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Matrix metalloproteinase (MMP)-2 activation by oxidative stress decreases aortic calponin-1 levels during hypertrophic remodeling in early hypertension

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ABSTRACT

Hypertension is characterized by maladaptive vascular remodeling and enhanced oxidative stress in the vascular wall. Peroxynitrite may directly activate latent matrix metalloproteinase (MMP)-2 in vascular smooth muscle cells (VSMC) by its S-glutathiolation. MMP-2 may then proteolyze calponin-1 in aortas from hypertensive animals, which stimulates VSMC proliferation and medial hypertrophy. Calponin-1 is an intracellular protein which helps to maintain VSMC in their differentiated (contractile) phenotype. The present study therefore investigated whether aortic MMP-2 activity is increased by oxidative stress in early hypertension and then contributes to hypertrophic arterial remodeling by reducing the levels of calponin-1. Male Wistar rats were submitted to the two kidney, one clip (2 K-1C) model of hypertension or sham surgery and were treated daily with tempol (18 mg/kg/day) or its vehicle (water) by gavage from the third to seventh day post-surgery. Systolic blood pressure (SBP) was daily assessed by tail-cuff plethysmography. After one week, aortas were removed to perform morphological analysis with hematoxylin and eosin staining and to analyze reactive oxygen-nitrogen species levels by dihydroethidium and immunohistochemistry for nitrotyrosine. MMP-2 activity was analyzed by in situ and gelatin zymography and its S-glutathiolation was analyzed by Western blot for MMP-2 of anti-glutathione immunoprecipitates. Calponin-1 levels were identified in aortas by immunofluorescence. SBP increased by approximately 50 mmHg at the first week in 2 K-1C rats which was unaffected by tempol. However, tempol ameliorated the hypertension-induced increase in arterial media-to-lumen ratio and hypertrophic remodeling. Tempol also decreased hypertension-induced aortic oxidative stress and the enhanced MMP-2 activity. S-glutathiolation may be a potential mechanism by which oxidative stress activates MMP-2 in aortas of 2 K-1C rats. Furthermore, calponin-1 was decreased in aortas from 2 K-1C rats and tempol prevented this. In conclusion, oxidative stress may contribute to the increase in aortic MMP-2 activity, possibly by S-glutathiolation, and this may result in calponin-1 loss and maladaptive vascular remodeling in early hypertension.

1. Introduction

Hypertrophic vascular remodeling, which generally occurs in conductance arteries to counteract the wall strain of hypertension, is described as a significant increase in vascular wall thickness followed by increased vascular smooth muscle cell (VSMC) proliferation and migration, generally from the medial to intimal layer. As a result, increased cross-sectional area (CSA) and media-to-lumen ratio (M/L) of aortas are used as important markers of this type of remodeling [1,2]. Oxidative stress and increased activity of pro-oxidants enzymes are typically associated with hypertension-induced proliferative signaling pathways that lead to vascular hypertrophic remodeling [3–5]. In fact, after treating VSMC with angiotensin II [6] or mechanical stretch [7], the NAD(P)H oxidase isoform 1 stimulates VSMC, through oxidative stress, to change their phenotype from contractile to synthetic and then start migration and remodeling. Oxidative stress is also pivotal to increase both the gene expression and the activity of matrix metalloproteinases (MMPs) in the vasculature, thus contributing to their capability to proteolyze both extra- and intracellular proteins in VSMC [8–11]. Increased activity of MMP-2 in aortas is usually associated with

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vascular hypertrophic and eutrophic remodeling that occur in conductance and resistance arteries in different models of hypertension [12–15]. When antioxidants are given to hypertensive rats or to isolated arteries treated with angiotensin II, exacerbated MMP-2 activity and maladaptive vascular remodeling are both reduced, thus showing a potential linkage between oxidative stress and MMP activity [9,16–18]. The mechanisms by which MMP-2 causes VSMC migration and proliferation during hypertrophic remodeling have been generally associated with its proteolytic action on extracellular proteins [1]. However, there are also different isoforms of MMP-2 that are retained inside cells and are activated by oxidative stress in cardiovascular injuries [19,20]. Peroxynitrite is the one of the most harmful reactive oxygen-nitrogen species that rapidly reacts with cysteinyl thiols in many proteins, which may alter their function. Peroxynitrite in the presence of glutathione disrupted the binding between the cysteinyl thiol in the autoinhibitory pro-peptide domain of MMP-2 with the zinc in its catalytic site, thus permitting MMP-2 activity even without the removal of its pro-peptide domain [21,22]. In fact, oxidative stress contributes to MMP-2-induced proteolysis of intracellular targets, such as troponin I [23] and titin [24] in cardiac myocytes and calponin [25] in VSMC, in cardiac ischemia and reperfusion injury and endotoxemia. Calponin-1, a 34 kDa protein, is an important marker of VSMC differentiation and is especially localized in the cytoskeleton, thus helping VSMC maintain a contractile phenotype [26]. We have shown that increased activity of MMP-2 contributed to the loss of calponin-1 in aortas, but not in mesenteric arteries [15], of two kidney-one clip (2 K-1C) hypertensive rats and this contributed to increase VSMC proliferation before hypertrophic remodeling is emerged in early hypertension [27]. However, it is unknown whether oxidative stress contributes to increased MMP-2 activity in VSMC causing calponin-1 loss and the hypertrophic vascular remodeling of hypertension. Therefore, we investigated for the first time whether MMP-2 activity is increased by oxidative stress in early hypertension and contributes to hypertrophic arterial remodeling by reducing the levels of calponin-1.

2. Materials and methods

2.1. Animals

We used male Wistar rats (180-200 g) from the colony maintained by the University of Sao Paulo that were kept at 25 °C and on a 12 h light/dark cycle with free access to chow and water. All experimental protocols were approved by the Ethics Committee on Animal Research of Ribeirao Preto Medical School, University of Sao Paulo (protocol #0015/2017) and were in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH) and Conselho Nacional de Controle de Experimentação Animal (CONCEA).

2.2. Two kidney-one clip (2 K-1C) surgery

The model of 2 K-1C hypertension was performed in rats by inserting a silver clip with 0.2 mm internal gap onto their left renal artery. The procedure was implemented under anesthesia with intraperitoneal injection of ketamine 100 mg/kg and xylazine 10 mg/kg. Sham animals were submitted to the same surgical procedure, but without the clip insertion. Anti-inflammatory flunixin (1.0 ml/kg in 0.9% saline) and the antibiotic oxytetracyclin (0.5 ml/kg) were each administered once intraperitoneally to the rats post-surgically. Body weight and the systolic blood pressure (SBP) were measured daily for seven days by tail-cuff plethysmography (CODA non-invasive blood pressure measuring equipment, Kent Scientific). Rats were considered hypertensive when the SBP increased ≥25 mmHg by the third day post-surgery compared to baseline (from 117.5 ± 1.4 to 149.9 ± 2.9, n = 10). Rats were treated daily with tempol (18 mg/kg/day, Sigma-Aldrich) [9] or vehicle (water) by gavage. Treatment started on the third day and was maintained for five days. This short time of treatment is effective in ameliorating the hypertrophic vascular remodeling in hypertension as described in previous studies by using doxycycline [15,27]. Rats were randomly allocated into four groups: Sham or 2 K-1C treated with vehicle and Sham or 2 K-1C treated with tempol. Tempol is a cell membrane-permeable nitroxide, which has a reducible (·N—O) group that metabolizes superoxide anion and other reactive oxygen species. Tempol mimics the activity of the superoxide dismutase in vitro and potentiates the effects of nitric oxide synthase. Tempol also reduces the formation of hydroxyl radicals produced by Fenton reactions and exerts similar effects of catalase, thus facilitating the metabolism of hydrogen peroxide [28].

2.2.1. Vascular morphometric analysis

Thoracic aorta was removed from the rats and then fixed in 10% phosphate-buffered formaldehyde (pH 7.0) for 24 h. After dehydration in 70% ethanol, they were embedded in paraffin, cut at 5 μm and stained with hematoxylin and eosin to first analyze the CSA, which is calculated by subtracting the aorta internal area from its external area, and then M/L, which is the ratio between the media layer thickness (M = external – internal diameter) per lumen diameter. The external and the internal diameters were calculated as the square root of 4 × the external area or 4 × internal area, respectively. Morphometric parameters were analyzed by ImageJ from NIH after images being captured by a light microscope at 25 × as previously described [12,29].

2.2.2. Aortic oxidative stress measured by in situ dihydroethidium (DHE) and immunohistochemistry of nitrotyrosine

For DHE, aortas were embedded in Tissue-Tek and then cryosectioned in 5 μm. The slides were incubated at 37 °C for 1 h with 10 μM DHE (#D7008; Sigma-Aldrich; in nm: excitation 535, emission 610) in a dark humidified chamber. Sections were washed with sodium phosphate buffer (PBS, pH 7.2–7.4) and images were captured at 400 × by a fluorescence microscope (Carl Zeiss Microscopy Ltd., Cambridge, UK). The red fluorescence of DHE is seen in the nuclei of VSMC and was quantified by adding fields selected around the marked nuclei by ImageJ, and the arithmetic mean was calculated for each slide. For immunohistochemistry, aortas were embedded in paraffin and cut into 5 μm sections. After heating the sections at 98 °C to induce antigen retrieval with citrate buffer (pH 6.0), 3% hydrogen peroxide and non-specific antibody blocking solution (#DAB500, Millipore) were used to inhibit endogenous peroxidase. Sections were incubated overnight at room temperature with anti-nitrotyrosine antibody (1:100; #06–284; Millipore) following by 2 h incubation with biotinylated goat anti-rabbit secondary antibody (#20782; Millipore). Nitrotyrosine staining was observed in a brown color after adding 3,3′-diaminobenzidine substrate to sections (Millipore #DAB150). Sections were also counterstained with hematoxylin and images were captured at 400 × using a light microscope (Carl Zeiss Microscopy Ltd.). The diaminobenzidine staining was quantified by 40 fields around the arterial media of aortas using the ImageJ and the arithmetic mean of six images was calculated for each sample. Rat liver was used as a positive control as it possesses a high basal signal for nitrotyrosine. As a negative control, aortas from Sham rats were incubated with buffer and diaminobenzidine, but not with the primary antibody.

2.2.3. MMP activity by gelatin and in situ zymography followed by immunofluorescence of MMP-2

To perform gelatin zymography, aortas were frozen in liquid nitrogen and then crushed by using a stainless-steel piston and cylinder that were previously cooled in liquid nitrogen. The resulting powder was diluted in RIPA buffer (#R0278; Sigma-Aldrich, St. Louis, Missouri, USA) containing protease inhibitors cocktail (#S8820; Sigma-Aldrich), 1 mM sodium orthovanadate (#S6508; Sigma-Aldrich) and 10 mM sodium fluoride (#201154; Sigma-Aldrich) overnight. Homogenates were centrifuged at approximately 15,300*g* for 20 min at 4 °C, and the

supernatants were collected. Bradford (#B6916; Sigma-Aldrich) protein assay was used to determine the protein concentration in aortas homogenates. Samples (15 μg protein each) were then prepared in non-reducing buffer (0.25 M Tris-HCl pH 6.8, 30% glycerol, 10% sodium dodecyl sulphate and 0.179 M bromophenol blue) and the electrophoresis was performed on 8% polyacrylamide gels containing 0.5 mg/ml gelatin for 2 h at 100 V. Two % Triton X-100 buffer was used to wash the gels for 30 min, twice, followed by incubation with 50 mM Tris-HCl buffer (10 mM $CaCl_2$ pH 7.4) overnight, at 37 °C. Coomassie Brillant Blue (0.05%) was used to stain the gels unless they are degraded by MMP (represented by the white bands). The 75, 72 and 64 kDa MMP-2 bands were visualized with ChemiDoc Imaging Systems (Biorad, California, USA) and quantified by ImageJ. Fetal bovine serum (FBS) was used as positive control to normalize the inter-gel activities of MMP-2.

To further analyze the activity of MMP-2, 5 μm aortic sections embedded in Tissue-Tek were incubated with dye-quenched gelatin at 1 mg/ml (#D12054; ThermoFisher) in 50 mM Tris-HCl buffer (1:10; 10 mM $CaCl_2$, pH 7.4) at 37 °C for 1 h, in dark humidified chambers. PBS (pH 7.2–7.4) was then used to wash the sections, which were next incubated with mouse monoclonal MMP-2 antibody (1:200; #MAB3308; Millipore, MA, USA) for 1 h, at room temperature, followed by 1 h incubation with mouse rhodamine conjugated secondary antibody (1:200; #AP181R; Millipore). Sections were fixed in 4% paraformaldehyde and finally washed with PBS. Images were captured at 400× using a fluorescence microscope (Carl Zeiss Microscopy Ltd., Cambridge, UK). Green and red fluorescence (from in situ zymography and MMP-2 immunofluorescence, respectively) were quantified by adding 40 fields around the media of aortas using the ImageJ and the arithmetic mean of 3 images was calculated for each sample [15]. Negative controls were performed by incubating the aortas with only PBS or only primary or secondary antibodies. Non-specific fluorescence was observed in the aortas (data not shown).

2.2.4. Measurement of S-glutathiolated MMP-2 levels by co-immunoprecipitation

To investigate whether 72 kDa MMP-2 may be activated by oxidative stress-induced S-glutathiolation, aorta samples (90 μg/sample) were diluted in 50 mM Tris-buffer containing 3.1 mM sucrose, pH 8.0. Samples were pre-cleared by incubating them with sepharose beads suspension (#17–5280-01, GE Healthcare, Sweden), previously diluted in Tris-sucrose buffer, for 1 h (1:1), at 4 °C. Samples were centrifuged at 15,300*g* for 20 min, at 4 °C, and the supernatants were first incubated with either mouse monoclonal anti-glutathione antibody [D8] (1:250; #ab19534; Abcam) or mouse monoclonal IgG_1 (1:100; negative control, #ab18443; Abcam) for 24 h. Fifty μl of sepharose suspension was used to incubate samples under agitation for 24 h at 4 °C. After centrifugation, precipitates were washed with Tris-sucrose buffer and centrifuged at 15,300*g* to be re-suspended in reducing buffer (0.25 M Tris-HCl pH 6.8, 30% glycerol, 0.6 M dithiothreitol, 10% sodium dodecyl sulphate and 0.179 M blue bromophenol). After heating at 95 °C for 5 min, samples were loaded onto 10% polyacrylamide gels at 100 V. A molecular weight markers set (#26619, Thermo Scientific, Waltham, MA, USA) was used to assist in the identification of the proteins of interest. After electrophoresis, gels were transferred to nitrocellulose membranes (GE Healthcare, UK) with a transfer buffer (20% v/v methanol, 48 mM Tris-base, 39 mM glycine and 0.04% w/v sodium dodecyl sulphate) by using a wet transfer system (#TE22 Mini Tank Transfer Unit, GE Healthcare, UK) for 1.5 h at 100 V. Membranes were blocked with 5% non-fat milk in TTBS buffer (NaCl 100 mM; Tris-Cl 100 mM; Tween 0.1%) for 1 h, and incubated overnight at 4 °C with mouse monoclonal MMP-2 antibody (1:1000, #MAB3308) followed by 1 h incubation with a polyclonal goat anti-mouse IgG HRP conjugate antibody (1:5000, #12–349, Millipore, USA). Bands were visualized by ECL chemiluminescence kit (GE Healthcare, UK) and the Amersham Imager 600 (GE Healthcare, UK). Band intensities were quantified using ImageJ [25].

2.2.5. In vitro effects of tempol on MMP-2 activity derived from HT1080 cancer cells

To verify whether tempol inhibits MMP-2 by directly inhibiting its enzymatic activity (instead of by reducing oxidative stress), HT1080 cell culture conditioned medium (which is well known to express copious amounts of MMP-2) was tested in the absence or presence of different concentrations of tempol in the gelatin zymography technique. Briefly, non-reducing buffer (0.25 M Tris-HCl pH 6.8, 30% glycerol, 10% sodium dodecyl sulphate and 0.179 M bromophenol blue) was added to conditioned medium from HT1080 cells (2.5 μg total protein) and then loaded onto 8% polyacrylamide gels *co*-polymerized with 0.5 mg/ml gelatin and electrophoresed for 2 h at 100 V. Two % Triton X-100 buffer was used to wash the gels for 30 min, twice. Gels were incubated overnight with different concentrations of tempol as follows: 0.5, 1.0 or 1.5 mM in Tris-HCl buffer (10 mM $CaCl_2$ pH 7.4) at 37 °C. Either only Tris-HCl buffer (10 mM $CaCl_2$, pH 7.4) or the pan-specific MMP inhibitor *o*-phenanthroline (0.05, 0.5 and 1.0 mM) was also used to incubate the gels as negative and positive controls, respectively. Coomassie Brillant Blue (0.05% v/v) was used to dye the gels and the 72 and 64 kDa MMP-2 bands were visualized with a densitometer (GS-800, Biorad, California, USA) and quantified by ImageJ [30].

2.2.6. Immunofluorescence of calponin-1 in aortas of hypertensive rats

Aorta sections were fixed in cold acetone for 5 min and then washed two times with 0.3% Triton X-100 and one time PBS (pH 7.2–7.4). Sections were blocked with 10% donkey serum (#S-30; Millipore) for 1 h and then were incubated overnight with a rabbit monoclonal calponin-1 antibody in humidified chambers (1:250, #ab46794; Abcam). Sections were incubated with secondary antibody conjugated to donkey anti-rabbit Alexa 488 (1:200; #A21206; Invitrogen, California, USA) for 1 h. Images were captured at 400× using a fluorescence microscope (Carl Zeiss Microscopy Ltd., Cambridge, UK) and the green fluorescence was quantified by ImageJ. Negative controls were performed by incubating the aortas with only PBS or only primary or secondary antibodies. Non-specific fluorescence was not observed in aortas (data not shown).

2.2.7. Statistical analyses

Data were analyzed by two-way ANOVA followed by the Bonferroni post-test by the GraphPad Prism Software 6.0 (La Jolla, CA, USA). *N* number varies between 4 and 10 for in vivo experiments and 3 for in vitro experiments. Statistically significant values were considered when $p < 0.05$. Data are presented as mean values ± standard error of the mean (SEM).

3. Results

3.1. Oxidative stress increases MMP-2 activity and contributes to hypertrophic arterial remodeling in early stages of hypertension

SBP was significantly higher in 2 K-1C rats one week post-surgery when compared to Sham (149.9 ± 2.9 vs. 117.5 ± 1.4; *$p < 0.05$, Fig. 1A). While tempol treatment for five days did not reduce the elevated SBP in 2 K-1C rats, it significantly restored the hypertrophic remodeling as represented by decreasing the M/L ratio (Fig. 1B, *$p < 0.05$ 2 K-1C + Tempol vs. 2 K-1C group), although it did not statistically reduce CSA. This effect is important as it is well-known that increased arterial M/L ratio is a critical predictive marker of development of cardiovascular events in hypertensive patients [31]. No changes in body weight were observed between the rats (Sham: 227.0 ± 6.7 g; Sham + Tempol: 230.0 ± 5.6 g; 2 K-1C: 222.5 ± 4.4 g; 2 K-1C + Tempol: 220.0 ± 6.3 g). Therefore, as it seems that oxidative stress contributes to hypertrophic arterial

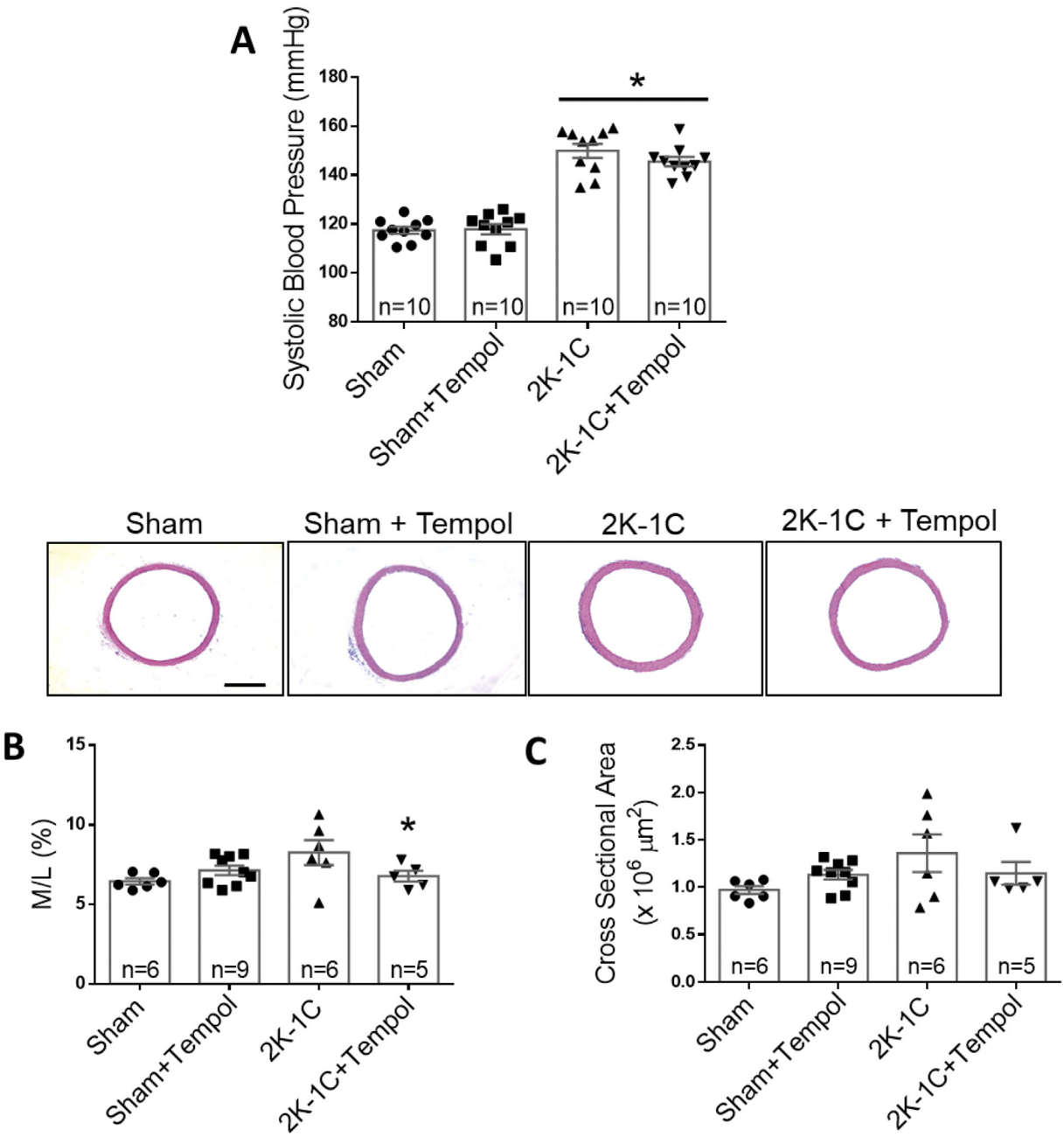

Fig. 1. Effects of tempol on systolic blood pressure (A) and morphological parameters of aortas (B) in 2 K-1C rats. Panel A shows systolic blood pressure on the seventh day measured by tail-cuff plethysmography (n = 10; *p < 0.05 vs. Sham and Sham + Tempol). Panels and graphs B and C show the values of aortic media/lm keep lumen (M/L) ratio and cross-sectional area (CSA) for all experimental groups (n = 5–9; *p < 0.05 2 K-1C + Tempol vs. 2 K-1C group). Bar is 200 µm.

remodeling in hypertension, we analyzed whether tempol decreases oxidative stress in aortas and the activity of MMP-2.

We first analyzed oxidative stress in situ by using DHE. Fig. 2A shows that tempol significantly reduced increased levels of reactive oxygen-nitrogen species throughout the aortic media of 2 K-1C (*p < 0.05 vs. Sham groups and 2 K-1C + Tempol). Furthermore, as peroxynitrite is a reactive oxygen-nitrogen species that reacts with tyrosine residues of many proteins, we analyzed nitrotyrosine immunoreactivity in aortas by immunohistochemistry. Tempol decreased the enhanced nitrotyrosine immunoreactivity in aortas of 2 K-1C rats (*p < 0.05 vs. Sham groups and 2 K-1C + Tempol; Fig. 2B). Since tempol is as a mimetic of superoxide dismutase it is predict to reduce the level of superoxide and therefore peroxynitrite in the arteries of hypertensive rats.

As oxidative stress contributes to regulate the expression and activity of MMP-2 in the vasculature in the chronic phases of hypertension [9], we analyzed here whether treatment with tempol also reduces the activity of MMP-2 in the first week of hypertension to contribute to reduce hypertrophic remodeling. By using gelatin zymography, we showed that the activity of aortic MMP-2 (represented by 75, 72 and 64 kDa bands) was increased in the aortas of 2 K-1C rats. Tempol slightly decreased 64 kDa MMP-2 activity in the 2 K-1C rats (p = 0.057, Fig. 3A). No MMP-9 bands were detected in the zymograms of aortas from 2 K-1C rats. Similarly, by in situ zymography and immunofluorescence for MMP-2, it is observed that tempol significantly decreased the augmented gelatinase activity in aortas of hypertensive rats (*p < 0.05 vs. Sham and 2 K-1C + Tempol, Fig. 3B). Part of the gelatinase activity may be a result of MMP-2 as the fluorescence of the merged images (represented by the yellow-green color in the last panel) is increased in 2 K-1C rats. To verify whether oxidative stress may

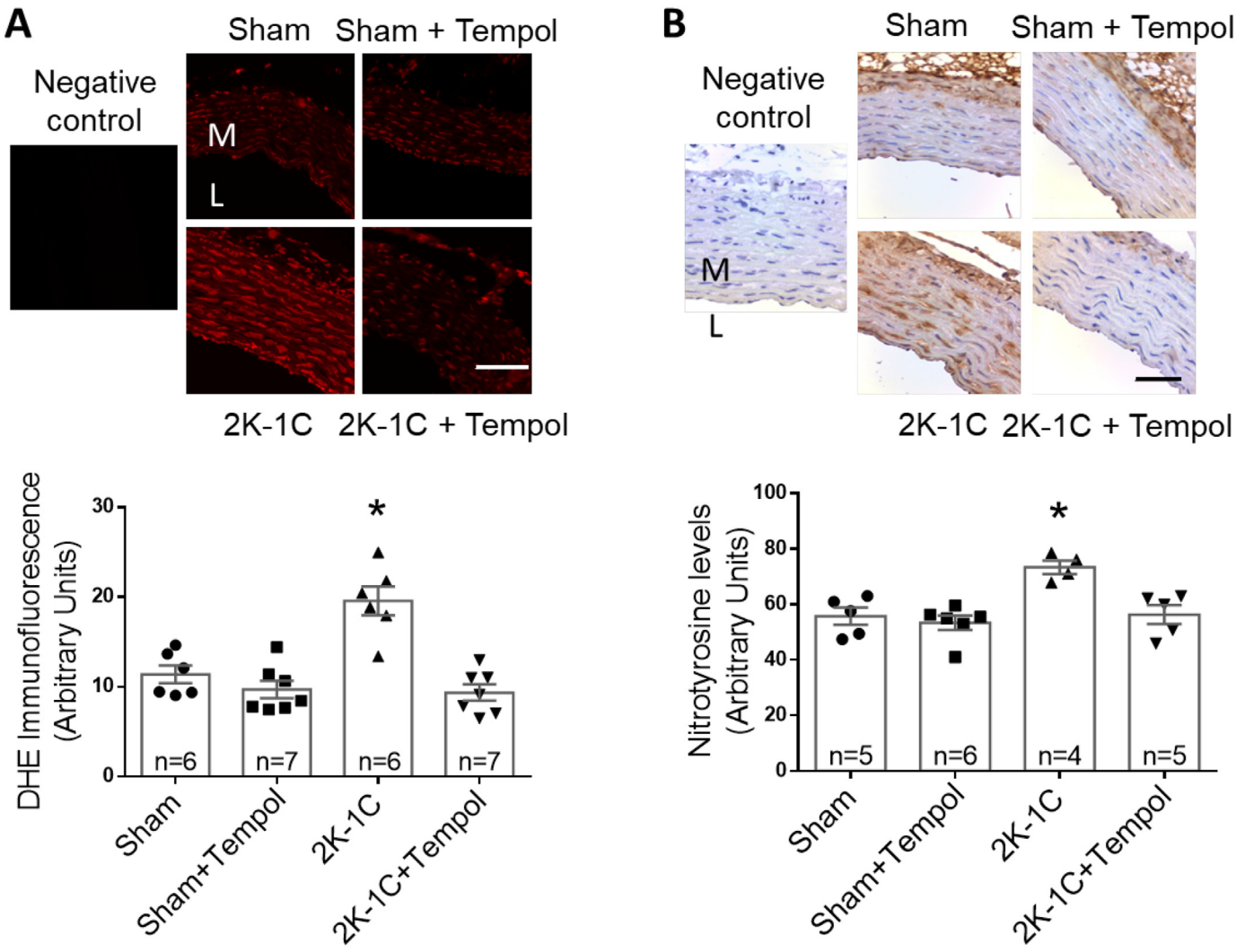

Fig. 2. Acute treatment with tempol reduces arterial oxidative stress in hypertensive rats. Panels and graph A show the representative photographs and the quantified values of DHE fluorescence in aortas of all groups (n = 6–7; *p < 0.05 vs. Sham groups and 2 K-1C + Tempol). Panels and graph B show representative photographs and the quantified values of diaminobenzidine intensity in aortas of all groups (n = 4–6; *p < 0.05 vs. Sham groups and 2 K-1C + Tempol). M = media, L = lumen. Bar is 20 μm.

contribute to activate MMP-2 in aortas of hypertensive rats by S-glutathiolation, Western blot for MMP-2 of anti-glutathione immunoprecipitates was performed. The 72 kDa S-glutathiolated form of MMP-2 is increased in aortas of 2 K-1C rats, while treatment with tempol decreases it (Fig. 3C). As the co-immunoprecipitation technique is not readily quantifiable, this experiment was repeated at least four times using different aortas from the groups and gave qualitatively similar results. As mentioned S-glutathiolation is an important post-translational mechanism by which peroxynitrite activates MMP-2 [21,22]. Immunohistochemistry for the tissue inhibitor of matrix metalloproteinase (TIMP)-2 was also performed in the aortas to verify whether oxidative stress may interfere with this TIMP to regulate MMP-2. In fact, no differences were observed in aortic TIMP-2 levels between the groups (data not shown).

It is also important to note that tempol per se does not directly inhibit MMP-2 activity. Fig. 4 shows that when tempol (0.5, 1.0 and 1.5 mM) was directly incubated with the gel from the gelatin zymogram separation of HT1080 cells conditioned medium, it did not inhibit MMP-2 activity whereas its activity was abolished by the MMP inhibitor *o*-phenanthroline (*p < 0.05 vs. Tris-HCl $CaCl_2$).

3.2. Increased MMP-2 activity by oxidative stress contributes to calponin-1 loss in early hypertension

To verify whether MMP-2 is indeed activated in the aortas by oxidative stress and leads to calponin-1 loss and hypertrophic remodeling in early hypertension, the levels of calponin-1 were analyzed by immunofluorescence. We observed loss of calponin-1 staining in the aortic media layer of 2 K-1C rats when compared to Sham and tempol prevented this (*p < 0.05 vs. Sham groups and 2 K-1C; Fig. 5). As calponin-1 is an exclusive intracellular protein and a potential proteolytic target of MMP-2 in aortas of hypertensive rats, these results suggest that MMP-2 may be activated by oxidative stress to contribute to decreased calponin-1 in aortas from hypertensive rats.

4. Discussion

We are showing for the first time that the acute treatment with tempol decreases the activity of MMP-2 in the vasculature of hypertensive rats and then helps to inhibit calponin-1 loss and the hypertrophic remodeling. S-glutathiolation may be a potential post-translational mechanism caused by oxidative stress to facilitate MMP-2 activity and its proteolytic effects in the vasculature during early

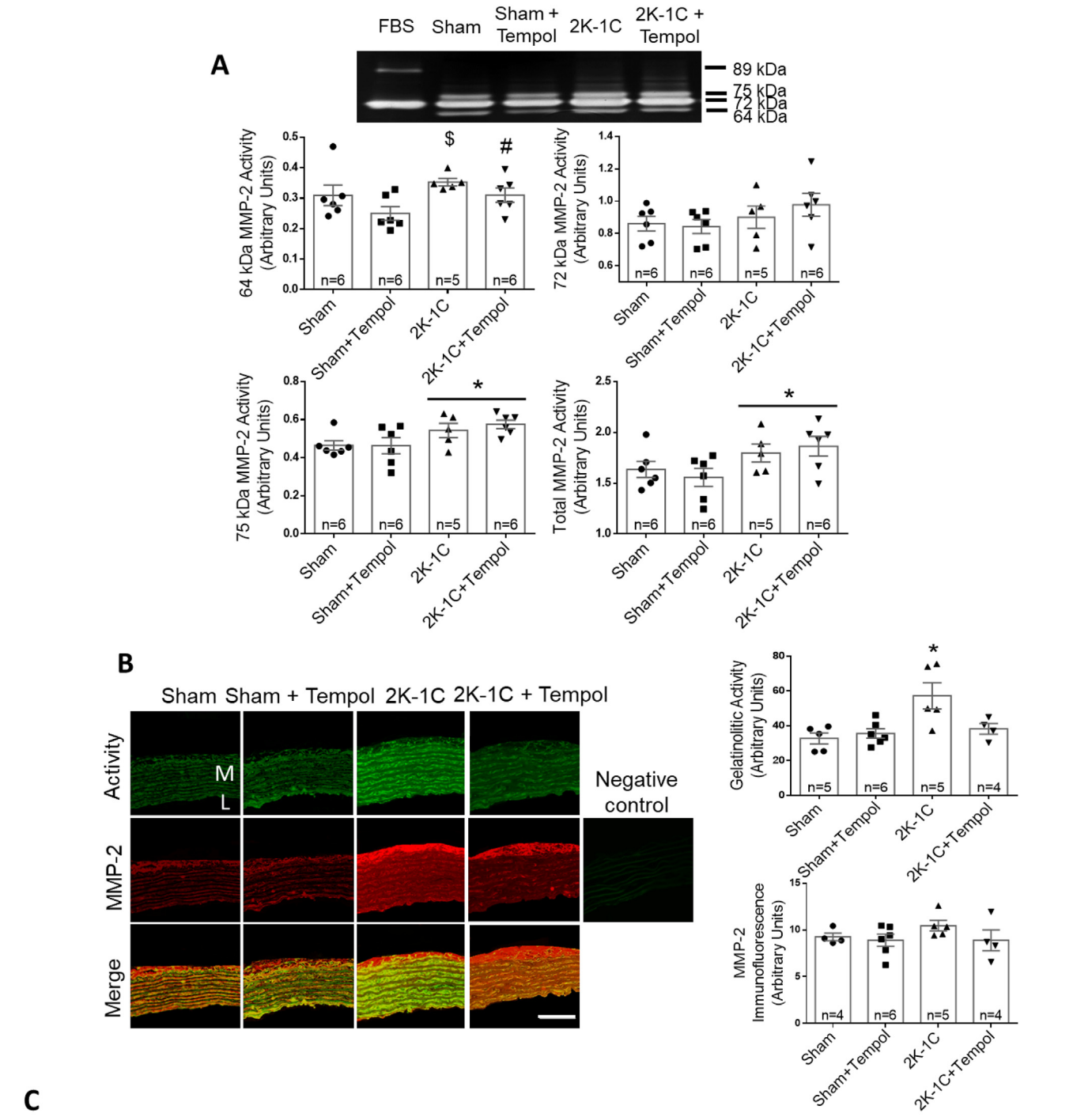

Fig. 3. Tempol reduces MMP-2 activity in aortas of hypertensive rats. Panel and graphs A show a representative gelatin zymogram and the quantified values of 75, 72 and 64 kDa MMP-2 activity in aortas of all groups (n = 5–6; *p < 0.05 vs. Sham and Sham + Tempol; $p = 0.0529 vs. Sham groups and 2 K-1C + Tempol; #p = 0.0571 vs. 2 K-1C). FBS = fetal bovine serum. Panel and graphs B show the representative photographs and the quantified values of fluorescence of gelatinase activity (green) and MMP-2 levels (red) in aortas (n = 4–6; *p < 0.05 vs. Sham groups and 2 K-1C + Tempol). M = media, L = lumen. Bar is 20 μm. Panel C shows representative images of the Western blot for MMP-2 of anti-glutathione immunoprecipitates in aortas from all groups. IgG was used as a negative control. This experiment was repeated at least four times using different aortas from the groups which gave qualitatively similar results. GSH: glutathione; IP: immunoprecipitation; WB: Western blot. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

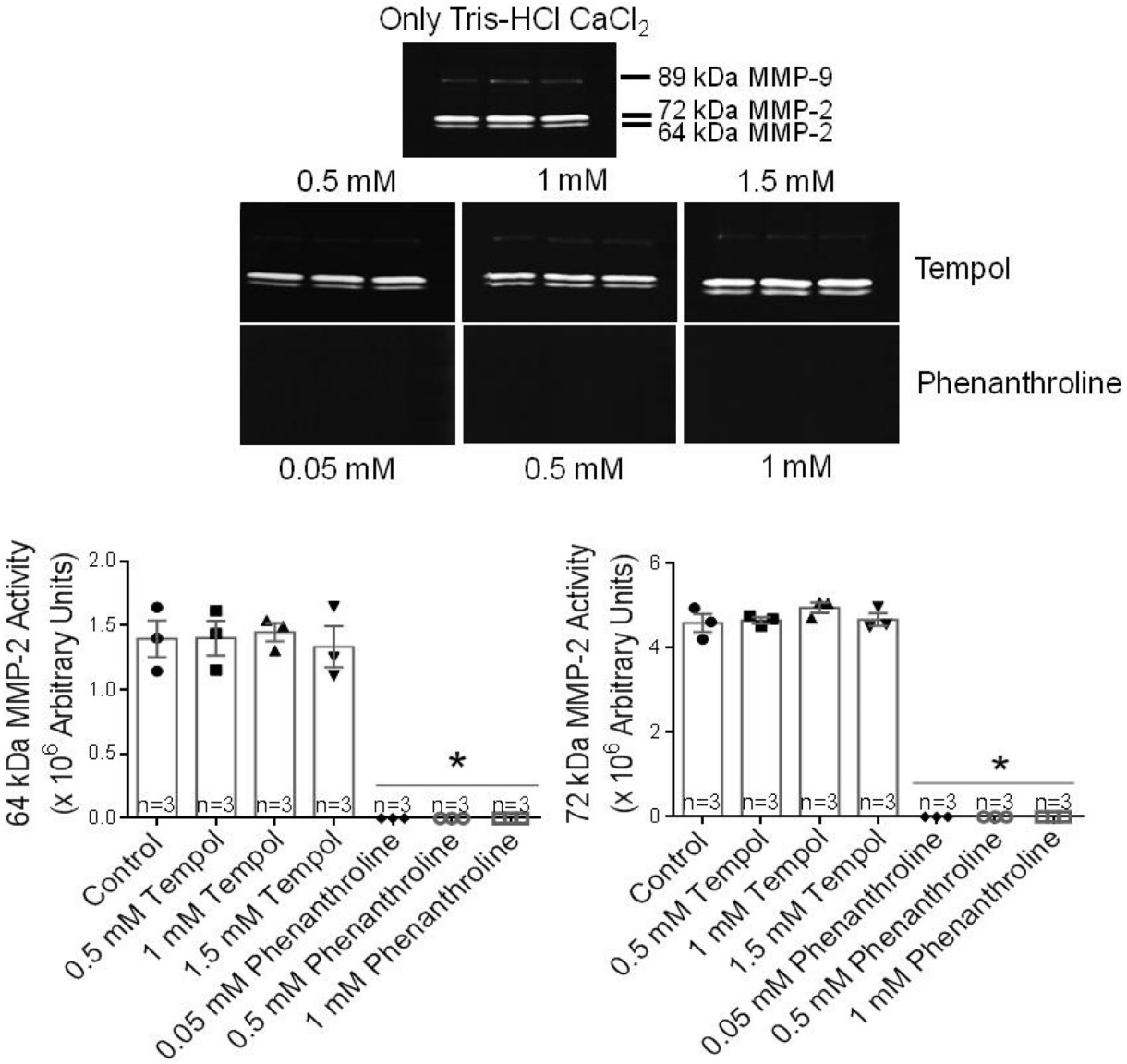

Fig. 4. In vitro effects of tempol on MMP-2 activity. Panels and graph show a representative image and the quantified values of different concentrations of tempol on MMP-2 activity from conditioned media of HT1080 cancer cells by gelatin zymographs (n = 3; *p < 0.05 vs. different concentrations of tempol and the control groups).

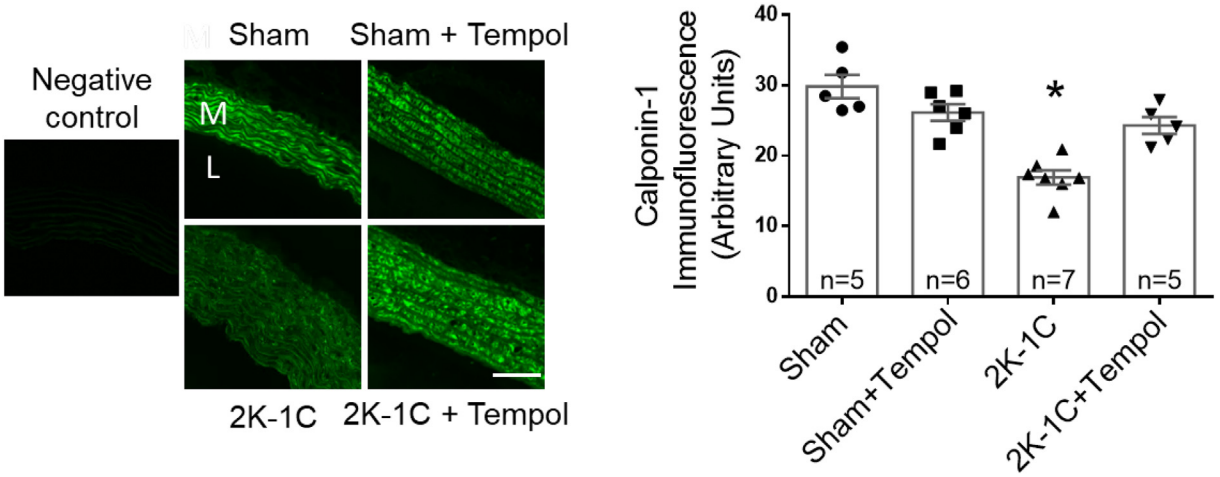

Fig. 5. Acute effects of tempol on calponin-1 levels in aortas of hypertensive rats. Panels and graph show representative photographs and the quantified values of the fluorescence of calponin-1 in aortas of all groups (n = 5–7; *p < .05 vs. Sham groups and 2 K-1C + Tempol). M = media, L = lumen. Bar is 20 μm.

hypertension.

Increased MMP-2 activity is notably related to the appearance of arterial maladaptive remodeling in hypertension as an adaptable mechanism to control wall tension. By proteolyzing extra- and intracellular components in VSMC, MMP-2 triggers several signaling pathways that led to cells proliferation and migration during arterial remodeling [1,2]. Therefore, inhibition of MMP activity with MMP inhibitors has been widely studied to reduce hypertension-induced severe maladaptive cardiovascular remodeling [13–15,32,33]. However, although arterial wall stretch of hypertension per se increases MMP-2 activity [7,34], oxidative stress generation is an important upstream mediator of its increased activity and expression in the vasculature, thus leading to increased media thickness [8,9]. For this reason, treatment with antioxidants, such as tempol and natural flavonols, have been shown to decrease MMP activity and vascular remodeling in chronic phases of hypertension [9,18]. Here, we are suggesting for the first time that acute treatment with tempol also contributed to decrease oxidative stress and MMP-2 activity at early stages of hypertension. Tempol decreased hypertension-induced arterial hypertrophic remodeling, although it did not decrease SBP. The beneficial effects of tempol on arterial remodeling occur independent of reducing hypertension, making it a valuable antioxidant to be used as a co-adjuvant to treat the cardiovascular complications of hypertension. However, when tempol is given chronically for 8 weeks to 2 K-1C rats, SBP was completely restored to basal levels [9]. Although tempol is a general antioxidant, it is successfully used to decrease oxidative stress in arteries of hypertensive humans [35] and several animal models of hypertension. Tempol was used here as the only antioxidant to reduce oxidative stress in hypertensive rats; however, the use of different types of antioxidants would also contribute to explore the sources of oxidative stress in this model of hypertension and needs further consideration.

Although oxidative stress contributes to increase the mRNA and protein levels of MMP-2, it may also elicit its activation. Low concentrations of peroxynitrite disrupt the binding between a cysteinyl thiol in the pro-peptide domain of 72 kDa MMP-2 with zinc in its catalytic site, allows it to be active and proteolyze diverse targets [21]. This post-translational alteration of MMP-2, known as S-glutathiolation, is a potential mechanism by which oxidative stress activates MMP-2. This is the first study suggesting that the increased MMP-2 activity in hypertensive aortas may be a result of its oxidative stress-induced activation by S-glutathiolation. When 72 kDa MMP-2 is treated with low concentrations of peroxynitrite in vitro, S-glutathiolation occurs at the Cys-65 and Cys-102 residues of the MMP-2 autoinhibitory domain as identified by mass spectrometry [21]. Furthermore, increased levels of S-glutathiolated 72 kDa MMP-2 were also observed in aortas of endotoxemic rats, which contributed to calponin-1 loss and hypocontractility [25]. As we here analyzed the S-glutathiolation of MMP-2 only by co-immunoprecipitation, more detailed studies need to be performed to confirm this hypothesis. Furthermore, we showed here that tempol decreased the accentuated 64 kDa MMP-2 activity in aortas of 2 K-1C rats; however, it is unknown at this moment whether 64 kDa MMP-2 is also found inside VSMC. In addition to reduce the activation of MMP-2 in the aortas of 2 K-1C rats, tempol also reduces MMP-2 activity in other tissues and different models of oxidative stress [36].

The intracellular forms of MMP-2 are derived from: (1) the signal sequence of canonical MMP-2 which is inefficient to direct MMP-2 into the secretory pathway, thus leaving about half of newly formed MMP-2 in the cytosol [19]; (2) an N-terminal splice variant that lacks some N-terminal amino acids of the MMP-2 signal sequence and thereby retains it within the cytosol [19]; and (3) a mitochondrial isoform of MMP-2 which is expressed during oxidative stress conditions [20]. In fact, the 72 kDa MMP-2 can be active within cardiac myocytes and VSMC where it contributes to the proteolysis of intracellular targets such as troponin I, titin [23,24] and calponin-1 [25] to lead to acute cardiovascular dysfunction. Therefore, to support the idea that MMP-2 may be intracellularly activated by oxidative stress in aortas of hypertensive rats, we also tested the levels of calponin-1, as it is an intracellular protein in VSMC and a potential target of intracellular MMP-2 [25,27]. We observed that the levels of calponin-1 were significantly reduced in aortas of hypertensive rats and, interestingly, treatment with tempol fully restored it. We also recently showed that treatment with doxycycline reduced the exacerbated MMP-2 activity in aortas of 2 K-1C rats and then contributed to prevent the loss of calponin-1 and VSMC proliferation [27]. However, this is the first time that we show that oxidative stress contributes to activate MMP-2 in the aortas of hypertensive rats to contribute to such proteolytic mechanism.

Calponin-1 is an actin-binding protein in the contractile machinery of VSMC that helps to retain them in a contractile phenotype. Loss of calponin-1 allows VSMC to switch to a synthetic phenotype and then migrate and proliferate to contribute to hypertrophic remodeling. We showed here that hypertension induced oxidative stress and increased MMP-2 activity, which contributed to the loss of calponin-1 in aortas and increased aortic M/L ratio. Although we did not observe any activity of MMP-9 in the aortas of hypertensive rats, it is important to note that all MMPs have a highly conserved PRCGVPD sequence in their autoinhibitory pro-peptide domain, thus making them susceptible to S-glutathiolation. In fact, peroxynitrite was also shown to activate MMP-1, MMP-8 and MMP-9 in vitro [37,38]. Moreover, both superoxide anion and H_2O_2 also enhanced the gelatinolytic activity of unpurified MMP-2 in smooth muscle cells [10].

5. Conclusions

In conclusion, acute treatment with tempol decreased both vascular oxidative stress and MMP-2 activity in early hypertension, thus contributing to prevent calponin-1 loss and hypertrophic remodeling. S-glutathiolation may be a potential posttranslational mechanism caused by oxidative stress to facilitate the activation of MMP-2 and its intracellular proteolytic effects in the vasculature.

Conflict of interest

All authors declare no conflict of interest.

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Author contributions

M.M.B.M. is an undergraduate student and J.M.P. is a PhD student who equally contributed to the execution of all experiments and data analysis. R.S. helped to analyze the data, write and revise the manuscript. Furthermore, in vitro experiments with HT1080 cells and tempol were executed by J.M.P. in the laboratory of R.S. M.M.C. designed the study, coordinated the experiments and wrote the manuscript.

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