Atorvastatin and sildenafil decrease vascular TGF-β levels and MMP-2 activity and ameliorate arterial remodeling in a model of renovascular hypertension

Danielle A. Guimarães a, Elen Rizzi a, Carla S. Ceron a, Alisson Martins-Oliveira a, Raquel F. Gerlach b, Sruti Shiva c, Jose E. Tanus-Santos a,⁎

a Department of Pharmacology, Ribeirao Preto Medical School, University of Sao Paulo, Brazil
b Department of Morphology, Estomatology and Physiology, Dental School of Ribeirao Preto, University of Sao Paulo, Brazil
c Vascular Medicine Institute, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA

A R T I C L E   I N F O
Article history:
Received 7 August 2015
Received in revised form 19 August 2015
Accepted 28 August 2015
Available online 31 August 2015

Keywords:
Atorvastatin
Hypertension
Matrix metalloproteinase
Sildenafil

A B S T R A C T
Imbalanced matrix metalloproteinase (MMP)-2 activity and transforming growth factor expression (TGF-β) are involved in vascular remodeling of hypertension. Atorvastatin and sildenafil exert antioxidant and pleiotropic effects that may result in cardiovascular protection. We hypothesized that atorvastatin and sildenafil alone or in association exert antiproliferative effects by down-regulating MMP-2 and TGF-β, thus reducing the vascular hypertrophy induced by two kidney, one clip (2K1C) hypertension.

Sham and 2K1C rats were treated with oral atorvastatin 50 mg/kg, sildenafil 45 mg/kg, or both, daily for 8 weeks. Blood pressure was monitored weekly. Morphologic changes in the aortas were studied. TGF-β levels were determined by immunofluorescence. MMP-2 activity and expression were determined by in situ zymography, gel zymography, Western blotting, and immunofluorescence. The effects of both drugs on proliferative responses of aortic smooth muscle cells to PDGF and on on MMP-2 activity in vitro were determined. Atorvastatin, sildenafil, or both drugs exerted antiproliferative effects in vitro. All treatments attenuated 2K1C-induced hypertension and prevented the increases in the aortic cross-sectional area and media/lumen ratio in 2K1C rats. Aortas from 2K1C rats showed higher collagen deposition, TGF-β levels and MMP-2 activity and expression when compared with Sham-operated animals. Treatment with atorvastatin and/or sildenafil was associated with attenuation of 2K1C hypertension-induced increases in these pro-fibrotic factors. However, these drugs had no in vitro effects on hr-MMP-2 activity.

Atorvastatin and sildenafil was associated with decreased vascular TGF-β levels and MMP-2 activity in renovascular hypertensive rats, thus ameliorating the vascular remodeling. These novel pleiotropic effects of both drugs may translate into protective effects in patients.

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1. Introduction

Vascular remodeling is critically involved in the pathogenesis of hypertension and is initiated by smooth muscle cell proliferation and migration that result in vascular hyperplasia and hypertrophy [1,2]. Indeed, upregulated MMP activity, especially MMP-2, has been implicated in vascular remodeling in hypertensive patients [3,4] and in animal models of hypertension [5–7]. In addition to its effects on the components of the extracellular matrix, MMP-2 activates latent TGF-β [8] thus further contributing to vascular pathogenetic mechanisms that impair vascular function [9]. Indeed, TGF-β is a major profibrotic factor implicated in extracellular matrix reorganization and collagen deposition during vascular fibrosis [10]. Therefore, drugs that inhibit MMP activity may contribute to TGF-β downregulation and prevent vascular remodeling in hypertension.

Statins inhibit 3-hydroxy-3-methylglutaryl (HMG)-coenzyme A reductase activity, an enzyme that plays a major role in the biosynthesis of cholesterol [11]. However, in the last few decades, growing evidence has accumulated to support the notion that...

http://dx.doi.org/10.1016/j.redox.2015.08.017
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2. Materials and methods

The present study was carried out in accordance with National Institutes of Health (NIH; USA) guidelines. All experimental protocols with animals were approved by our Institutional Animal Care and Use Committee of the Ribeirão Preto Medical School, University of São Paulo.

2.1. Isolation of rat aortic smooth muscle cells

Rat aortic smooth muscle cells (RASMCs) were isolated from Sprague-Dawley rats (6–8 weeks of age) as previously described [23]. The rats were anesthetized with an intraperitoneal injection of pentobarbital. The aorta was isolated and immersed in 20% fetal bovine serum-Dulbecco’s modified Eagle’s medium (DMEM) containing 1000 U/mL heparin. Fat and connective tissue were removed and the aorta was incubated in DMEM (serum-free) with collagenase type II (2 mg/mL) for 45 min at 37 °C. After removal of the endothelium, the vessel was cut lengthwise, and the smooth muscle cells were removed mechanically.

2.2. Experiments with rat aortic smooth muscle cells to examine antiproliferative effects of atorvastatin or sildenafil in the presence of platelet-derived growth factor

RASMCs were examined by microscopy and used between passages 5 and 8. Approximately 20,000 cells/well were plated in 24-well plates with 1 mL DMEM and F-12 HAM’S medium with 10% fetal bovine serum. The RASMCs were pre-treated with vehicle (Control, untreated cells), atorvastatin (0.1, 1, or 10 μM) and sildenafil (0.1, 1, or 10 μM) for 1 h and stimulated with platelet-derived growth factor-BB (PDGF-BB) 1 ng/mL for 24 h [24].

2.3. Assessment of cell proliferation

Growth-arrested RASMCs (serum starvation for 24 h) were subject to experimental conditions in the presence of 5 μCi/mL 3H-thymidine (NESS) for 24 h. 3H-thymidine incorporation into trichloroacetic acid-precipitated DNA was quantified by scintillation spectrometry as described [24].

2.4. Renovascular (2K1C) hypertension animal model and treatments

Male Wistar rats weighing from 180 to 200 g were maintained under the animal house conditions (12 h light/dark cycle at 25 °C), and allowed free access to rat chow and water. Under anesthesia (ketamine 100 mg/kg and xylazine 10 mg/kg i.p.), 2K1C hypertension was performed by placing 0.2 mm silver

statins exert beneficial cardiovascular effects independent of their effects on cholesterol levels [12]. Similarly, phosphodiesterase-5 inhibitors, particularly sildenafil, which are usually prescribed to patients with erectile dysfunction or with pulmonary hypertension, have also been shown to exert protective effects that may justify their use in patients with other cardiovascular diseases [13]. In fact, there is evidence that both statins and phosphodiesterase-5 inhibitors may inhibit pro-fibrotic mechanisms, it is not known whether atorvastatin or sildenafil attenuates the vascular remodeling associated with hypertension, or whether the combination of these drugs increases the possible effects associated with each drug alone.

In this study, we aimed at testing the hypothesis that both atorvastatin and sildenafil, inhibit proliferative mechanisms involving MMP-2 and TGF-β expression [18,19]. Interestingly, some studies suggest that the combination of a statin with sildenafil further increases the protective effects of each drug alone [20–22]. However, although there is some evidence that both statins and phosphodiesterase-5 inhibitors may inhibit pro-fibrotic mechanisms, it is not known whether atorvastatin or sildenafil further increases the protective effects of each drug alone.
As normotensive controls, sham-operated rats underwent the same surgical procedure without 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 $\geq 160$ mm Hg two weeks after the surgery.

The 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 $^2$; 2K1C and Sham groups that received sildenafil 45 mg/kg $^1$; and 2K1C and Sham groups that received the combination of atorvastatin 45 mg/kg and sildenafil 50 mg/kg per day. The treatments were started two weeks after 2K1C surgery and were maintained for eight additional weeks. All treatments were given by oral gavage. The animals were killed by decapitation after 10 weeks of hypertension 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 $-70$ $^\circ$C until used for biochemical measurements.

**2.5. Morphometric analysis of the aorta and assessment of aortic collagen content**

The thoracic aortas were carefully removed and cleaned of connective tissue and fat. After that, the aortas were fixed in 4% phosphate-buffered paraformaldehyde (pH 7.4) for 24 h, followed by 70% ethanol (at least 24 h) and then embedded in paraffin. The blocks of paraffin were cut at four micrometer thick slices and stained with hematoxylin and eosin (H&E). The morphometric parameters including media cross-sectional area (CSA) and media to lumen diameter (M/L) were quantified as previously described using ImageJ Program (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2011) $^5$.

Trichrome staining (Gomori) was used to determine the collagen content in the aortic media layer with light microscopy (DMLB; Leica, Bensheim, Germany) and the image was captured at 400 $\times$. These structural analyses in the media were evaluated by two skilled blinded observers. The evaluation of collagen surface was scored qualitatively as absent (0), low (1), moderate (2), or strong (3) in the study groups. Each score reflects changes in
the intensity and extension of staining.

### 2.6. Assessment of TGF-β by immunofluorescence

The aortas were frozen in Tissue-tek O.C.T. compound and 4-μm thick cryostat sections were incubated with antibody against TGF-β1 (polyclonal rabbit anti-TGF-β1; 1:500, ab92486, Abcam, USA) at room temperature in dark humidified chambers for 1 h. Slices were washed 3 times with cold PBS and anti-rabbit rhodamine conjugated secondary antibody (1:200, AP187R, Millipore, USA) was added for 1 h. Immunofluorescence images were viewed with a fluorescent microscope (Leica Imaging Systems Ltd., Cambridge, England) and the images were captured at 400×. Red fluorescence intensity was evaluated by using ImageJ Program (National Institutes of Health) in 40 fields selected around the vessel circumference (interassay coefficient of variation less than 3%), and the arithmetic of 40 fields was calculated for each slide [26].

### 2.7. Measurement of aortic MMP-2 levels by gelatin zymography

Gelatin zymography was performed as previously described [27]. Frozen aortic tissue samples (approximately 30 mg) were homogenized with cold RIPA-buffer on ice. The protein concentration in the supernatant was performed with Bradford protein assay. Tissue extracts diluted 1:1 with 2× sample buffer were subjected to electrophoresis on 7% SDS-PAGE co-polymerized with gelatin (0.1%). After electrophoresis, the gels were soaked in a 2% Triton X-100 solution for 30 min twice at room temperature. Then, the gels were incubated in Tris–HCl buffer (10 mmol L⁻¹ CaCl₂, pH 7.4) overnight, at 37 °C. The staining was carried out for 3 h with Coomassie Brilliant Blue G-250 (0.05%) and destained with 25% methanol and 7% acetic acid for 2 h. Gelatinolytic activity was detected as unstained bands against the blue background of stained gelatin, and quantified by densitometry using a Kodak Electrophoresis Documentation and Analysis System (EDAS) 290 (Kodak, Rochester, NY). Intergel analysis was possible after normalization of the gelatinolytic activity with an internal standard (fetal bovine serum).

### 2.8. Assessment of aortic gelatinolytic activity by in situ zymography and aortic MMP-2 levels by immunofluorescence

In situ gelatinolytic activity in the media of frozen thoracic
The aorta was performed as previously described [28]. Frozen 4 μm sections were incubated with dye-quenched (DQ) Gelatin (E12055, Molecular Probes, Oregon 411, USA) diluted 1:20 for 30 min in dark humidified chambers. The images were examined with fluorescent microscopy (Leica Imaging Systems Ltd., Cambridge, England) and captured at 400×. The intensity of the green fluorescent signal was evaluated by using ImageJ Program (NIH – National Institute of Health).

To co-localized aortic gelatinolytic activity with MMP-2 expression immuno-fluorescence for MMP-2 was performed. After DQ gelatin, the sections were rinsed 3× with cold PBS and incubated with mouse monoclonal MMP-2 antibody (1:500; MAB3308, Millipore, USA) for 1 h. Slices were then incubated with anti-mouse rhodamine conjugated secondary antibody (1:200, AP181R, Millipore, USA) Sections were examined with fluorescent microscopy (Leica Imaging Systems Ltd., Cambridge, England) and the image was captured at 400×. The intensity of the red fluorescent signal was evaluated by using ImageJ Program [28].

2.9. Assessment of aortic MMP-2 expression by Western blot analysis

Aortic samples of about 30 mg were lysed with cold RIPA-buffer. The protein concentration of tissue homogenate was determined with Bradford protein assay. 40 μg of protein extracts were separated by 12% polyacrylamide gels. Then, the proteins were transferred to nitrocellulose membranes (GE Healthcare, Madison, WI, USA). The membranes were blocked for 1 h at room temperature with TBS-T (NaCl 100 mM; Tris–Cl 100 mM; Tween 0.1%) containing 5% BSA (bovine serum albumin) and incubated overnight at 4 °C with primary antibody against MMP-2 (1:1000; MAB3308, Millipore, USA). The membranes were then incubated with 1:1000 horseradish peroxidase (HRP)-secondary goat anti-
rabbit antibody (AP132P, Millipore, USA) for 1 h. The protein bands were revealed with ECL chemiluminescence kit (GE Healthcare). MMP-2 expression was normalized with respect to β-actin expression (1:1000; MAB1501, Millipore, USA).

2.10. Assessment of direct in vitro effects of atorvastatin or sildenafil on MMP-2 activity

To examine whether atorvastatin, sildenafil or both drugs (at concentrations varying from 0.1 to 10 μM) inhibit MMP-2 activity in vitro, we measured the direct effects of these drugs on recombinant human MMP-2 (rhMMP-2) activity obtained as previously described [29]. Proteolytic activity of 300 ng of rhMMP-2 was measured in absence or presence of these drugs by using the Gelatinolytic Activity Kit (E12055, Molecular Probes, OR, USA) in a microplate spectrofluorometer (at λexcitation 495, λemission 515 nm; Gemini EM, Molecular Devices, Sunnyvale, CA, USA) after 30 min of incubation at 37 °C, as previously described [28]. Phenanthroline (0.1 mM) was used as a positive control for MMP-2 activity inhibition.

2.11. Statistical analysis

Results are expressed as means ± S.E.M. Comparisons between groups were assessed by two-way analysis of variance (ANOVA) followed by the Bonferroni correction and by the Kruskal–Wallis test or t test as appropriate using GraphPad Prism software. A probability value < 0.05 was considered significant.

3. Results

3.1. Both atorvastatin and sildenafil inhibit PDGF-BB-stimulated

![Fig. 6. Representative sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gelatin zymogram of aortic samples (Panel (a)). Molecular weights of MMP-2 bands (75, 72 and 64 kDa MMP-2) were identified after electrophoresis on 7% SDS–PAGE. STD: internal standard. Panel (b) shows the quantification of each molecular weight form (75, 72 and 64 kDa) of MMP-2 in the aortic extracts. Data are expressed as mean ± S.E.M. *P < 0.05 versus Sham Vehicle group; **P < 0.05 versus 2K1C Vehicle group. (Sham + Vehicle: n = 18; Sham + ATORVA: n = 9; Sham + SILD: n = 9; Sham + ATORVA+SILD: n = 9; 2K1C + vehicle: n = 18; 2K1C + ATORVA: n = 10; 2K1C + SILD: n = 9; 2K1C + ATORVA+SILD: n = 10).](image-url)
RASMCs proliferation

PDGF-BB is a major inducer of smooth muscle cell proliferation and migration and plays an important role in the pathogenesis of vascular diseases including hypertension [30]. The RASMCs were stimulated with PDGF-BB for 24 h and both atorvastatin (1 and 10 μM) and sildenafil (0.1, 1 and 10 μM) significantly inhibited PDGF-BB-induced cell proliferation (all P < 0.05; Fig. 1).

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

We found similar baseline SBP in all groups and no significant changes in SBP were seen in the groups of Sham-operated animals treated with Vehicle or with the drugs (Fig. 2A; P > 0.05). While SBP increased progressively in 2K1C rats treated with vehicle (207 ± 4 mm Hg after 10 weeks), treatment with atorvastatin, sildenafil, or both drugs exerted similar antihypertensive effects (SBP = 148 ± 7, 156 ± 5, and 138 ± 4 mm Hg, respectively; P < 0.05, Fig. 2A).

No significant differences were found with respect to body weight gain (P > 0.05; Fig. 2B).

3.3. Treatment with atorvastatin or sildenafil, or the combination of drugs, ameliorated the vascular remodeling in 2K1C rats

2K1C hypertension was associated with vascular hypertrophy as revealed by increased aortic cross-sectional area (CSA) and increased media to lumen (M/L) ratio in hypertensive rats compared to Sham-operated animals (both P < 0.05; Fig. 3A–C). Interestingly, while no effects were found in Sham-operated animals treated with drugs, both atorvastatin or sildenafil, or the combination of drugs blunted the morphological alterations induced by 2K1C hypertension (all P < 0.05; Fig. 3A–C).

In agreement with the morphological results, increased collagen surface area was found in 2K1C hypertensive rats treated with vehicle as compared with Sham-operated rats (P < 0.05; Fig. 4A and B). Again, while no effects were found in Sham-operated animals treated with drugs, both atorvastatin or sildenafil, or the combination of drugs blunted the increases in collagen surface area induced by 2K1C hypertension (all P < 0.05; Fig. 4A and B).

3.4. Both atorvastatin or sildenafil, or the combination of drugs, blunted the increases in aortic expression of TGF-β induced by hypertension

Hypertension increased the aortic expression of TGF-β in 2K1C rats treated with vehicle compared with Sham-operated rats (P < 0.05; Fig. 5A and B). Again, while no significant differences were found in Sham-operated animals treated with drugs, both atorvastatin or sildenafil, or the combination of drugs blunted the increases in aortic TGF-β content induced by 2K1C hypertension (all P < 0.05; Fig. 5A and B).

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**Fig. 7.** Effects of treatments on in situ gelatinolytic activity and MMP-2 levels detected by immunofluorescence in the aortas. Panel (a) shows representative photographs of in situ gelatinolytic activity (400 ×), MMP-2 detected by immunofluorescence, and their co-localization (merge) in the aortas. Panel (b) shows the quantification of aortic in situ gelatinolytic activity detected as bright green fluorescence. Panel (c) shows the quantification of aortic MMP-2 levels detected by immunofluorescence as bright red fluorescence. Data are expressed as mean ± S.E.M. *P < 0.05 versus Sham Vehicle group; **P < 0.05 versus 2K1C Vehicle group. (Sham + Vehicle: n = 6; Sham + ATORVA: n = 7; Sham + SILD: n = 8; Sham + ATORVA + SILD: n = 7; 2K1C + vehicle: n = 5; 2K1C + ATORVA: n = 7; 2K1C + SILD: n = 8; 2K1C + ATORVA + SILD: n = 8).
2K1C hypertension increased the aortic levels of the 3 isoforms of MMP-2 when compared with Sham-operated animals \((P<0.05; \text{Fig. 7A and B})\). No significant effects on arterial remodeling were observed in rats treated with either atorvastatin or sildenafil, nor the combination, either alone or in combination, attenuated PDGF-BB-induced arterial remodeling. Supporting the antiproliferative effects reported here, both atorvastatin and sildenafil inhibited vascular smooth muscle cells proliferation stimulated under other conditions, possibly as a result of interference with regulation of cell cycle [31,32]. Importantly, while previous studies have already shown that both atorvastatin [33] or sildenafil [17,34] may revert cardiac remodeling, this is the first study to show that both drugs exert beneficial, pleiotropic effects in hypertension.

Our findings showed clear antiproliferative effects of both atorvastatin and sildenafil, which attenuated PDGF-BB-induced RASMCs proliferation in vitro and blunted hypertension-induced media hypertrophy. Supporting the antiproliferative effects reported here, both atorvastatin and sildenafil inhibited vascular smooth muscle cells proliferation stimulated under other conditions, possibly as a result of interference with regulation of cell cycle [31,32]. Importantly, while previous studies have already shown that both atorvastatin [33] or sildenafil [17,34] may revert cardiac remodeling, this is the first study to show that both drugs blunt the vascular remodeling induced by renovascular hypertension.

The 2K1C hypertension model critically depends on the activation of the renin–angiotensin system, with increased angiotensin-converting enzyme activity and angiotensin II levels, which promotes oxidative stress and proliferative mechanisms leading to arterial remodeling [35]. In agreement with the present results, previous studies have notably implicated enhanced vascular MMP-2 activity and TGF-β levels as major players in the collagen deposition and other proliferative and profibrotic alterations associated with vascular remodeling of 2K1C hypertension [6,7,26]. In fact, MMP-2 activates TGF-β, which interacts with its receptors to promote classic profibrotic pathways that lead to increased synthesis of collagen and other components of the extracellular matrix [8,10]. Moreover, angiotensin II, which is implicated in the pathogenesis of 2K1C hypertension, stimulates TGF-β expression and activity in vascular smooth muscle cells [36]. Then, angiotensin II-induced vascular remodeling depends partly on TGF-β stimulation. Although unproven, the improvement of the vascular

3.5. Treatment with atorvastatin or sildenafil, or the combination of both, decreased aortic MMP-2 levels and activity and reduced in situ aortic gelatinolytic activity

A representative zymogram of aortic extracts displaying bands corresponding to the three usual molecular weights of MMP-2 (75 kDa, 72 kDa, and 64 kDa) is shown in Fig. 6A. As expected, 2K1C hypertension increased the aortic levels of the 3 isoforms of MMP-2 when compared with Sham-operated animals treated with vehicle (all \(P<0.05; \text{Fig. 6A and B})\). While the drugs exerted no effects in Sham-operated animals, both atorvastatin and sildenafil, alone or in combination, attenuated 2K1C hypertension-induced increases in aortic 72 kDa and 64 kDa MMP-2 levels (all \(P<0.05; \text{Fig. 6A and B})\). No significant effects were found on the aortic levels of the 75 kDa MMP-2 levels.

To confirm aortic zymogram findings, we assessed aortic MMP-2 levels by immunofluorescence and in situ gelatinolytic aortic activity. Hypertension increased aortic in situ gelatinolytic activity, as revealed by increased green fluorescence in the media layer in the 2K1C+Vehicle group compared with Sham-operated animals \((P<0.05; \text{Fig. 7A and B})\), and the increased in situ gelatinolytic activity co-localized with increased aortic MMP-2 expression assessed by immunofluorescence \((P<0.05; \text{Fig. 7A and C})\). Interestingly, both atorvastatin or sildenafil, or the combination, blunted both increases in aortic in situ gelatinolytic activity and MMP-2 levels indeed by hypertension (all \(P<0.05; \text{Fig. 7A–C})\).

To further confirm these findings, we assessed aortic MMP-2 expression by Western blot analysis. In agreement with the results reported above, 2K1C hypertension increased aortic MMP-2 expression more than three-fold \((P<0.05; \text{Fig. 8A and B})\), and treatment with atorvastatin or sildenafil, or both, significantly attenuated hypertension-induced increases in aortic MMP-2 expression (all \(P<0.05; \text{Fig. 8A and B})\).

3.6. Lack of direct inhibitory effects of atorvastatin or sildenafil on in vitro MMP-2 activity

Given the positive results shown above, we examined the possibility that atorvastatin or sildenafil could directly inhibit MMP-2. While phenanthroline (non-selective Zn \(^{2+}\) chelating MMP inhibitor) inhibited rhMMP-2 activity by more than 80%, neither atorvastatin, nor sildenafil, either alone of combined, affected MMP-2 activity \((P>0.05; \text{Fig. 9})\).

4. Discussion

The present study shows that both atorvastatin or sildenafil inhibit proliferative mechanisms and therefore attenuate the vascular remodeling associated with hypertension. To our knowledge, this is the first study to show that atorvastatin or sildenafil prevent aortic hypertrophy caused by 2K1C hypertension, and that this effect possibly involves lowered expression and activity of TGF-β and MMP-2. Our findings may suggest a mechanism by which these drugs exert beneficial, pleiotropic effects in hypertension.

Our findings showed clear antiproliferative effects of both atorvastatin and sildenafil, which attenuated PDGF-BB-induced RASMCs proliferation in vitro and blunted hypertension-induced media hypertrophy. Supporting the antiproliferative effects reported here, both atorvastatin and sildenafil inhibited vascular smooth muscle cells proliferation stimulated under other conditions, possibly as a result of interference with regulation of cell cycle [31,32]. Importantly, while previous studies have already shown that both atorvastatin [33] or sildenafil [17,34] may revert cardiac remodeling, this is the first study to show that both drugs blunt the vascular remodeling induced by renovascular hypertension.
alterations after treatments with atorvastatin or sildenafil shown in the present study probably results of inhibition of proinflammatory pathways, as suggested by the lower TGF-β levels that we found in the aortas from hypertensive rats treated with atorvastatin or sildenafil. The lower aortic TGF-β levels, in turn, may reflect decreased MMP-2 expression and activity with both drugs [8, 10]. This suggestion is supported by previous studies showing that atorvastatin blunts the increases in TGF-β expression in response to angiotensin II infusion in rats [16]. Moreover, atorvastatin prevents collagen secretion by smooth muscle cells [37, 38]. Similarly, sildenafil reduced TGF-β expression in the DOCA-salt hypertension model, thus allowing regression of renal injury [19]. Activated MMPs proteolyze a variety of extracellular matrix components including collagen, and therefore favor pathological vascular remodeling by mechanisms that add to those activated by TGF-β [1, 39]. The increased MMP-2 levels and gelatinolytic activity that we found in 2K1C hypertension confirms previous studies and may reflect increased oxidative stress resulting from enhanced angiotensin II signaling [5, 7, 40, 41]. Because we found in a previous study that both atorvastatin or sildenafil completely blunted 2K1C hypertension-induced oxidative stress under the same conditions of the present study [42], it is possible that antioxidant effects exerted by both drugs may have inhibited hypertension-induced increases in vascular MMP-2 expression and activity, as shown with other antioxidant compounds [28]. Similar antioxidant effects of sildenafil leading to lower MMP activity has been shown under other conditions [18, 20], and the same has also been reported with atorvastatin [14, 15, 43]. Moreover, the inhibition of vascular remodeling associated with lower MMP-2 expression and activity that we found are not explained by direct inhibition of MMP-2 by either atorvastatin or sildenafil, as demonstrated by lack of significant effects of both drugs on rhMMP-2 activity that we found in the present study.

Both atorvastatin and sildenafil upregulate intracellular signaling pathways involved in the vascular nitric oxide (NO) signaling [44]. Atorvastatin increases endothelial NO synthase expression and activity, thus increasing NO production [42, 44], whereas sildenafil inhibits phosphodiesterase-5, increasing cyclic GMP tissue levels [45]. Interestingly, while the combination of both drugs resulted in improved effects in previous studies [20–22], the same was not true in our 2K1C hypertension model and vascular remodeling, and therefore pathways activated by cGMP may not be critically involved in the effects reported here. Alternatively, since atorvastatin and sildenafil activate the same pathway, the effects on intracellular cGMP levels were not amplified when both drugs were associated. Consistent with our findings, both drugs produced similar antiangiogenic effects in other animal models of hypertension [46–48], and in hypertensive patients [49, 50].

Some limitations of the present study should be taken into consideration. The aortas used in the histological analysis in the animal models of hypertension [46, 48] were fixed by simple immersion into the fixative solution, and not after fixation, which could avoid arterial retraction upon excision of the aorats [51]. While arterial retraction tends to increase vascular cross-sectional area as compared to in vivo conditions, this effect is probably not relevant when comparing aortas from treated and untreated animals. Another limitation is that the doses of sildenafil or atorvastatin used in the present study are more than one order of magnitude above those used to treat patients. However, experimental studies in rats usually require such doses given significant differences in many pharmacologic parameters when rats are compared to humans.

In conclusion, treatment with atorvastatin and sildenafil was associated with decreased vascular TGF-β levels and MMP-2 activity in renovascular hypertensive rats and prevention of vascular remodeling. These findings show novel beneficial pleiotropic effects of atorvastatin and sildenafil in a relevant hypertension model and that may translate into clinical protective effects in hypertensive patients. The similarity of biochemical effects associated with both drugs may underlie the lack of sinergism between them.

Acknowledgments

This study was funded by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). The authors thank Sandra de Oliveira Conde for excellent technical assistance.

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