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RESEARCH PAPER

Spironolactone and hydrochlorothiazide exert antioxidant effects and reduce vascular matrix metalloproteinase-2 activity and expression in a model of renovascular hypertension

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Background and purpose: Increased oxidative stress and up-regulation of matrix metalloproteinases (MMPs) may cause structural and functional vascular changes in renovascular hypertension. We examined whether treatment with spironolactone (SPRL), hydrochlorothiazide (HCTZ) or both drugs together modified hypertension-induced changes in arterial blood pressure, aortic remodelling, vascular reactivity, oxidative stress and MMP levels and activity, in a model of renovascular hypertension.
Experimental approach: We used the two-kidney,one-clip (2K1C) model of hypertension in Wistar rats. Sham-operated or hypertensive rats were treated with vehicle, SPRL (25 mg·kg^{-1}·day^{-1}), HCTZ (20 mg·kg^{-1}·day^{-1}) or a combination for 8 weeks. Systolic blood pressure was monitored weekly. Aortic rings were isolated to assess endothelium-dependent and -independent relaxations. Morphometry of the vascular wall was carried out in sections of aorta. Aortic NADPH oxidase activity and superoxide production were evaluated. Formation of reactive oxygen species was measured in plasma as thiobarbituric acid-reactive substances. Aortic MMP-2 levels and activity were determined by gelatin and *in situ* zymography, fluorimetry and immunohistochemistry.
Key results: Treatment with SPRL, HCTZ or the combination attenuated 2K1C-induced hypertension, and reversed the endothelial dysfunction in 2K1C rats. Both drugs or the combination reversed vascular aortic remodelling induced by hypertension, attenuated hypertension-induced increases in oxidative stress and reduced MMP-2 levels and activity.
Conclusions and implications: SPRL or HCTZ, alone or combined, exerted antioxidant effects, and decreased renovascular hypertension-induced MMP-2 up-regulation, thus improving the vascular dysfunction and remodelling found in this model of hypertension.
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Keywords: hydrochlorothiazide; matrix metalloproteinases; reactive oxygen species; renovascular hypertension; spironolactone

Abbreviations: CSA, cross-sectional area; DHE, dihydroethidium; DMEM, Dulbecco's modified Eagle's medium; MDA, malondialdehyde; M/L, media-to-lumen diameter; MMPs, metalloproteinases; MTT, thiazolyl blue tetrazolium bromide; NAD(P)H, nicotinamide adenine dinucleotide phosphate; RASMC, rat aortic smooth muscle cell; ROS, reactive oxygen species; SBP, systolic blood pressure; SNP, sodium nitroprusside; TBARS, thiobarbituric acid-reactive substances; TIMP, tissue inhibitor of metalloproteinases; VSMC, vascular smooth muscle cell; 2K1C, two-kidney, one-clip hypertension

Introduction

Increased oxidative stress results from an imbalance between the production and inactivation of reactive oxygen species (ROS) by endogenous antioxidant systems (Oliveira-Sales *et al.*, 2008), and is involved in many pathophysiological conditions affecting the cardiovascular system, including atherosclerosis and hypertension (Warnholtz *et al.* 1999; Rueckschloss *et al.*, 2003). Of relevance for the present study, hypertension is associated with impaired vascular function and morphological changes in the vasculature, which involve up-regulation of metalloproteinases (MMPs) (Castro *et al.*, 2008; 2009; Wang *et al.*, 2009). These enzymes are zinc-dependent endopeptidases with a major role in the

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remodelling of the extracellular matrix, and can be activated by serine proteases, other MMPs and ROS (Cao *et al.*, 1995; Fu *et al.*, 2008). In fact, increased oxidative stress and enhanced MMP activities have been reported in plasma and in vascular tissues, both in clinical studies and in animal models of hypertension (Bouvet *et al.*, 2005; Martinez *et al.*, 2006; 2008; Schulz, 2007; Castro *et al.*, 2008; Rizzi *et al.*, 2009). Supporting these findings, antioxidants or MMP inhibitors were shown to prevent the impairment of vascular function and vascular remodelling associated with hypertension (Castro *et al.*, 2008; 2009; Wang *et al.*, 2009).

Two-kidney, one-clip hypertension (2K1C) is associated with increased angiotensin II and aldosterone levels. Both mediators produce mitogenic effects critically involved in the development of the structural and functional vascular changes of hypertension including endothelial dysfunction, vascular hypertrophy and deposition of extracellular matrix (Min *et al.*, 2005). These mediators increase vascular superoxide anion production by activating vascular NAD(P)H oxidases, reduce nitric oxide (NO) availability (Lerman *et al.*, 2005; Rude *et al.*, 2005) and up-regulate MMPs (Rude *et al.*, 2005; Johar *et al.*, 2006). Interestingly, these alterations were attenuated by spironolactone (SPRL), an aldosterone antagonist which produced antioxidant effects (Virdis *et al.*, 2002; Nakano *et al.*, 2005; Rude *et al.*, 2005; Sartorio *et al.*, 2007) and reduced MMP activities (Rude *et al.*, 2005). Therefore, it is possible that SPRL can prevent the increases in vascular MMP levels and the vascular dysfunction and remodelling associated with hypertension. In the present study, we examined this possibility.

Hydrochlorothiazide (HCTZ) is widely used in the therapy of hypertension. While this diuretic produces antihypertensive effects that could decrease transmural pressure activation of MMPs (Chesler *et al.*, 1999), it may cause or exacerbate volume depletion, and thus increase angiotensin II and aldosterone levels (Koenig *et al.*, 1991), possibly up-regulating MMPs (Rude *et al.*, 2005; Johar *et al.*, 2006). Therefore, we have also examined whether the combination of HCTZ and SPRL produces antihypertensive effects associated with more complete prevention of 2K1C hypertension-induced increases in vascular MMPs, vascular dysfunction and remodelling (Castro *et al.*, 2008; Martinez *et al.*, 2008) compared with the effects produced by HCTZ alone.

Methods

Animals and treatments

All animal care and experimental procedures in this study complied with the guidelines of the Faculty of Medicine of Ribeirao Preto, University of Sao Paulo, and the principles published by the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male Wistar rats (180–200 g) obtained from the colony at University of São Paulo were maintained on 12 h light/dark cycle at 25°C with free access to rat chow and water.

2K1C hypertension was induced by clipping the left renal artery with a silver clip (0.2 mm) in anaesthetized rats (ketamine 100 $mg \cdot kg^{-1}$ and xylazine 10 $mg \cdot kg^{-1}$ i.p.). Sham-operated rats underwent the same surgical procedure except for the clip placement. Treatment was started 2 weeks after surgery and maintained for 8 weeks. The animals were randomly assigned to one of eight groups: 2K1C and sham groups that received tap water, 2K1C and sham groups that received SPRL 25 $mg \cdot kg^{-1} \cdot day^{-1}$ (Aldactone; Laboratórios Pfizer Ltda, Guarulhos, São Paulo, Brazil), 2K1C and sham groups that received HCTZ 20 $mg \cdot kg^{-1} \cdot day^{-1}$ (Clorana; Sanofi-Synthelabo) and 2K1C and sham groups that received SPRL 25 $mg \cdot kg^{-1} \cdot day^{-1}$ plus HCTZ 20 $mg \cdot kg^{-1} \cdot day^{-1}$. The drugs were given by gavage (Virdis *et al.*, 2002; Pu *et al.*, 2003; Nobre *et al.*, 2006), and distilled water was used as vehicle. Body weight and tail systolic blood pressure (SBP) were assessed weekly by tail-cuff plethysmography. To minimize the effects of stress induced by this method on blood pressure measurement, the animals were trained for a week before surgery. At the end of the experimental period, the animals were anaesthetized and killed by decapitation. For lipid peroxide determination, plasma was prepared by centrifugation of blood. The thoracic aorta was removed and placed in chilled buffer.

Vascular reactivity

Vascular reactivity was assessed in aortic rings as previously described (Castro *et al.*, 2008; Martinez *et al.*, 2008).

Morphometric analysis of the vascular wall

Aortas embedded in paraffin blocks were sliced and stained with haematoxylin and eosin. Media cross-sectional area (CSA), media-to-lumen diameter (M/L) and the number of vascular smooth muscle cells (VSMCs) were quantified as previously described (Dao *et al.*, 2001).

Assessment of vascular ROS and lipid peroxide levels in plasma

Dihydroethidium (DHE) was used to evaluate *in situ* ROS production by fluorescence microscopy as previously described (Hao *et al.*, 2006; Viel *et al.*, 2008). In addition, NADPH-dependent superoxide production was measured in aortic rings by using a luminescence technique as previously described (Janiszewski *et al.*, 2002).

Plasma lipid peroxide levels were determined by measuring thiobarbituric acid-reactive substances (TBARS) using a fluorometric method (Hao *et al.*, 2006; Viel *et al.*, 2008).

In vitro *effects of SPRL and HCTZ on ROS*

To assess possible direct antioxidant effects produced by SPRL (1, 10 and 100 $\mu mol \cdot L^{-1}$) (Xiao *et al.*, 2000; Min *et al.*, 2005; Sonder *et al.*, 2006), HCTZ (1, 10 and 100 $\mu mol \cdot L^{-1}$) (Calder *et al.*, 1993; Zhu *et al.*, 2005; Sladkova *et al.*, 2007) or combination of both drugs (at the same concentrations), we examined whether these drugs affect ROS formation by leucocytes and aortas using a tube chemiluminescence reader. Radical formation by leucocytes and aortic fragments was determined by chemiluminescence using luminol (200 $\mu mol \cdot L^{-1}$) or lucigenin (5 $\mu mol \cdot L^{-1}$), respectively, in 1 mL Hank's solution at 37°C. The leucocytes were stimulated with phorbol 12-myristate 13-acetate (100 $nmol \cdot L^{-1}$), and the aortic fragments were stimulated with menadione (10 $\mu mol \cdot L^{-1}$), an

intracellular ROS generator, as previously described (Heumuller *et al*., 2008). Apocynin 0.06 mmol·L^{-1} was used as positive control to ROS inhibition.

Rat aortic smooth muscle cell line A7r5 (RASMC; from the Rio de Janeiro Cell Bank (UFRJ, Rio de Janeiro, Brazil), generously provided by Dr Jamil Assreuy (UFSC, Santa Catarina, Brazil) were grown in Dulbecco's modified Eagle's medium (DMEM) and incubated with SPRL (1 μmol·L^{-1}) (Jaffe and Mendelsohn, 2005; Jaffe *et al*., 2007), HCTZ (1 μmol·L^{-1}) (Rabkin, 1993; Calder *et al*., 1994) or combination of both drugs (at the same concentrations) in the presence or absence of angiotensin II 0.1 μmol·L^{-1} (Janiszewski *et al*., 2002; Touyz *et al*., 2003) during 24 h. The NADH/NADPH oxidase activity was measured in the cellular homogenates using a luminescence assay as previously described (Zafari *et al*., 1998; Janiszewski *et al*., 2002). Cell viability was assessed after each experiment using thiazolyl blue tetrazolium bromide (MTT).

Measurement of aortic MMP-2 levels by gelatin zymography
Gelatin zymography was performed as previously described (Martinez *et al*., 2006; 2008; Castro *et al*., 2008).

Gelatinolytic activity assay and in situ *zymography*
Net MMP activities in the aortic homogenates and *in situ* MMP activity in media and intimae of frozen thoracic aorta were measured as previously described (Castro *et al*., 2009). Aortic MMP activity was co-localized with aortic MMP-2 expression by immunofluorescence. After DQ gelatin, tissue sections were incubated with MMP-2 primary mouse anti-human monoclonal antibody (MAB3308, Chemicon, Temecula, CA, USA). Sections were examined with fluorescent microscopy (Leica Imaging Systems Ltd, Cambridge, England), and images were captured at a magnification of ×400.

Immunohistochemistry
MMP-2 and tissue inhibitor of metalloproteinase-2 (TIMP-2) levels in thoracic aorta were assessed by immunohistochemistry as previously described (Castro *et al*., 2009).

In vitro *effects of SPRL and HCTZ on MMP-2 activity*
To examine whether SPRL (1, 10 and 100 μmol·L^{-1}) (Xiao *et al*., 2000; Min *et al*., 2005; Sonder *et al*., 2006), HCTZ (1, 10 and 100 μmol·L^{-1}) (Calder *et al*., 1992; 1993; Zhu *et al*., 2005; Sladkova *et al*., 2007) or the combination of the drugs at the same concentrations directly inhibited MMP-2 activity *in vitro*, we measured human recombinant MMP-2 activity (R&D Systems Inc., Minneapolis, MN, USA) in the absence or presence of the drugs as previously described (Castro *et al*., 2009). Phenanthroline (0.05 and 0.1 mmol·L^{-1}) was used as a control for MMP-2 inhibition.

Statistical analysis
Results are expressed as means ± SEM between groups. Comparisons were assessed by two-way or one-way ANOVA followed by Tukey test using GraphPad Prism software. A P value <0.05 was considered significant.

Materials
For the anaesthetic mixture, ketamine was purchased from Agener União (Pouso Alegre, Minas Gerais, Brazil), and xylazine from Laboratory Calier do Brazil (Osasco, São Paulo, Brazil). The PMA, MTT, menadione, apocyanin, DHE, angiotensin II, luminol/lucigenin and TBARS chemicals were purchased from Sigma (St Louis, MO, USA).

Results

SBP levels and body weight
The baseline SBP was similar in the eight experimental groups before surgery, and no significant changes in SBP were seen in sham + vehicle and sham + treatment groups (Figure 1A). SBP increased in the 2K1C + vehicle group after the first week (Figure 1A). All treatments attenuated the increases in SBP in hypertensive rats (final blood pressure (all $P < 0.05$; Figure 1A). Lower SBP levels were found in 2K1C + HCTZ and 2K1C + SPRL + HCTZ groups compared with the 2K1C + SPRL group ($P <$ 0.05; Figure 1A). No significant differences in body weight were observed among the experimental groups (Figure 1B).

Treatment ameliorates vascular dysfunction, and attenuates vascular remodelling in 2K1C rats
Vasorelaxation induced by ACh (10^{-11}–10^{-5} M) and sodium nitroprusside (SNP; 10^{-11}–10^{-6} M) is shown in Figure 1C and D respectively. An impaired response to ACh was observed in 2K1C + vehicle group ($P < 0.05$; Figure 1C), without significant alterations in the responses to SNP (Figure 1D). Treatment with each drug or the combination normalized ACh-induced vasorelaxation in 2K1C aortic rats (all $P < 0.05$; Figure 1C).

Renovascular hypertension was associated with arterial wall hypertrophy, with significant increases in number of VSMCs, increased aortic CSA and increased M/L ratio (Figure 2A,B; all $P < 0.05$). Figure 2A,B shows that each drug or the combination of drugs decreased these alterations. Aortic morphology was not affected by any treatment in sham groups.

Effects of treatments on vascular ROS production and lipid peroxide levels
Higher ROS levels were found in the media of thoracic aorta from the 2K1C + vehicle group when compared with the sham groups ($P < 0.05$; Figure 3A,B). Treatment of 2K1C rats with each drug or with the combination of both drugs blunted 2K1C hypertension-induced increases in oxidative stress ($P < 0.05$; Figure 3A,B).

Higher NAD(P) H oxidase activity was found in the aortas from hypertensive rats ($P < 0.05$; Figure 3C) compared with the sham groups. Treatment with either SPRL or HCTZ significantly inhibited NAD(P)H oxidase activity in aortas of hypertensive rats ($P < 0.05$; Figure 3C). Unfortunately, technical problems precluded us from studying the aortas from

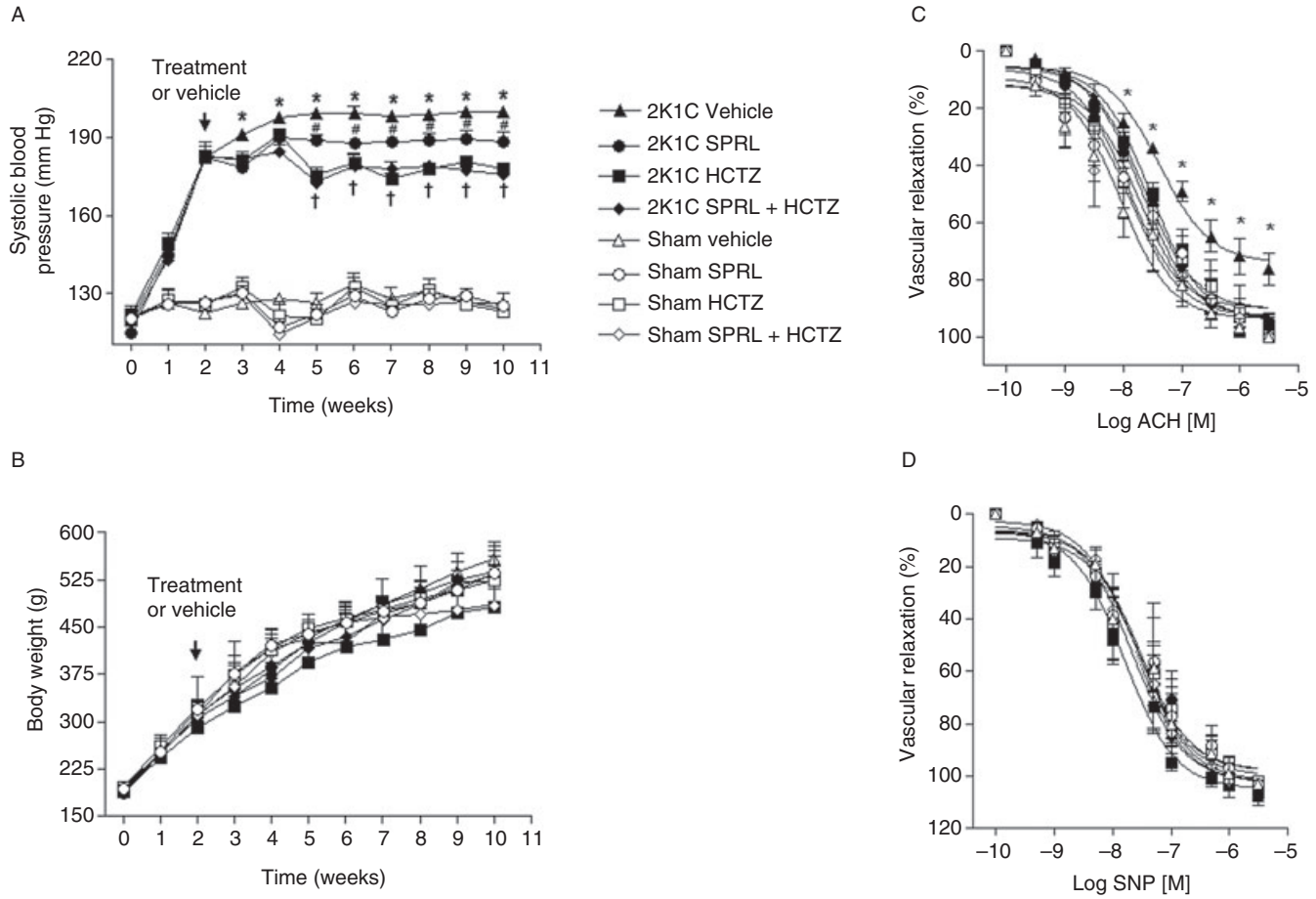

Figure 1 SBP measured by tail-cuff method (A, $n = 12$) and body weight (B, $n = 12$) along the 10 weeks of study. Treatment was with HCTZ, SPRL or a combination of the two drugs. (C and D) Endothelial cell-dependent vasorelaxation induced by ACh ($n = 5$–6/group) and endothelial cell-independent relaxation induced by SNP ($n = 5$–6/group) in rat aortic ring preparations respectively. Values are expressed as mean ± SEM. *$P < 0.05$ for 2K1C + vehicle group versus the four sham-operated groups. #$P < 0.05$ for 2K1C + SPRL group versus the other groups. †$P < 0.05$ for 2K1C + HCTZ and 2K1C + SPRL + HCTZ groups versus the other groups.

the 2K1C + SPRL + HTZ group, and we do not have NAD(P)H oxidase activity results for this treatment group.

The 2K1C + vehicle group presented higher malondialdehyde (MDA) levels, compared with the sham groups ($P < 0.05$; Figure 3D). Treatment of 2K1C rats with SPRL or with the combination of drugs (but not with HCTZ alone) was associated with lower MDA levels compared with those found in the 2K1C + vehicle group ($P < 0.05$; Figure 3D).

In vitro *effects of SPRL and HCTZ on ROS*

In vitro studies showed higher NAD(P)H oxidase activity in the RASMC incubated with angiotensin II ($P < 0.05$; Figure 3E) compared with vehicle. SPRL (1 $\mu mol \cdot L^{-1}$), HCTZ (1 $\mu mol \cdot L^{-1}$) and the combination of both drugs (at the same concentrations) significantly inhibited NAD(P)H oxidase activity in the RASMC ($P < 0.05$; Figure 3E). The cellular viability was 100% in all treatments (data not shown). However, SPRL, HCTZ or their combination produced no such significant antioxidant effects on leucocytes and on the aortas ($P > 0.05$; Figure 3F and G, respectively), except for HCTZ at 100 $\mu mol \cdot L^{-1}$, which produced a small antioxidant effect. Conversely, apocynin produced significant antioxidant effects, both on leucocytes and on the aortas (Figure 3F,G; both $P < 0.05$).

Levels and activity of MMP-2 in hypertensive rats

A representative zymogram of aortic extracts is presented in Figure 4A, which shows the molecular weights of MMP-2 bands. Aortas from 2K1C rats showed higher levels of three MMP-2 forms (75, 72 and 64 kDa) compared with the four sham-operated groups ($P < 0.05$; Figure 4B). Treatment with SPRL (but not with HCTZ or with the combination) attenuated 2K1C hypertension-induced increases in MMP-2 (72 kDa). Treatment with SPRL, HCTZ or with the combination attenuated 2K1C hypertension-induced increases in MMP-2 (64 kDa) and total MMP-2 levels ($P < 0.05$; Figure 4B).

In addition, the *in situ* gelatinolytic MMP activities were measured, and enhanced green fluorescence was found in the endothelium and in the media of thoracic aorta from 2K1C + vehicle group compared with other study groups ($P < 0.05$; Figure 5A,B). The gelatinolytic activity co-localized with aortic MMP-2 expression (Figure 5A) by immunofluorescence. In parallel with the *in situ* gelatinolytic MMP activities, the red

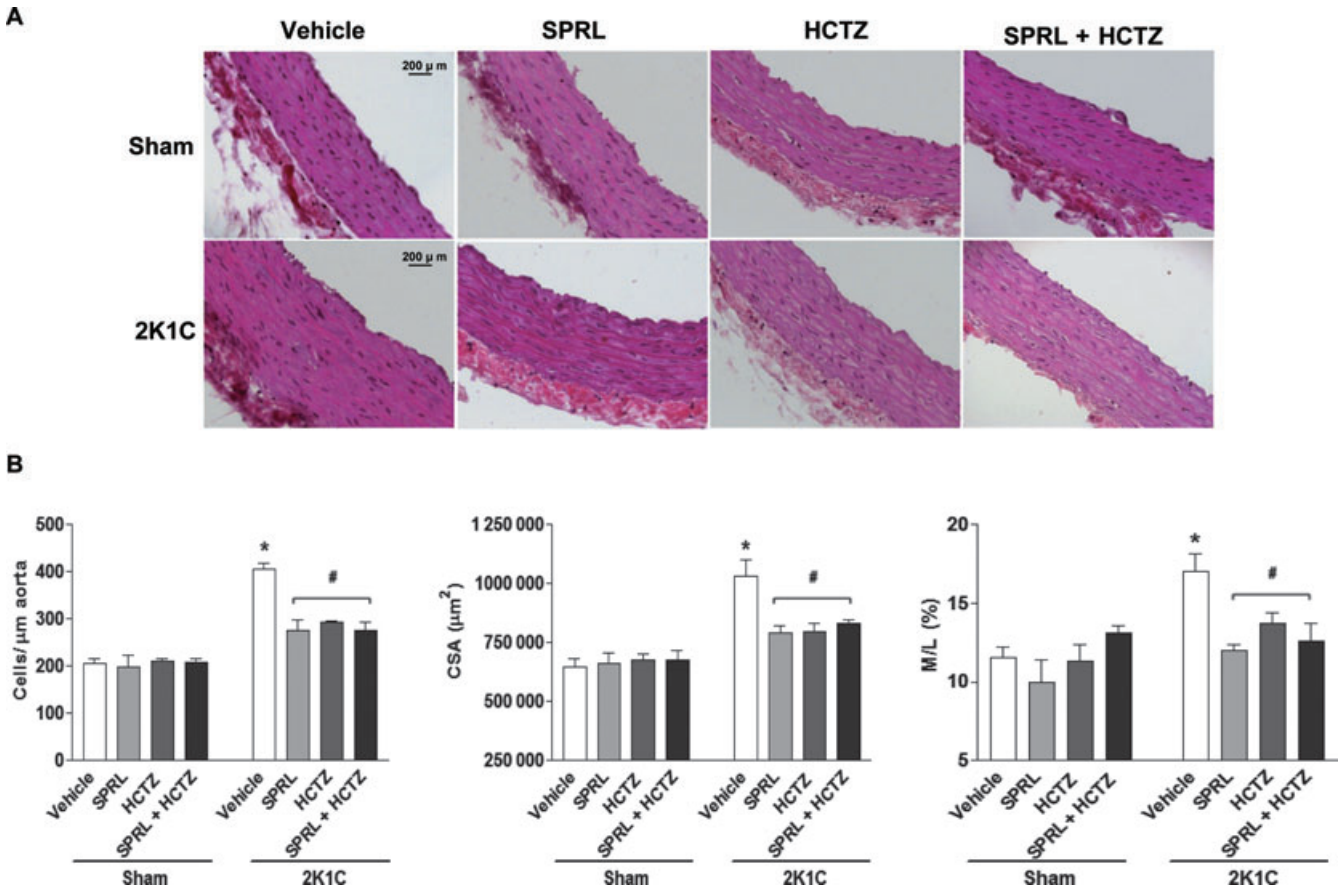

Figure 2 Structural alterations induced in the aortas by 2K1C hypertension and effects of treatment with HCTZ, SPRL or a combination of the two drugs. (A) Representative photographs of aortic samples (×400) stained by haematoxylin and eosin. (B) The values for VSMCs number per length of aorta, thoracic aorta medial CSA and M/L. Values are expressed as mean + SEM ($n = 5$ per group). *$P < 0.05$ for 2K1C + vehicle group versus the four sham-operated groups. #$P < 0.05$ for 2K1C + vehicle group versus 2K1C + treatment groups.

fluorescence was also higher in the 2K1C + vehicle group compared with the other study groups ($P < 0.05$; Figure 5C).

To further confirm these findings, we have also assayed net MMP activity in aortic tissue homogenates with a gelatinolytic activity assay. Total aortic net MMP activity was significantly higher in the 2K1C + vehicle group compared with the other study groups ($P < 0.05$; Figure 5D).

TIMP-2 levels and ratio of MMP-2/TIMP-2 in 2K1C rats

Representative immunohistochemistry photomicrographs showing MMP-2 and TIMP-2 levels in the aortas from rats are shown in Figure 6A. While higher MMP-2 levels were found in the 2K1C + vehicle group compared with the other study groups ($P < 0.05$; Figure 6A,B), no significant differences were found in TIMP-2 levels ($P > 0.05$; Figure 6A,C), thus leading to higher MMP-2/TIMP-2 ratios in the 2K1C + vehicle group compared with the other study groups ($P < 0.05$; Figure 6D).

In vitro *effects of SPRL and HCTZ on MMP-2 activity*

As shown in Supporting Information Figure S1, SPRL, HCTZ or the combination of both drugs had no significant effects on human recombinant MMP-2 activity, even when studied at very high concentrations. Conversely, phenanthroline inhibited MMP-2 activity ($P < 0.05$).

Discussion and conclusions

The major findings of the present study were: (i) treatment of 2K1C hypertension with SPRL, HCTZ or the combination produced antihypertensive effects, and prevented the functional and structural vascular alterations associated with this hypertension model; (ii) while SPRL produced minor antihypertensive effects compared with HCTZ or the combination of drugs, treatment with both drugs or the combination produced antioxidant effects and blunted MMP-2 up-regulation in 2K1C hypertensive rats; and (iii) the antioxidant effects produced by SPRL, HCTZ or the combination probably resulted from direct inhibition of vascular NADPH oxidase activity. This is the first study to evaluate the effects of these drugs on MMPs in this model of hypertension.

It is well known that increased blood pressure induces long-lasting changes in the arterial vascular wall (Safar *et al.*, 1998), which may result from the interplay of several mechanisms (Intengan and Schiffrin, 2001). Interestingly, SPRL induced negligible antihypertensive effects in the present study, which

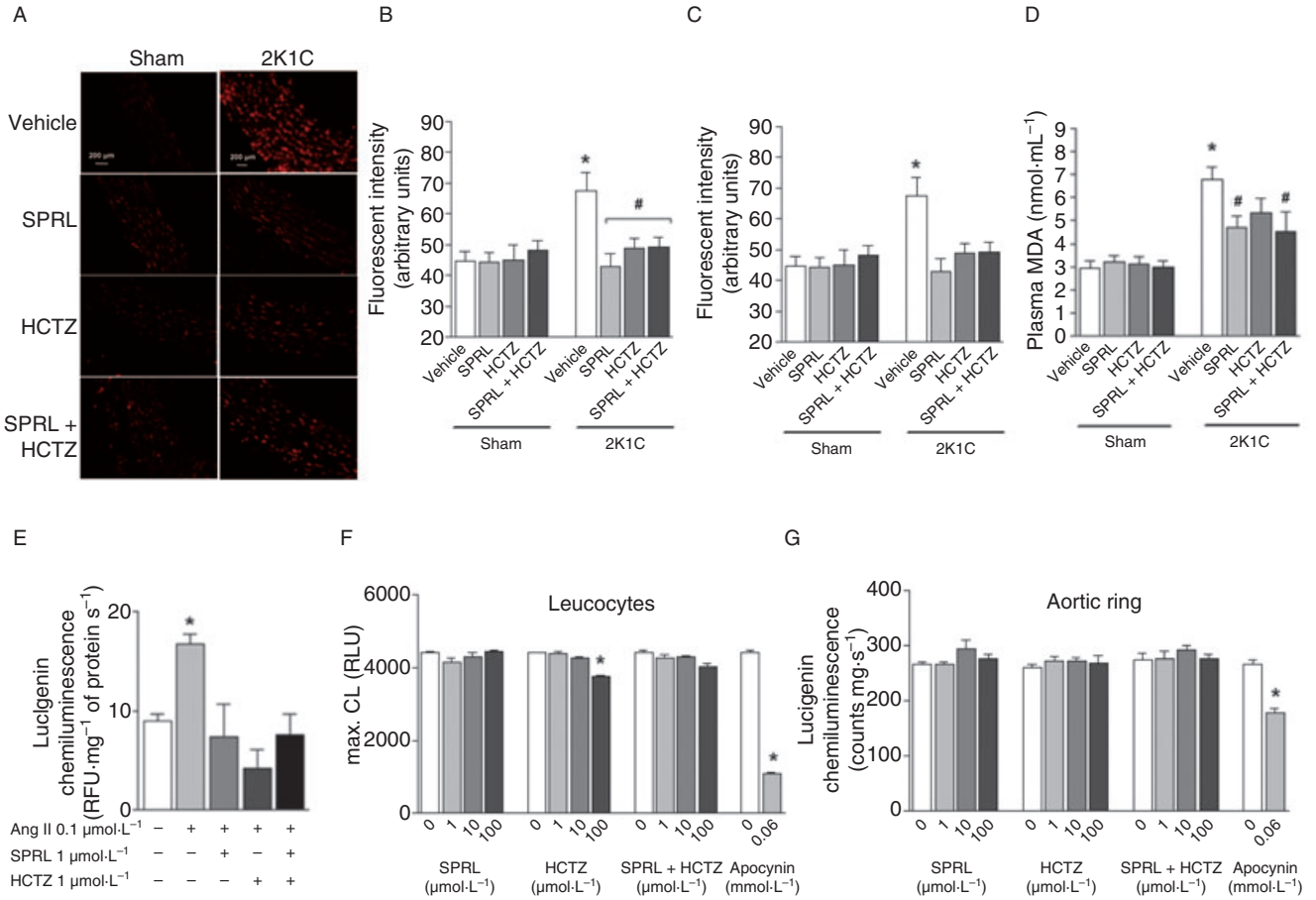

Figure 3 Vascular ROS production (A–C), lipid peroxide levels in plasma (D) and *in vitro* effects of SPRL and HCTZ (E–G). (A) Photomicrographs (×400) with red fluorescence in DHE aortic samples. (B) The quantification of aortic fluorescence in each experimental group ($n = 4$ per group). (C) NADPH-dependent superoxide production measured in the aortic rings ($n = 5$ per group). (D) TBARS concentration in plasma samples expressed in terms of MDA ($n = 10$ per group). (E) NADPH-dependent superoxide production measured in RASMC line A7r5 ($n = 3$ per group). (F) Intensity of ROS generation by leucocytes stimulated with PMA (100 nmol·L^{-1}) and assessed by maximum chemiluminescence (max. CL.). (G) Generation of ROS by aortic vessels stimulated with menadione (5 μmol·L^{-1}), and assessed by chemiluminescence. At least six independent experiments were performed in duplicate. Data are shown as mean ± SEM. (B–E) *$P < 0.05$ for 2K1C + vehicle versus all the other groups. #$P < 0.05$ for 2K1C + SPRL, and 2K1C + HCTZ versus 2K1C + vehicle group. (F and G) *$P < 0.05$ versus corresponding vehicle.

were associated with complete functional and morphological normalization of the vascular wall, thus suggesting that aldosterone is a major mediator in the pathogenesis of 2K1C hypertension-induced functional and morphological alterations. These findings are in agreement with earlier work showing that chronic treatment with mineralocorticoid antagonists ameliorated aortic remodelling and endothelium-dependent relaxations in spontaneously hypertensive (SHR) rats and in heart failure (Thai *et al.*, 2006; de las Heras *et al.*, 2007), and also improved the endothelial dysfunction in the early phase after myocardial infarction (Sartorio *et al.*, 2007). Moreover, SPRL improved angiotensin II-induced structural and functional vascular alterations (Virdis *et al.*, 2002). While the effects produced by SPRL may differ significantly from those produced by the more selective antagonist eplerenone, our findings are consistent with the idea that blockade of mineralocorticoid receptors attenuates the cardiovascular remodelling in hypertension, independently of blood pressure normalization (Burla *et al.*, 2007). In addition, SPRL produced antioxidant effects and inhibited MMP-2 up-regulation associated with 2K1C hypertension, thus suggesting that antioxidant mechanisms may have had major effects on MMP-2 regulation, and blunted the development of the structural and vascular changes in 2K1C hypertension, independently of the antihypertensive effects produced by SPRL. These findings are supported by previous findings showing that antioxidant drugs prevented the increases in aortic MMP-2, and the functional and structural alterations induced by 2K1C hypertension (Castro *et al.*, 2009).

While SPRL produced minor antihypertensive effects, the combination of HCTZ and SPRL improved the antihypertensive effects produced by SPRL. However, this improved antihypertensive effect was not associated with greater effects on the vascular function or structure. Correspondingly, we found no additional beneficial effects on oxidative stress or on MMP-2 up-regulation when both drugs were given to hypertensive rats. These findings suggest that blockade of mineralocorticoid receptors may have completely blunted critical alterations associated with 2K1C hypertension. In fact, it is possible that blockade of these receptors by SPRL may have also counteracted deleterious consequences of HCTZ-induced volume depletion, which include increased angiotensin II and

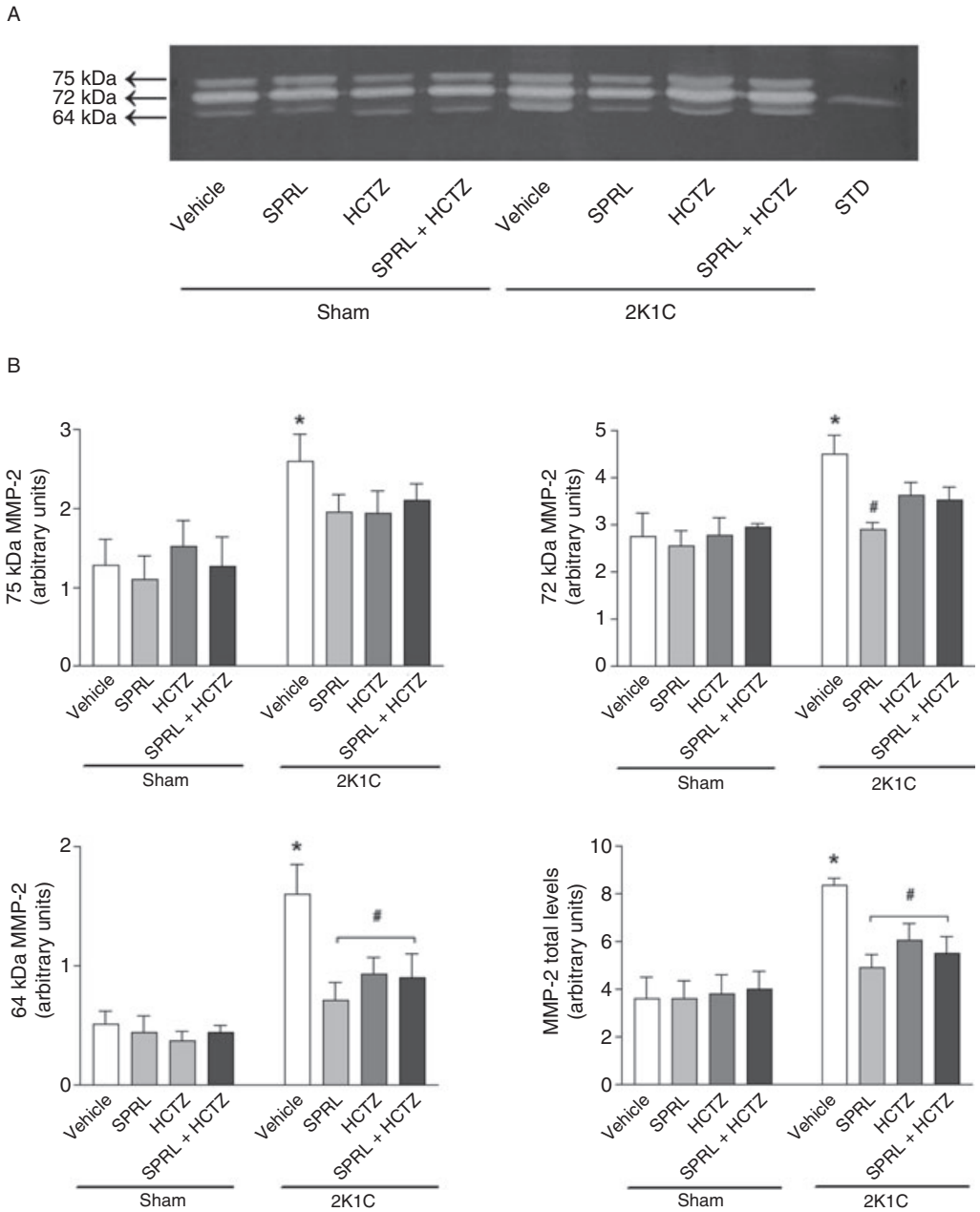

Figure 4 Representative sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gelatin zymogram of aortic samples (A). Molecular weights of MMP-2 bands (75, 72 and 64 kDa MMP-2) were identified after electrophoresis on 12% SDS–PAGE. Std: internal standard. (B) Values for each molecular weight form of MMP-2 in the aorta extracts. Values are expressed as mean ± SEM ($n = 8$ per group). *$P < 0.05$ 2K1C + vehicle versus four sham groups. #$P < 0.05$ versus the 2K1C + vehicle group.

aldosterone levels (Koenig *et al.*, 1991), thus resulting in oxidative stress and MMP up-regulation (Rude *et al.*, 2005; Johar *et al.*, 2006). Moreover, the antagonism of mineralocorticoid receptors has been shown to prevent or attenuate profibrotic mechanisms including increased expression of collagen, fibronectin, MMP-2 and MMP-9 activities (Rude *et al.*, 2005).

Interestingly, we found that HCTZ decreased the structural and functional alterations in 2K1C hypertension. While our findings confirm previous results showing beneficial vascular effects caused by HCTZ in SHR (Mougenot *et al.*, 2005), they are in contrast with previous findings showing that the same dose of HCTZ did not prevent arterial media thickening in stroke-prone SHR (Contard *et al.*, 1993). It is possible that these differences result from significant differences between the animal models used to show how HCTZ may affect the vascular function and structure. In spite of these differences, the antioxidant effects produced by HCTZ may help to explain the beneficial effects produced by this diuretic.

The treatment with HCTZ inhibited the increases in vascular ROS levels and NAD(P)H oxidase activity induced by hypertension, but not the increases in plasma MDA concentrations. Because NAD(P)H oxidase is a major source of ROS (Griendling *et al.*, 2000), the reduction in the vascular NAD(P)H oxidase activity is consistent with the lower *in situ*

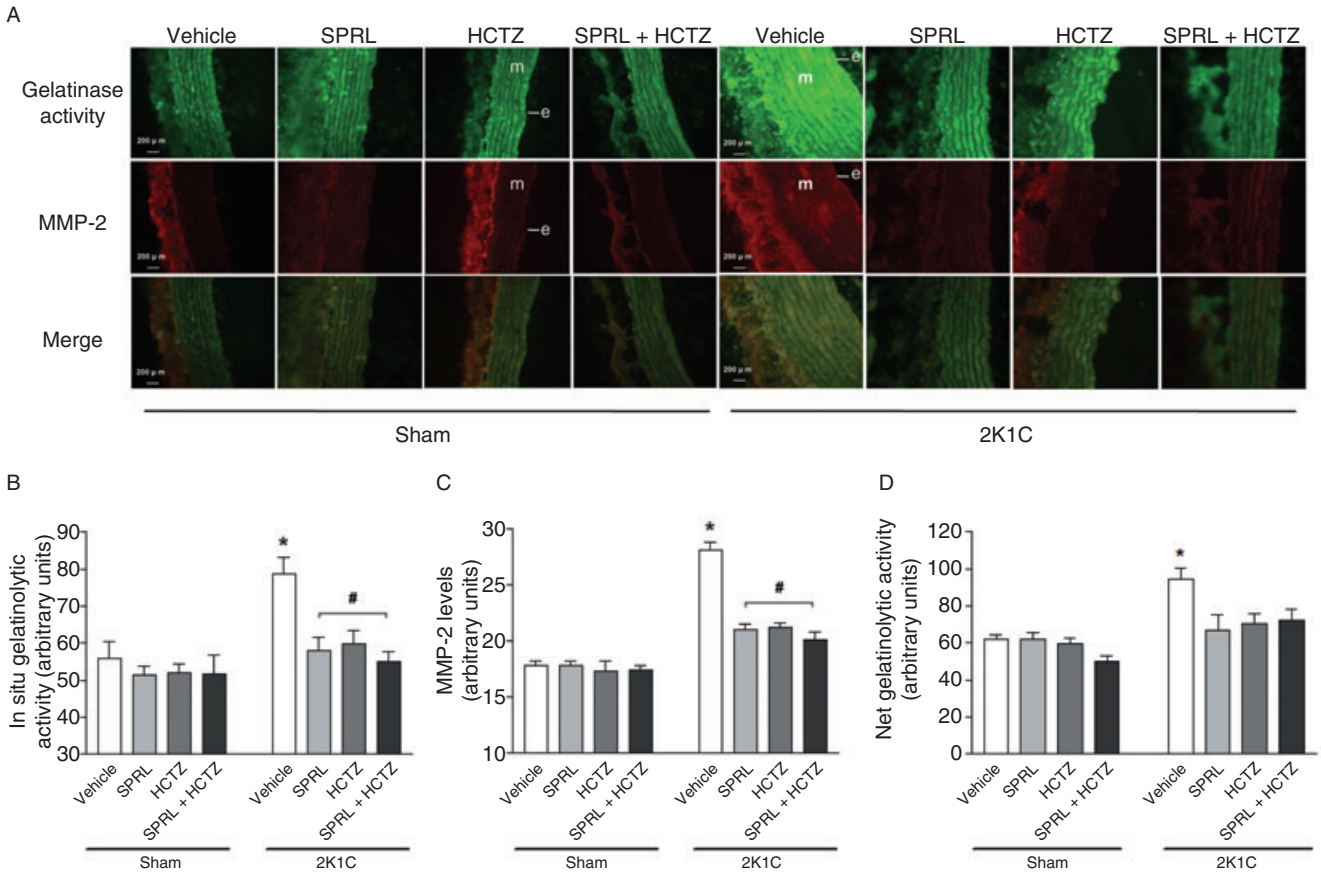

Figure 5 Treatment effects on *in situ* gelatinase activity on MMP-2 levels and on gelatinolytic activity in the aortas. (A) Representative photographs of gelatinase activity (×400), MMP-2 levels and their co-localization in the aortas (e, endothelium; m, media). (B) Mean gelatinolytic activity in aortas in each study group as assessed by the measurement of bright green fluorescence. (C) Mean MMP-2 levels in the aortas in each study group C as assessed by bright red fluorescence. (D) Net MMP in aortas in each study group. Data are shown as mean ± SEM. **P* < 0.05 for 2K1C + vehicle group versus the four sham-operated groups. #*P* < 0.05 for 2K1C + vehicle group versus 2K1C + treatment groups.

ROS concentrations that we found after all treatments. The lack of significant effects of HCTZ on MDA levels may be due to lack of specificity associated with this method, which is explained by the fact that TBA reacts with a variety of compounds, such as sugars, amino acids, aldehydes and bilirubin (Knight *et al*., 1988; Valenzuela, 1991).

It is possible that antioxidant effects produced by HCTZ increase NO bioavailability, thus explaining the improvement in endothelial-dependent function reported here. The antioxidant effects may also have blunted the increases in MMP-2 activity, thus preventing the vascular remodelling, as previously reported in this model (Martinez *et al*., 2008; Castro *et al*., 2009). To our knowledge, this is the first study showing that HCTZ may protect against the vascular remodelling of hypertension through mechanisms that lower MMP-2 activity in the vessels. Of note, we have excluded direct antioxidant or direct inhibitory effects produced by HCTZ or SPRL in the present study.

There is recent evidence indicating that MMP-2 modulates vascular contractility (Fernandez-Patron *et al*., 1999; 2000; Martinez *et al*., 2004). While precise mechanisms have not yet been described, our results suggest that a reduction in vascular MMP-2 levels improves ACh-induced, endothelium-dependent responses in hypertensive rats. Conversely, the lack of significant improvement of SNP-induced, endothelium-independent responses suggests that the endothelial effects are probably of major importance. The antioxidant effects produced by SPRL and HCTZ may have contributed to the improvements of endothelial dysfunction in hypertensive animals (Touyz and Schiffrin, 2004).

MMPs are regulated by gene transcription, post-translational activation and interaction with their endogenous inhibitors (TIMPs). Confirming previous findings (Castro *et al*., 2009), we found increased net MMP-2 activity in hypertensive rats, as revealed by increased MMP-2/TIMP-2 ratio, in comparison with normotensive rats. The lack of significant differences in TIMP-2 expression among the study groups suggests that the differences in MMP activities reported here did not reflect alterations in TIMP-2 concentrations. Clearly, these findings suggest that both SPRL and HCTZ do not affect MMP activities by modifying TIMP concentrations.

The increased aortic NAD(P)H oxidase activity that we found in 2K1C hypertensive rats may be the main mechanism responsible for the increased oxidative stress, which is very important for the maintenance of 2K1C hypertension

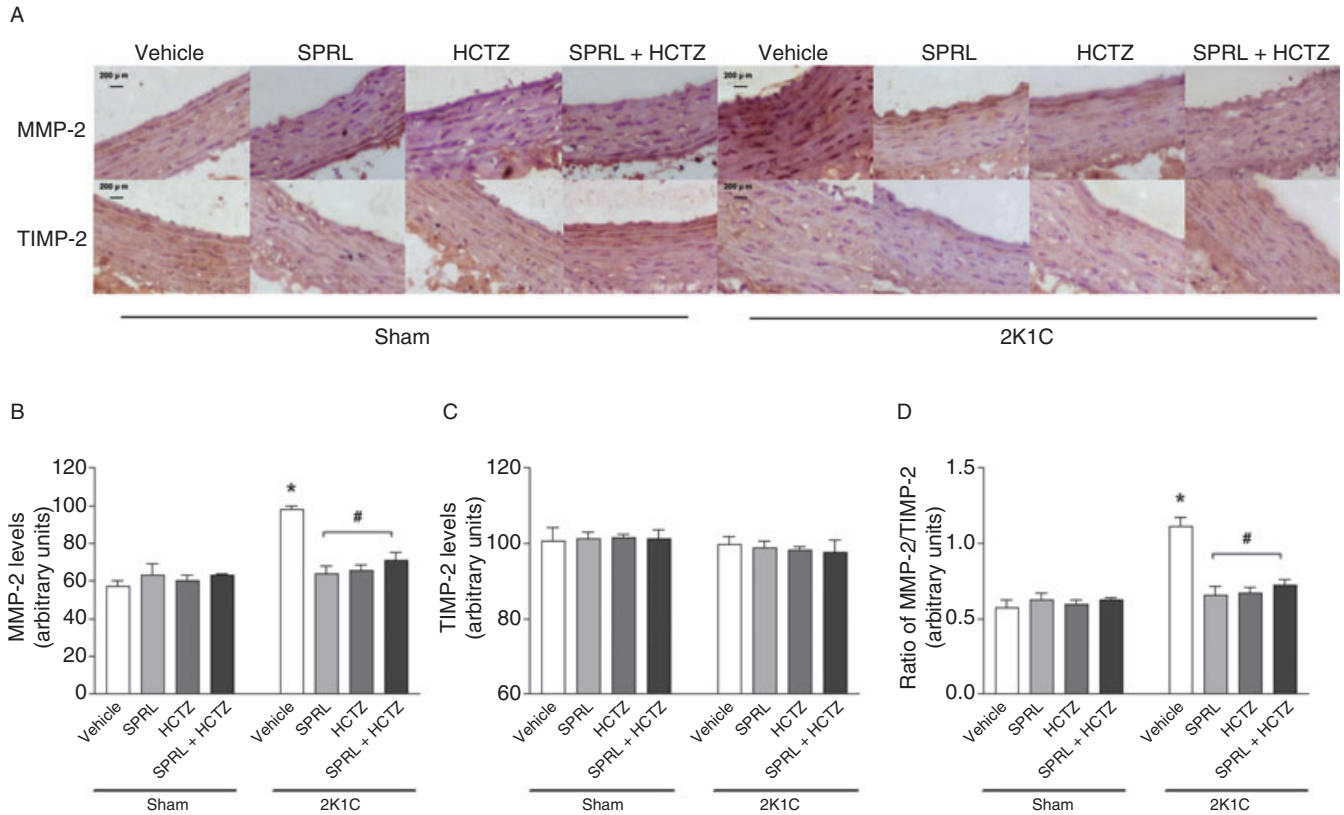

Figure 6 (A) Representative photographs of immunostaining of MMP-2 and TIMP-2 performed in the endothelium and media of hypertensive-treated rats (×400). (B) Quantification of brown staining of MMP-2 and TIMP-2 (four to five sections per aorta). (C) MMP-2/TIMP-2 ratio. Data are shown as mean ± SEM. *$P < 0.05$ for 2K1C + vehicle group versus four sham groups. #$P < 0.05$ for 2K1C + vehicle group versus 2K1C + treatment groups.

(Lerman *et al.*, 2005). In this regard, the antioxidant effects produced by both SPRL and HCTZ found in the present study are of special interest. While previous studies have shown that mineralocorticoid antagonists decrease NAD(P)H oxidase activity (Virdis *et al.*, 2002; Di Zhang *et al.*, 2008) in hypertension, the present study is the first to show that SPRL or HCTZ decreases NAD(P)H oxidase activity in 2K1C hypertension. These findings are consistent with the lower superoxide production that we found by using the DHE method, and with the lower plasma MDA levels in hypertensive rats treated with SPRL or HCTZ. Further supporting this suggestion, both drugs inhibited the increases in NADPH oxidase activity caused by stimulation of RASMCs with angiotensin II. Together, our findings strongly suggest that SPRL and HCTZ inhibit the major source of vascular ROS.

A surprising finding of the present study was that the combination of treatments was not more effective. In our view, this is probably because either SPRL or HCTZ, or the combination of treatments inhibited vascular NADPH oxidase activity and produced relevant antioxidant effects.

In conclusion, we found that SPRL, HCTZ or a combination of these two drugs produced small antihypertensive effects, reversed the endothelial dysfunction and decreased levels of vascular ROS found in 2K1C hypertension probably by inhibiting NADPH oxidase and inhibiting the activity of MMP-2. The beneficial vascular effects produced by these drugs are apparently not crucially dependent on their antihypertensive effects, and suggest that these drugs may prevent the vascular alterations found in 2K1C hypertension, and thus may help to prevent organ damage in hypertension. Clinical studies should be carried out to validate these findings in hypertensive patients.

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Conflict of interest

No conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 *In vitro* effects of antioxidants on human recombinant MMP-2 activity. Human recombinant MMP-2 activity was measured using a gelatinolytic activity kit in the absence or presence of SPRL (0, 1, 10 and 100 $\mu mol \cdot L^{-1}$), HCTZ (0, 1, 10 and 100 $\mu mol \cdot L^{-1}$) or combination (0, 1, 10 and 100 $\mu mol \cdot L^{-1}$). Phenanthroline (Phe) and EDTA were used as positive controls for MMP-2 activity inhibition ($P < 0.01$ versus the respective zero concentration). Data are shown as means ± SEM of three experiments done in duplicate.

Appendix S1 SPRL and HCTZ produce antioxidant effects, and reduce vascular MMP-2 activity and expression in 2K1C hypertension.

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