changes were found in the Sham+vehicle and in the Sham+treatment groups (Fig. 1A). SBP increased progressively in the 2K1C+Vehicle group (final SBP = 200 ± 4 mmHg). The treatment of 2K1C rats with atorvastatin, sildenafil, or both drugs exerted similar antihypertensive effects (final SBP = 148 ± 6, 156 ± 2, and 138 ± 4 mmHg, respectively; Fig. 1A; $P < 0.05$). Similar body weight gain was found in the eight experimental groups (Fig. 1B; $P > 0.05$).

Effects of drug treatments on aortic and plasma nitrite concentrations

To examine the effects of drug treatments on the vascular stores of NO, nitrite concentrations were assessed in aortic and in plasma samples. Treatment with atorvastatin significantly increased nitrite concentrations in the aortas from 2K1C hypertensive animals by approximately 70% ($P < 0.05$), whereas this statin only marginally ($P = 0.085$) increased nitrite concentrations in Sham-operated animals (Fig. 2A). In contrast with atorvastatin, treatment with sildenafil did not affect aortic nitrite concentrations in both Sham-operated and in 2K1C hypertensive rats ($P > 0.05$; Fig. 2A).

To further confirm the effects of treatments on NO storage, we measured nitrite concentrations in plasma samples. Interestingly, the changes in plasma nitrite concentrations were similar to those found in the aortic tissue. While atorvastatin treatment increased plasma nitrite concentrations by approximately 70% to 110% in 2K1C and in Sham-operated animals, sildenafil increased plasma nitrite concentrations only in Sham-operated animals (all $P < 0.05$; Fig. 2B).

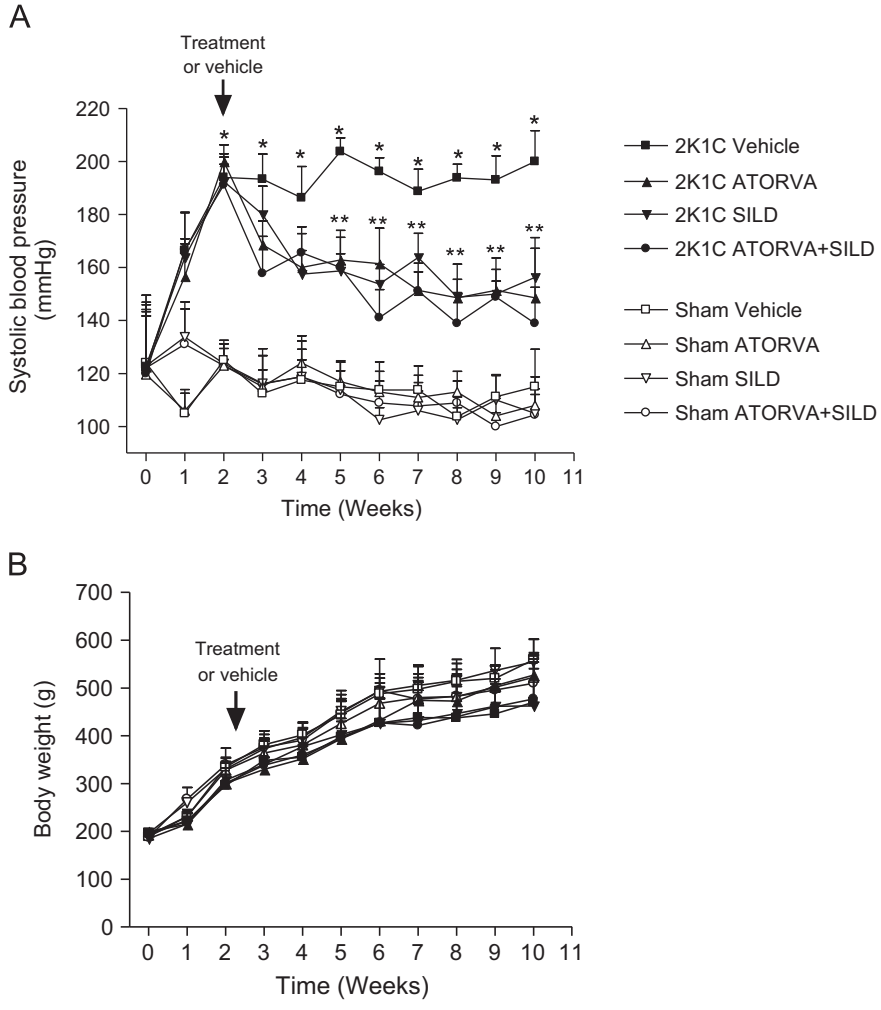

Fig. 1. Systolic blood pressure (mmHg) measured by tail-cuff method (panel A) and body weight (panel B) in the eight experimental groups along 10 weeks of study ($n = 7$–10/group). Data are shown as mean ± S.E.M. *$P < 0.01$ *versus* Sham Vehicle group; **$P < 0.01$ *versus* 2K1C Vehicle group.

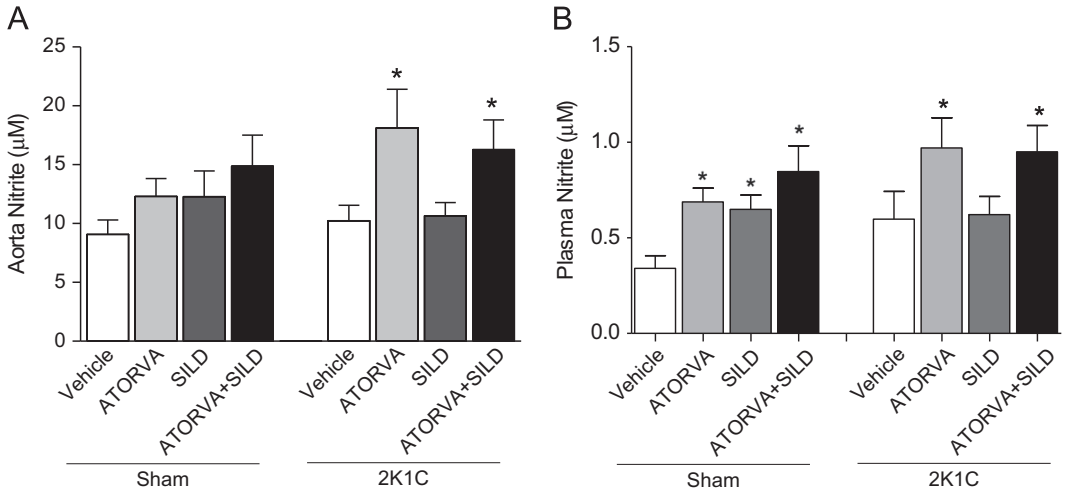

Fig. 2. Nitrite concentrations (μM; $n=7$–9/group) in the aortas (panel A) and in plasma samples (μM; $n=4$–5/group) from rats at the end of treatment. Nitrite concentrations were assessed by reductive chemiluminescence. Data are shown as mean ± S.E.M. $^{*}P<0.05$ *versus* respective Vehicle group.

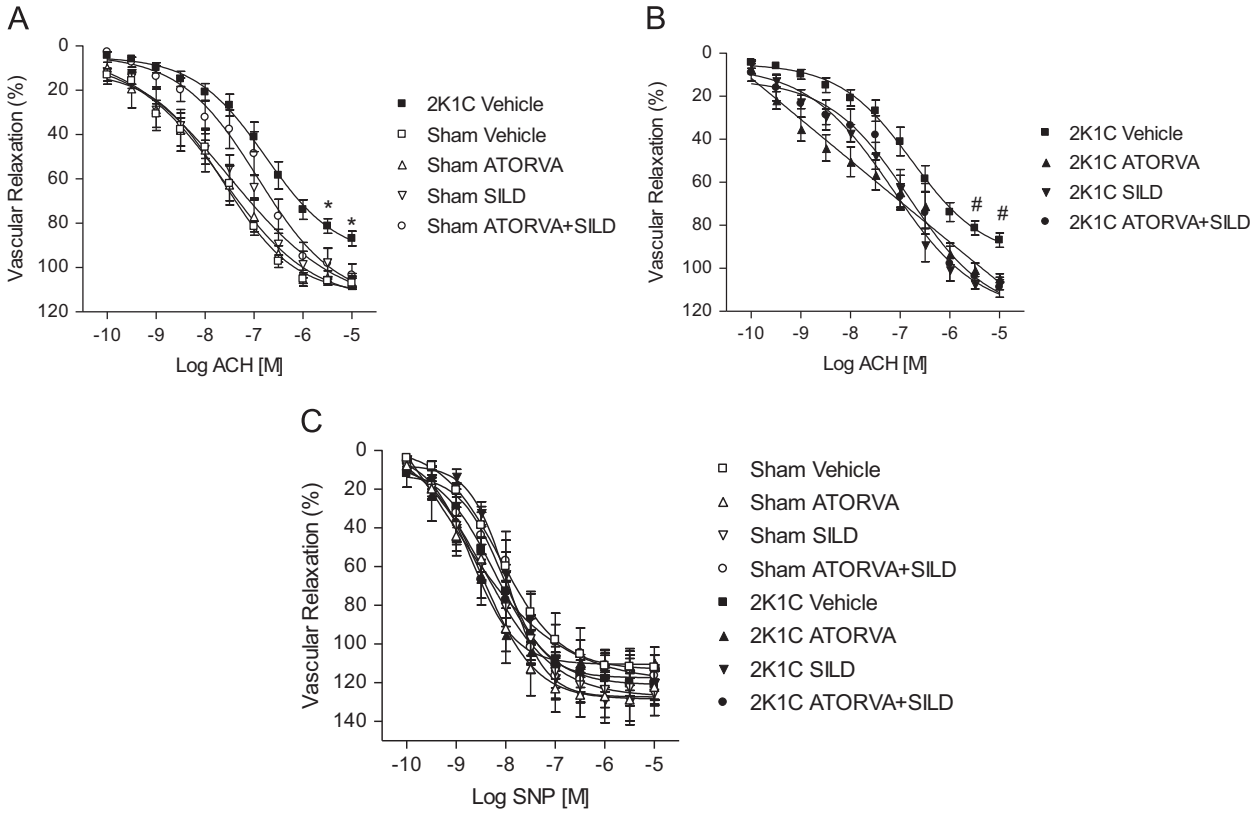

Fig. 3. Endothelial cell-dependent vasorelaxation induced by acetylcholine (panels A and B) and endothelial cell-independent relaxation induced by sodium nitroprusside. Rat aortic ring preparations were studied after precontraction with phenylephrine (10^{-7} M) ($n=6$–10/group). Data are shown as mean ± S.E.M. $^{*}P<0.05$ *versus* Sham Vehicle group (panel A); $^{\#}P<0.05$ *versus* 2K1C treated groups (panel B).

No significant interactions were found between atorvastatin and sildenafil treatments with respect to aortic or plasma nitrite concentrations ($P>0.05$; Fig. 2A and B).

Effects of treatments on vascular function

To examine whether increased vascular NO storage as a result of drug treatment would translate into improved vascular function, we examined the vascular responses to acetylcholine. Fig. 3A shows impaired endothelial cell-dependent responses to acetylcholine in 2K1C hypertensive rats as compared with the Sham-operated groups, as revealed by a right shift in the concentration–dependent responses and decreased maximum response to acetylcholine in hypertensive rats ($P<0.05$; Fig. 3A). Treatment with atorvastatin or sildenafil, or both, restored the vascular responses to acetylcholine ($P<0.05$; Fig. 3B).

No significant changes were found in the aortic responses to sodium nitroprusside when 2K1C hypertensive rats were compared with Sham-operated controls, and drug treatments had no effects on the responses to this NO-donor drug ($P>0.05$; Fig. 3C).

No significant interactions were found when both drug treatment were combined ($P > 0.05$; Fig. 3A–C).

Effects of treatments on 2K1C-induced increases in oxidative stress

Treatment with atorvastatin or sildenafil could contribute to increased NO bioavailability by decreasing hypertension-induced oxidative stress. To examine the possible antioxidant effects of both drugs, we used three different methods. First, we used the fluorescent superoxide indicator DHE to study the effects of the treatments on 2K1C hypertension-induced increases in ROS production in aortic tissues. Fig. 4A and B shows higher vascular ROS levels in 2K1C hypertensive rats compared with Sham-operated groups ($P < 0.05$). While drug treatments exerted no effects in Sham-operated groups, both drugs abolished 2K1C-induced increases in vascular ROS production ($P < 0.05$; Fig. 4A and B).

To confirm antioxidant effects exerted by both drugs, we measured the NADPH oxidase activity in the aortas from rats. This is important because vascular ROS are produced in endothelial, adventitial, and vascular smooth muscle cells and derive primarily from NADPH oxidase activity. Fig. 4C shows that hypertension was associated with increased NADPH oxidase activity when compared with Sham-operated groups, and both drugs significantly decreased NADPH oxidase activity in hypertensive rats ($P < 0.05$).

To further validate the antioxidant effects found in vascular tissue, we measured plasma lipid peroxide levels. Higher malondialdehyde (MDA) levels were found in 2K1C+Vehicle group as compared with the Sham-operated groups ($P < 0.05$; Fig. 4D). Both drug treatments were associated with lower MDA levels in 2K1C hypertensive rats ($P < 0.05$; Fig. 4D).

The combination of drugs did not improve the antioxidant effects of each individual drug treatment ($P > 0.05$; Fig. 4A–D).

Effects of treatments on vascular nitrotyrosine levels

Diffusion-limited reaction between superoxide and NO results in peroxinitrite formation [29], and this highly reactive molecule increases tissue nitrotyrosine levels. Therefore, we examined whether antioxidant effects exerted by atorvastatin or sildenafil could prevent hypertension-induced increases in vascular nitrotyrosine levels. While higher vascular nitrotyrosine levels were found in 2K1C hypertensive rats ($P < 0.05$), both drug treatments attenuated this alteration (Fig. 5A and B; $P < 0.05$). No further attenuation was found when drug treatments were combined (Fig. 5A and B; $P > 0.05$).

Discussion

This is the first study to show that atorvastatin, but not sildenafil, increased vascular nitrite concentrations in hypertensive rats. We demonstrated for the first time that both drugs exerted similar antioxidant and antihypertensive effects, and prevented

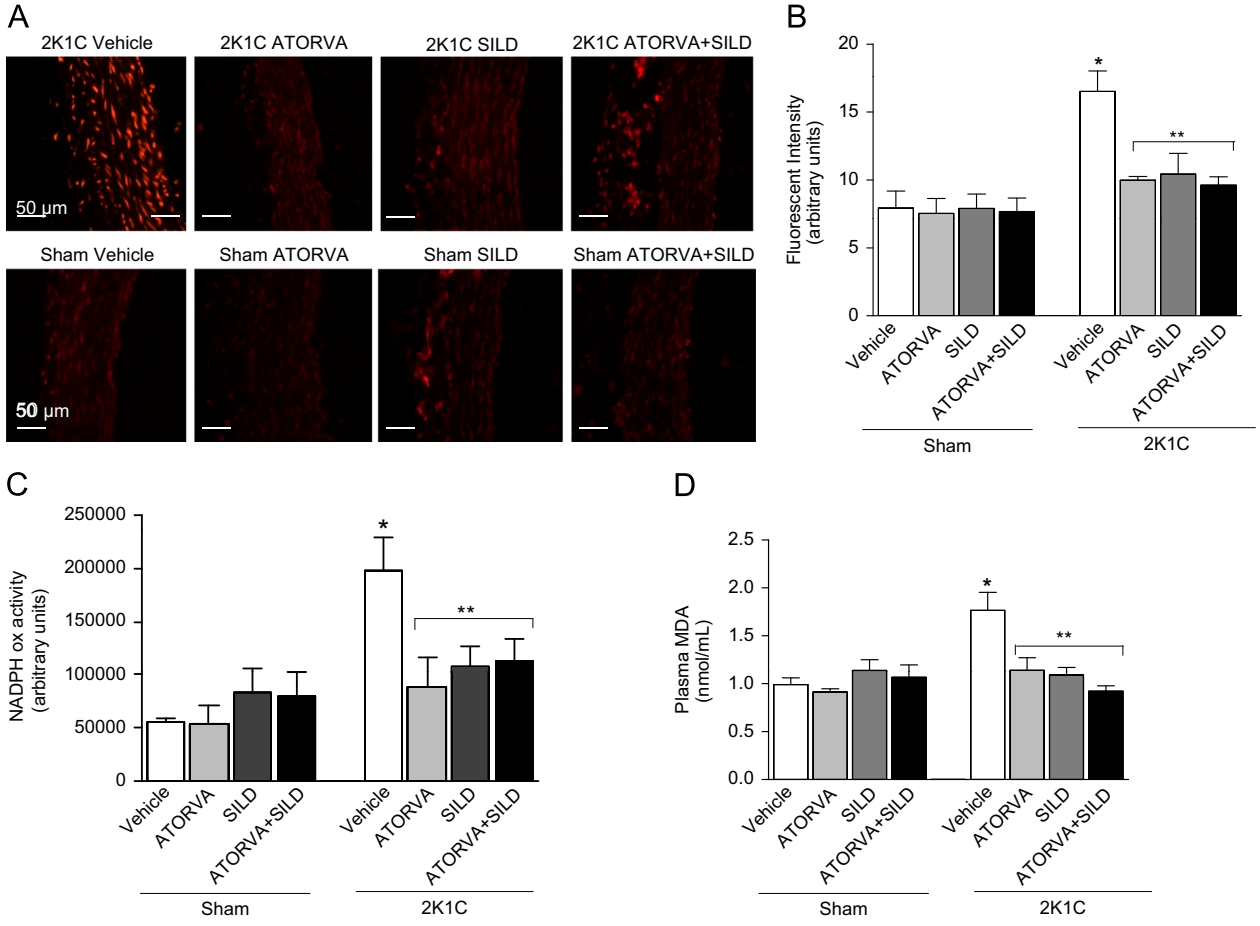

Fig. 4. Effects of drug treatments on vascular oxidative stress. Panel A shows representative photomicrographs (x400) with red fluorescence of DHE aortic samples from hypertensive and Sham-operated rats. Panel B shows the quantification of aortic fluorescence ($n=8$/group). Panel C shows NADPH-dependent superoxide production measured as lucigenin chemiluminescence in the aortic rings ($n=6$–8/group). Panel D shows lipid peroxide levels measured in plasma samples and expressed in terms of MDA ($n=10$/group). Data are shown as mean ± S.E.M. *$P < 0.05$ *versus* Sham Vehicle group; **$P < 0.05$ *versus* 2K1C Vehicle group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

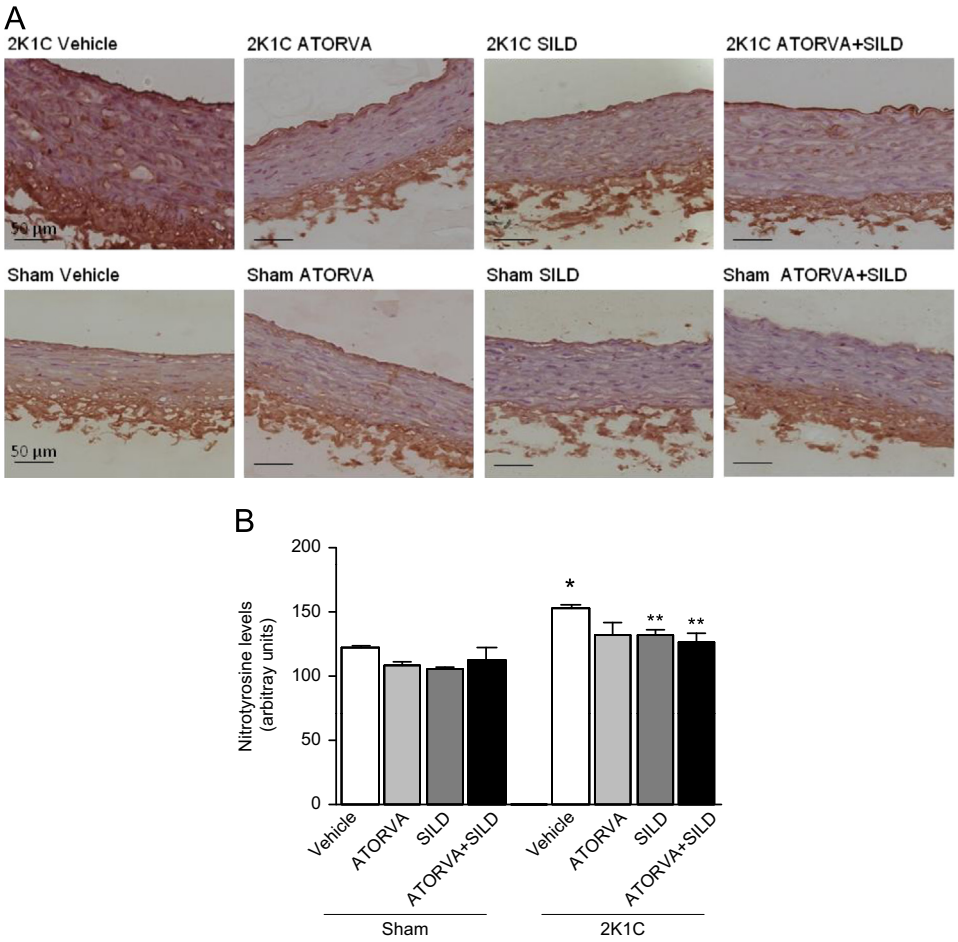

Fig. 5. Effects of drug treatments on aortic nitrotyrosine levels. Panel A shows representative photomicrographs (x400) of immunostaining of nitrotyrosine performed in the aortas. Panel B shows the quantification of brown staining of nitrotyrosine ($n=4$ per group). Data are shown as mean ± S.E.M. *$P<0.05$ *versus* Sham Vehicle group; **$P<0.05$ *versus* 2K1C Vehicle group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

2K1C hypertension-induced endothelial dysfunction. No significant additive effects were found when both drugs were combined.

Increased production of angiotensin II in 2K1C hypertension activates NADPH oxidase, increases vascular ROS, and causes vascular dysfunction [30], as we reported here. Both atorvastatin and sildenafil exerted antihypertensive effects in the 2K1C hypertension model, and this effect was consistently associated with antioxidant effects, as revealed by results obtained with three independent assays to evaluate vascular and systemic oxidative stress. Further confirming these results, both drugs at least tended to lower vascular nitrotyrosine levels in hypertensive rats. These results may explain the improved vascular function in 2K1C hypertensive rats treated with either atorvastatin or sildenafil. Indeed, our findings support previous studies showing similar effects of atorvastatin [21] in spontaneously hypertensive rats (SHR). In agreement with our findings, the authors showed that atorvastatin reduced systolic blood pressure by approximately 20 mmHg and improved endothelial dysfunction as a result of lower vascular ROS levels [21]. It is possible that some quantitative differences in the antihypertensive effects (40 mmHg decrease in SBP with atorvastatin in the present study *versus* 20 mmHg in the previous study [21]) may reflect differences in study design or animal model.

The responses we found with sildenafil are similar to those previously shown in rats with NO depletion-induced hypertension [22] or in the SHR model [31]. The antihypertensive effects and the prevention against endothelial dysfunction of 2K1C hypertension with atorvastatin or sildenafil probably resulted of antioxidant mechanisms shared by both drugs. The increased ROS levels formed in the vasculature from hypertensive animals react with NO generating peroxinitrite and reduce tissue NO activity [9]. Our results suggest that this critical mechanism may have been blunted by both drugs, thus improving endothelial-dependent vascular responses and lowering blood pressure. Together, our findings and those previously reported strongly suggest that both atorvastatin and sildenafil exert significant pleiotropic effects, independent of the canonical mechanism explaining their *in vivo* effects.

Tissue and circulating nitrite levels have been widely measured in experimental and clinical studies because nitrite plays important roles in signaling and regulation of blood flow [4,32,33]. In fact, low nitrite levels indicate endothelial dysfunction and are associated with cardiovascular risk factors [34]. Consistent with our results, circulating nitrite levels are usually below 1 µM, whereas tissue concentrations may achieve 10–20 µM [24,35]. The significant differences between circulating and tissue nitrite concentrations are very relevant because there is evidence that nitrite conversion into NO occurs predominantly in tissues [7], and therefore pharmacological approaches that increase vascular nitrite concentrations may offer improved cardiovascular protection.

Low vascular or plasma nitrite levels could indicate endothelial dysfunction contributing to hypertension. However, we found similar aortic or plasma nitrite concentrations in 2K1C hypertensive rats compared to sham operated controls. While this finding could suggest that the 2K1C hypertension model may not be the most appropriate model to use since there was no reduction in vascular or plasma nitrate levels, there are clear time-dependent pathophysiological alterations in this hypertension model, with significant activation of

the renin-angiotensin system during the initial weeks [36], which is apparently normalized in the following weeks [37]. Time-dependent changes in nitrite concentrations in 2K1C hypertension may explain the lack of significant differences that we found with respect to nitrite concentrations. Indeed, we have consistently found lower nitrite [27,38], nitrate [38], and nitroso species [39] concentrations in 2K1C hypertensive rats compared to controls after 4 weeks of 2K1C hypertension, but not after 8 weeks of hypertension. While it might have been more interesting to determine the effects of atorvastatin and sildenafil on nitrite levels at an earlier phase of 2K1C hypertension, we wanted to avoid this phase of transition from high to low renin-angiotensin system activity [36,37]. Adding complexity to this issue, aortic inducible NOS (iNOS) expression doubled after 8 weeks of 2K1C hypertension [25], and it is not clear how increased iNOS expression may affect nitrite levels in 2K1C hypertension.

Interestingly, while both atorvastatin and sildenafil exerted antioxidant effects and improved endothelial dysfunction, we found increased vascular and plasma nitrite levels only in animals treated with atorvastatin. While this particular result could suggest that increasing tissue nitrite levels is not relevant to improve endothelial dysfunction or to lower blood pressure, we have not compared long term effects of both drugs in this hypertension model. For example, although we have not examined vascular hypertrophy or remodeling in the present study, it is possible that increased vascular nitrite levels translate into less vascular remodeling of hypertension, independent of drug-induced antihypertensive effects. This suggestion is supported by recent findings showing that nitrite promotes NO generation in vessels and in smooth muscle cells, and decreases cell proliferation [40]. Further studies are required to examine whether statin-induced increases in vascular nitrite concentrations really attenuate vascular growth in hypertension models and in patients. Moreover, both atorvastatin and sildenafil showed antioxidant effects, and therefore it is probable that the increases in vascular nitrite concentrations do not necessarily reflect antioxidant effects.

Previous studies reported that combining sildenafil and atorvastatin improves vascular responses more than each drug alone [18–20]. While our present results do not support this suggestion, it is possible that differences between animal models may explain the lack of interaction reported here. While the doses used in the present study were based on previous studies [21,22], it is possible that maximum effects have been achieved with each drug, thus preventing us to detect significant interactions between treatments.

In conclusion, we demonstrate that atorvastatin and sildenafil exert antioxidant effects, improve endothelial function, and lower blood pressure in 2K1C hypertension. While both drugs showed similar effects, only atorvastatin increased vascular NO stores. This effect may translate into improved long-term protective effects against cardiovascular events in hypertension.

References

[1] S. Moncada, A. Higgs, The L-arginine-nitric oxide pathway, N. Engl. J. Med. 329 (1993) 2002–2012.

[2] G.D. Thomas, W. Zhang, R.G. Victor, Nitric oxide deficiency as a cause of clinical hypertension: promising new drug targets for refractory hypertension, J. Am. Med. Assoc. 285 (2001) 2055–2057.

[3] L. Ghiadoni, S. Taddei, A. Virdis, Hypertension and endothelial dysfunction: therapeutic approach, Curr. Vasc. Pharmacol. 10 (2012) 42–60.

[4] M.T. Gladwin, A.N. Schechter, D.B. Kim-Shapiro, R.P. Patel, N. Hogg, S. Shiva, R.O. Cannon 3rd, M. Kelm, D.A. Wink, M.G. Espey, E.H. Oldfield, R.M. Pluta, B.A. Freeman, J.R. Lancaster Jr., M. Feelisch, J.O. Lundberg, The emerging biology of the nitrite anion, Nat. Chem. Biol. 1 (2005) 308–314.

[5] J.O. Lundberg, M.T. Gladwin, A. Ahluwalia, N. Benjamin, N.S. Bryan, A. Butler, P. Cabrales, A. Fago, M. Feelisch, P.C. Ford, B.A. Freeman, M. Frenneaux, J. Friedman, M. Kelm, C.G. Kevil, D.B. Kim-Shapiro, A.V. Kozlov, J.R. Lancaster Jr, D.J. Lefer, K. McColl, K. McCurry, R.P. Patel, J. Petersson, T. Rassaf, V.P. Reutov, G.B. Richter-Addo, A. Schechter, S. Shiva, K. Tsuchiya, E.E. van Faassen, A.J. Webb, B.S. Zuckerbraun, J.L. Zweier, E. Weitzberg, Nitrate and nitrite in biology, nutrition and therapeutics, Nat. Chem. Biol. 5 (2009) 865–869.

[6] K. Cosby, K.S. Partovi, J.H. Crawford, R.P. Patel, C.D. Reiter, S. Martyr, B.K. Yang, M.A. Waclawiw, G. Zalos, X. Xu, K.T. Huang, H. Shields, D.B. Kim-Shapiro, A.N. Schechter, R.O. Cannon 3rd, M.T. Gladwin, Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation, Nat. Med. 9 (2003) 1498–1505.

[7] H. Li, H. Cui, T.K. Kundu, W. Alzawahra, J.L. Zweier, Nitric oxide production from nitrite occurs primarily in tissues not in the blood: critical role of xanthine oxidase and aldehyde oxidase, J. Biol. Chem. 283 (2008) 17855–17863.

[8] S. Shiva, Nitrite: a physiological store of nitric oxide and modulator of mitochondrial function, Redox Biol. 1 (2013) 40–44.

[9] R.M. Touyz, A.M. Briones, Reactive oxygen species and vascular biology: implications in human hypertension, Hypertens. Res. 34 (2011) 5–14.

[10] H. Cai, K.K. Griendling, D.G. Harrison, The vascular NAD(P)H oxidases as therapeutic targets in cardiovascular diseases, Trends Pharmacol. Sci. 24 (2003) 471–478.

[11] M.M. Castro, E. Rizzi, L. Figueiredo-Lopes, K. Fernandes, L.M. Bendhack, D.L. Pitol, R.F. Gerlach, J.E. Tanus-Santos, Metalloproteinase inhibition ameliorates hypertension and prevents vascular dysfunction and remodeling in renovascular hypertensive rats, Atherosclerosis 198 (2008) 320–331.

[12] M.M. Castro, E. Rizzi, G.J. Rodrigues, C.S. Ceron, L.M. Bendhack, R.F. Gerlach, J.E. Tanus-Santos, Antioxidant treatment reduces matrix metalloproteinase-2-induced vascular changes in renovascular hypertension, Free Radic. Biol. Med. 46 (2009) 1298–1307.

[13] M. Endres, U. Laufs, Effects of statins on endothelium and signaling mechanisms, Stroke 35 (2004) 2708–2711.

[14] J. Davignon, Beneficial cardiovascular pleiotropic effects of statins, Circulation (2004)III39–III43.

[15] C. Vlachopoulos, N. Ioakeimidis, K. Rokkas, C. Stefanadis, Cardiovascular effects of phosphodiesterase type 5 inhibitors, J. Sex. Med. 6 (2009) 658–674.

[16] C.A. Dias-Junior, D.C. Souza-Costa, T. Zerbini, J.B. da Rocha, R.F. Gerlach, J.E. Tanus-Santos, The effect of sildenafil on pulmonary embolism-induced oxidative stress and pulmonary hypertension, Anesth. Analg. 101 (2005) 115–120.

[17] J.E. Tanus-Santos, Antioxidant effects of phosphodiesterase-5 inhibitors, Cardiovasc. Res. 100 (2013) 170.

[18] Q.M. Wang, Y. Wei, Y. Zheng, C. Waeber, Efficacy of combined atorvastatin and sildenafil in promoting recovery after ischemic stroke in mice, Am. J. Phys. Med. Rehabil. 92 (2013) 143–150.

[19] M.M. Castro, E. Rizzi, R.R. Rascado, S. Nagassaki, L.M. Bendhack, J.E. Tanus-Santos, Atorvastatin enhances sildenafil-induced vasodilation through nitric oxide-mediated mechanisms, Eur. J. Pharmacol. 498 (2004) 189–194.

[20] E.M. Neto-Neves, C.A. Dias-Junior, J.A. Uzuelli, R.P. Pereira, F. Spiller, P.G. Czaikoski, J.E. Tanus-Santos, Sildenafil improves the beneficial hemodynamic effects exerted by atorvastatin during acute pulmonary thromboembolism, Eur. J. Pharmacol. 670 (2011) 554–560.

[21] S. Wassmann, U. Laufs, A.T. Baumer, K. Muller, K. Ahlbory, W. Linz, G. Itter, R. Rosen, M. Bohm, G. Nickenig, HMG-CoA reductase inhibitors improve endothelial dysfunction in normocholesterolemic hypertension via reduced production of reactive oxygen species, Hypertension 37 (2001) 1450–1457.

[22] S.E. Ferreira-Melo, J.C. Yugar-Toledo, O.R. Coelho, I.M. De Luca, J.E. Tanus-Santos, S. Hyslop, M.C. Irigoyen, H. Moreno Jr., Sildenafil reduces cardiovascular remodeling associated with hypertensive cardiomyopathy in NOS inhibitor-treated rats, Eur. J. Pharmacol. 542 (2006) 141–147.

[23] M. Feelisch, T. Rassaf, S. Mnaimneh, N. Singh, N.S. Bryan, D. Jourd'Heuil, M. Kelm, Concomitant S-, N-, and heme-nitros(yl)ation in biological tissues and fluids: implications for the fate of NO *in vivo*, FASEB J. 16 (2002) 1775–1785.

[24] N.S. Bryan, T. Rassaf, R.E. Maloney, C.M. Rodriguez, F. Saijo, J.R. Rodriguez, M. Feelisch, Cellular targets and mechanisms of nitros(yl)ation: an insight into their nature and kinetics *in vivo*, Proc. Natl. Acad. Sci. USA 101 (2004) 4308–4313.

[25] S.B. Cau, D.A. Guimaraes, E. Rizzi, C.S. Ceron, L.L. Souza, C.R. Tirapelli, R.F. Gerlach, J.E. Tanus-Santos, Pyrrolidine dithiocarbamate down-regulates vascular matrix metalloproteinases and ameliorates vascular dysfunction and remodelling in renovascular hypertension, Br. J. Pharmacol. 164 (2011) 372–381.

[26] C.S. Ceron, M.M. Castro, E. Rizzi, M.F. Montenegro, V. Fontana, M.C. Salgado, R.F. Gerlach, J.E. Tanus-Santos, Spironolactone and hydrochlorothiazide exert antioxidant effects and reduce vascular matrix metalloproteinase-2 activity and expression in a model of renovascular hypertension, Br. J. Pharmacol. 160 (2010) 77–87.

[27] M.F. Montenegro, L.C. Pinheiro, J.H. Amaral, D.M. Marcal, A.C. Palei, A.J. Costa-Filho, J.E. Tanus-Santos, Antihypertensive and antioxidant effects of a single daily dose of sodium nitrite in a model of renovascular hypertension, Naunyn Schmiedebergs Arch. Pharmacol. 385 (2012) 509–517.

[28] C.M. Prado, M.A. Rossi, Circumferential wall tension due to hypertension plays a pivotal role in aorta remodelling, Int. J. Exp. Pathol. 87 (2006) 425–436.

[29] D.G. Harrison, M.C. Gongora, T.J. Guzik, J. Widder, Oxidative stress and hypertension, J. Am. Soc. Hypertens. 1 (2007) 30–44.

[30] O. Jung, J.G. Schreiber, H. Geiger, T. Pedrazzini, R. Busse, R.P. Brandes, gp91phox-containing NADPH oxidase mediates endothelial dysfunction in renovascular hypertension, Circulation 109 (2004) 1795–1801.

[31] K. Yaguas, R. Bautista, Y. Quiroz, A. Ferrebuz, H. Pons, M. Franco, N.D. Vaziri, B. Rodriguez-Iturbe, Chronic sildenafil treatment corrects endothelial dysfunction and improves hypertension, Am. J. Nephrol. 31 (2010) 283–291.

[32] M.T. Gladwin, N.J. Raat, S. Shiva, C. Dezfulian, N. Hogg, D.B. Kim-Shapiro, R.P. Patel, Nitrite as a vascular endocrine nitric oxide reservoir that contributes to hypoxic signaling, cytoprotection, and vasodilation, Am. J. Physiol. Heart Circ. Physiol. 291 (2006) H2026–H2035.
[33] M.T. Gladwin, J.H. Crawford, R.P. Patel, The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation, Free Radic. Biol. Med. 36 (2004) 707–717.
[34] P. Kleinbongard, A. Dejam, T. Lauer, T. Jax, S. Kerber, P. Gharini, J. Balzer, R.B. Zotz, R.E. Scharf, R. Willers, A.N. Schechter, M. Feelisch, M. Kelm, Plasma nitrite concentrations reflect the degree of endothelial dysfunction in humans, Free Radic. Biol. Med. 40 (2006) 295–302.
[35] J.L. Zweier, H. Li, A. Samouilov, X. Liu, Mechanisms of nitrite reduction to nitric oxide in the heart and vessel wall, Nitric Oxide 22 (2010) 83–90.
[36] A. Martins-Oliveira, M.M. Castro, D.M. Oliveira, E. Rizzi, C.S. Ceron, D. Guimaraes, R.I. Reis, C.M. Costa-Neto, D.E. Casarini, A.A. Ribeiro, R.F. Gerlach, J.E. Tanus-Santos, Contrasting effects of aliskiren *versus* losartan on hypertensive vascular remodeling, Int. J. Cardiol. 167 (2013) 1199–1205.
[37] C.S. Ceron, E. Rizzi, D.A. Guimaraes, A. Martins-Oliveira, S.B. Cau, J. Ramos, R.F. Gerlach, J.E. Tanus-Santos, Time course involvement of matrix metalloproteinases in the vascular alterations of renovascular hypertension, Matrix Biol. 31 (2012) 261–270.
[38] M.F. Montenegro, J.H. Amaral, L.C. Pinheiro, E.K. Sakamoto, G.C. Ferreira, R.I. Reis, D.M. Marcal, R.P. Pereira, J.E. Tanus-Santos, Sodium nitrite down-regulates vascular NADPH oxidase and exerts antihypertensive effects in hypertension, Free Radic. Biol. Med. 51 (2011) 144–152.
[39] M.F. Montenegro, E.M. Neto-Neves, C.A. Dias-Junior, C.S. Ceron, M.M. Castro, V.A. Gomes, A. Kanashiro, J.E. Tanus-Santos, Quercetin restores plasma nitrite and nitroso species levels in renovascular hypertension, Naunyn Schmiedebergs Arch. Pharmacol. 382 (2010) 293–301.
[40] M.J. Alef, R. Vallabhaneni, E. Carchman, S.M. Morris Jr, S. Shiva, Y. Wang, E.E. Kelley, M.M. Tarpey, M.T. Gladwin, E. Tzeng, B.S. Zuckerbraun, N.O. Nitrite-generated, circumvents dysregulated arginine/NOS signaling to protect against intimal hyperplasia in Sprague-Dawley rats, J. Clin. Invest. 121 (2011) 1646–1656.