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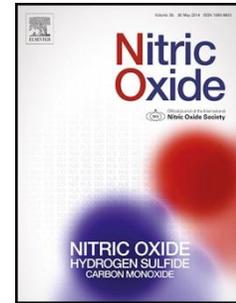
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**Curcumin improves endothelial dysfunction and vascular remodeling in 2K-1C
hypertensive rats by raising nitric oxide availability and reducing oxidative stress**

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Highlights

- Consumption of polyphenol-rich foods has demonstrated their beneficial role in the prevention and treatment of hypertension.
- Curcumin, a principle polyphenolic compound of turmeric, blunts the increase in blood pressure and improves endothelial function and vascular remodeling in 2kidney-1clip renovascular hypertensive rats.
- The mechanisms of curcumin involve an increase in nitric oxide bioavailability and antioxidant activity, together with a decrease in angiotensin converting enzyme, MMP-2 and MMP-9 levels.
- Supplementation of curcumin in the daily diet may be useful for the prevention of hypertension.

Abstract

Oxidative stress plays a role in maintaining high arterial blood pressure and contributes to the vascular changes that lead to hypertension. Consumption of polyphenol-rich foods has demonstrated their beneficial role in the prevention and treatment of hypertension. Curcumin (CUR), a phenolic compound present in the rhizomes of turmeric, possesses cardiovascular protective, anti-inflammatory and antioxidant properties. The present study was designed to investigate the protective effect of CUR on 2 kidney-1clip (2K-1C)-induced hypertension, endothelial dysfunction, vascular remodeling and oxidative stress in male Sprague-Dawley rats. Sham operated or 2K-1C rats were treated with CUR at dose of 50 or 100 mg/kg/day (or vehicle). After 6 weeks of treatment, CUR ameliorated hemodynamic performance in 2K-1C hypertensive rats ($P < 0.05$), by reducing blood pressure, increasing hindlimb blood flow and decreasing hindlimb vascular resistance. Hemodynamic restoration was associated with a reduction in plasma angiotensin converting enzyme level. Endothelium-dependent vasorelaxation, in response to acetylcholine, of aortic rings isolated from 2K-1C hypertensive rats-treated with CUR was significantly increased ($P < 0.05$). CUR also attenuated hypertension-

induced oxidative stress and vascular structural modifications. These effects were associated with elevated plasma nitrate/nitrite, upregulated eNOS expression, downregulated p47phox NADPH oxidase and decreased superoxide production in the vascular tissues. The overall findings of this study suggest the mechanisms responsible for the antihypertensive action of CUR in 2K-1C hypertension-induced endothelial dysfunction and vascular remodeling involve the improvement NO bioavailability and a reduction in oxidative stress.

Keywords: curcumin, 2K-1C hypertension, endothelial dysfunction, nitric oxide, oxidative stress, vascular remodeling

Introduction

Hypertension is a risk factor for cardiovascular disease that is strongly associated with vascular dysfunction and remodeling [1-3]. In both animal models and in man, increased blood pressure has been associated with oxidative stress in the vascular system which, in turn, may be both a cause and an effect of hypertension [4]. Reactive oxygen species (ROS) play a physiological role in controlling endothelial function, vascular tone and cardiac function, and a pathophysiological role in inflammation, hypertrophy, proliferation, apoptosis, migration, fibrosis and angiogenesis, all of which are important processes contributing to endothelial dysfunction, increased contraction of vascular smooth muscle and structural remodeling causing increased peripheral resistance and elevated blood pressure [5-7].

Previous studies have demonstrated that a group of zinc-endopeptidases called matrix metalloproteinases (MMPs) play an important role in vascular dysfunction and remodeling in many types of cardiovascular disease including hypertension [8-10]. Increased MMP activation leads to the degradation of extracellular matrix (ECM) proteins in the blood vessels and promotes the migration and proliferation of vascular smooth muscle cells (VSMCs) [11]. ROS-

induced MMP-2 and MMP-9 activation have been of particular interest in investigations into the role of vascular remodeling in hypertension [8,12-13].

In 2K-1C renovascular hypertension, development and maintenance of increased pressure is not only due to angiotensin II (Ang II)-induced vasoconstriction, released through activation of the renin angiotensin aldosterone system (RAAS), but is also associated with oxidative stress, endothelial dysfunction and vascular remodeling [14]. It has been demonstrated that excessive vascular NADPH oxidase expression, induced by Ang II, leads to increased superoxide production ($O_2^{\cdot-}$) and contributes to endothelial nitric oxide synthase (eNOS) downregulation and endothelial dysfunction [14-15]. Moreover, Ang II activation in 2K-1C hypertension also promotes vascular remodeling as indicated by arterial wall thickening and increased MMP-2 expression and activity [9,16].

Previous studies have shown that increased production of oxidants, reduced nitric oxide (NO) bioavailability, and reduced activity of antioxidants in the vascular system are involved in hypertension and cardiovascular disease [17-18]. Interestingly, treatment with tempol and the polyphenol-rich plant *Euterpe oleracea* Mart. were associated with decreased concentration of $O_2^{\cdot-}$ in vascular tissues and inhibition of MMP-2-induced vascular changes in 2K-1C hypertension [12,19]. These results and those of many other studies in which polyphenols have been used in the treatment of cardiovascular disease [20] suggest that administration of antioxidants may be useful for the treatment of hypertension.

Polyphenols play an important role in the maintenance of health and prevention of diseases. CUR (diferuloylmethane) is an active ingredient of polyphenolic curcuminoids extracted from the rhizomes of turmeric (*Curcuma longa* Linn) of the Zingiberaceae family. CUR has been well recognized as a dietary spice for centuries and its pharmacological activity

have been studied in various animal models and clinical investigations into, among others, its anti-inflammatory, anti-cancer, anti-diabetic, anti-hypertensive, anti-dementia and antioxidant properties [21-26]. Previous studies have demonstrated that CUR inhibits VSMC migration and proliferation and collagen synthesis in vascular cell cultures stimulated with platelet-derived growth factor and in the experimental model of L-NAME-induced hypertension [27-28]. Furthermore, recent studies have shown that CUR reduces oxidative stress in a rat model of L-NAME hypertension [23,27]. However, there are no reports about the activity of CUR in 2K-1C hypertension-induced endothelial dysfunction and vascular remodeling. Therefore, the aim of this study was to determine whether the antioxidant CUR could reverse the hypertension, endothelial dysfunction, vascular structural changes and remodeling induced in 2K-1C hypertensive rats.

Material and Methods

Animals and Treatments

We used the model of 2K-1C renovascular hypertension in male Sprague-Dawley rats. The experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee of Khon Kaen University (AEKKU 66/2555). The CUR (total curcuminoids > 98% by HPLC) was generously provided by the Research and Development Institute, The Government Pharmaceutical Organization (Bangkok, Thailand).

Male Sprague-Dawley rats weighing between 160 and 180 g, obtained from the National Laboratory Animal Center, Mahidol University, Salaya (Nakornpathom, Thailand), were used in this study. The animals were housed at The Northeast Laboratory Animal Center (Khon Kaen University, Thailand) and maintained on a 12-h dark/light cycle at room temperature (25 ± 2 °C) with free access to standard rat chow and water. After one week of acclimatization, rats were

anesthetized with pentobarbital sodium (60 mg/kg i.p.). Hypertension was induced by clipping the left renal artery with a silver clip (spacing, 0.2 mm). Sham-operated rats underwent the same surgical procedure except for the clip placement. After recovery from surgery for five days, animals were randomly assigned to five groups of sixteen animals in each. Ten animals were used for functional and biochemical evaluations and the aortas of the remaining six were fixed in formaldehyde and used for morphometric analysis. Groups 1 and 2 were sham operated rats that received propylene glycol (PG), a vehicle, at 1.5 ml/kg/day (group 1), and CUR 100 mg/kg/day by gavage (group 2). Groups 3-5 were clipped rats that received PG alone, CUR 50 and 100 mg/kg/day by gavage, respectively. The doses of CUR were based on the results of a previous study which showed that they were sufficient to reduce blood pressure in L-NAME hypertensive rats [23]. Based on the use of body surface area for dose translation from rat to human [29], the high dose of CUR (100 mg/kg) used in this study corresponds approximately to a 950 mg dose for a 60 kg person. It has been reported that oral administration of CUR at dose up to 8 g/day for 3 months did not cause any toxicity in humans [30].

Treatment was started after the five day post-surgery recovery period and maintained for six weeks. Systolic blood pressure (SBP) was measured on the day before renal artery clipping (regarded as baseline data), five days after surgery and weekly during treatment, using tail-cuff plethysmography (Blood pressure analyzer, model 179; IITC Life Science Inc., CA, USA).

Hemodynamic Measurements

After six weeks of treatment, rats were anesthetized with pentobarbital sodium (60 mg/kg; i.p.) and a tracheotomy was performed to allow spontaneous breathing. The femoral artery was cannulated and connected to a pressure transducer for monitoring blood pressure (BP) and heart rate (HR), using the Acqknowledge data acquisition system, (BIOPAC Systems Inc.,

California, USA). Hindlimb blood flow (HBF) was continuously measured by placing a cuff type electromagnetic flow probe (4 mm internal circumference) around the abdominal aorta, accessed through a laparotomy, just below the branches of renal arteries. The electromagnetic flow probe was connected to an electromagnetic flowmeter (Carolina Medical Electronics, North Carolina, USA). Hindlimb vascular resistance (HVR) was calculated from the mean arterial pressure (MAP) divided by HBF. Blood samples were withdrawn from the abdominal aorta for assays of angiotensin converting enzyme (ACE) level and oxidative stress makers. Following the hemodynamic measurements, the aortas and carotid arteries were rapidly excised from the animals and used for vascular reactivity testing, analysis of eNOS and p47^{phox} NADPH oxidase expression, and O₂^{•-} production.

Vascular Reactivity Testing

The isolated thoracic aortas were cleaned of surrounding fat and connective tissues, and cut into rings 4 mm in length. The aortic rings were mounted for isometric tension recording in the chambers of an organ bath containing modified Krebs salt solution of the following concentration (mM): NaCl 119, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.18, Glucose 11, NaHCO₃ 25 and CaCl₂·2H₂O 2.5, which was maintained at 37 °C, pH 7.4, and bubbled with 95% O₂ and 5% CO₂. The system was connected to an isometric force transducer and aortic responses were recorded on a computer using LabChart V 7.0 (PowerLab System, AD Instruments, Australia). A load of 1 g was initially applied to the aortic ring which was equilibrated for 90 min. To examine endothelium-dependent and endothelium-independent relaxation, the cumulative concentration-response curves to ACh and sodium nitroprusside (SNP) ranging from 10⁻⁹ to 10⁻⁵ M were obtained after pre-contraction with phenylephrine (1 μM).

Assays of O₂^{•-} Production, Malondialdehyde, Protein Carbonyl and Nitrate/Nitrite

The isolated carotid arteries were immediately placed in ice-cold Krebs salt solution and dissected free of fat and adhering tissue. The arterial segments were cut into rings 4 mm in length and incubated with Krebs salt solution. $O_2^{\cdot -}$ production in the carotid artery was measured using a lucigenin-enhanced chemiluminescence method as previously described [23]. Plasma malondialdehyde (MDA) was determined by measuring thiobarbituric acid reactive substances and oxidizing protein damage was assessed by measuring the formation of carbonyl groups, by reaction with 2,4-dinitrophenylhydrazine, again following a previously described method [31]. The level of plasma nitrate/nitrite, the end products of NO metabolism, was measured as previously described [23].

Assay of Angiotensin Converting Enzyme Activity

The level of ACE activity in plasma was determined using the *o*-phthalaldehyde (OPA)-chromogenic reaction for histidyl-leucine following a previously described method [32] with slight modifications. In brief, 25 μ L sample of plasma and 50 μ L of 15 mM Hip-His-Leu solution were mixed in 100 μ L buffer (20 mM sodium borate and 300 mM NaCl, pH 8.3), and incubated at 37 °C for 30 min. Background absorbance was determined from a plasma sample diluted in 150 μ L buffer. The color reaction was formed by adding OPA reagent (1 mM OPA and 1 mM 2-meraptoethanol in buffer containing 0.1M sodium borate and 0.2 M NaOH, pH 12). After incubation at room temperature for 20 min, the absorbance was measured at 390 nm with a spectrophotometer (Ultrospec 6300 *pro*. Bichrom Ltd., UK). Results were calibrated according to a standard curve of ACE solution (15- 120 mU/mL).

Western Blot Analysis

Western blotting was performed on aortic homogenates in order to detect the protein expressions of eNOS and the p47^{phox} NADPH oxidase subunit following previously described

methods [23,33]. In brief, the rat thoracic aortas were homogenized in cell lysis buffer (Cell Signaling Technology, Inc., MA, USA) and centrifuged at 4 °C and 12000 rpm for 30 min. The supernatant was collected and the protein content was analyzed by the Bradford dye-binding method [34]. A total of 30 µg of protein per sample was separated on 10% sodium dodecyl sulfate polyacrylamide gel by an electrophoresis system. The proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane, blocked with 5% skimmed milk in Tris buffer saline with 0.1% Tween-20 and incubated overnight with primary antibody of either mouse monoclonal anti-eNOS (1:2000 dilution; BD Biosciences, CA, USA) or mouse monoclonal anti-p47^{phox} (1:1500 dilution; Santa Cruz Biotechnology, Indian Gulch, CA, USA). The membranes were repeatedly washed with TBS and incubated for 2 h at room temperature with the secondary antibody horseradish peroxidase goat anti-mouse IgG (1:2000 dilution; Santa Cruz Biotechnology). The blots were incubated in the enhanced chemiluminescent substrate solution (Thermo Fisher Scientific Inc., IL, USA). The intensity of specific eNOS or p47^{phox} NADPH oxidase and β-actin bands were imaged and captured using a digital imaging system for quantitative imaging of gels and blots (Imagequant 400, GE Healthcare Pittsburgh, PA, USA). The intensity of the bands was normalized to β-actin expression from the same sample. The intensities were expressed as percentages of those from the aorta of normal controls.

Morphometric Analysis and Composition of the Vascular Wall

The animals were sacrificed by an overdose of anesthetic drug, thoracic aortas were cleaned of loosely adhering fibrous tissue and fixed with 4% phosphate-buffered formaldehyde. The vessels were cut to 5 mm in length, and embedded vertically in paraffin blocks using standard histological procedures. Five-µm-thick sections were cut and stained with hematoxylin and eosin (H&E), Picrosirius Red, and Miller's elastic stain to determine the number of VSMCs

and the area fraction of collagen and elastin in the aortic media layer, these being taken as measures of their concentration in the specimens. Medial cross-sectional area (CSA) was calculated by subtracting the lumen internal area (A_i) from the external area (A_e), which was measured in tissue sections ($\times 40$). The external radius (R_e) and the internal radius (R_i) were calculated as the square root of A_e/π and A_i/π , respectively. Medial thickness (M) was calculated as R_e minus R_i . Finally, media to lumen ratio (M/L) was calculated as the wall thickness divided by radius of the lumen [12].

The stained sections were examined with light microscopy (Nikon ECLIPSE Ni-u, Nikon Instruments Inc., NY, USA) and the images were captured at $\times 200$ with a digital microscope camera (Nikon DS-Ri1 Camera). Twelve images from three non-consecutive sections per animal were captured and used to count the number of VSMCs and measure the lumen and medial areas and the areas within the media of stained collagen and elastin by means of image analysis software (Image-Pro Plus, Media Cybernetics, MD, USA). The number of VSMCs was obtained by counting their nuclei of in the sections stained with H&E. The area fraction of collagen or elastin in the aortic medial layer was assessed by automatically counting thresholded pixels stained with Picrosirius Red or Miller's elastic staining and dividing by the total number of medial pixels. All measurements were made by one observer and preliminary observations of intra-observer repeatability showed a coefficient of variation of less than 5% for all estimations of thresholded area.

Immunohistochemistry

To determine the amount of smooth muscle α -actin (SMA) and the levels of MMP-2 and MMP-9 in the thoracic aortas, the de-waxed aortic sections were stained with antibodies specific to each: (ab5694; Abcam, for SMA, ab37150; Abcam, for MMP-2 and AB19016; Millipore, for

MMP-9) and the R.T.U. Vectastain ABC kit (Vector Laboratories, Inc., CA, USA) as recommended by the manufacture. The percentage of immunohistochemically stained SMA, MMP-2 or MMP-9 in the aortic wall was quantified in a similar way as collagen and elastin, by counting the thresholded pixels stained for SMA, MMP-2 or MMP-9 using the Image-Pro Plus Program.

Assessment of the immunoreactivity for both MMP-2 and MMP-9 was quantified by grading the immunohistochemical staining intensity [12] into one of 3 categories: weak (+1) for light brown to yellow, moderate (+2) for brown, and strong (+3) for dark brown. The level of MMP-2 or MMP-9 was expressed in arbitrary units, obtained by multiplying the percentage of area fraction and the intensity score.

Statistical Analysis

Results are expressed as mean \pm standard error of the mean (SEM), and n refers to the number of animals used. Multiple comparisons between groups were made with one-way analysis of variance (ANOVA) followed by Student Newman–Keuls post-hoc test. A probability value < 0.05 was considered significant.

Results

CUR Attenuates Hypertension and Hemodynamic Disturbances

Systolic blood pressure (SBP) was significantly increased in 2K-1C rats after renal artery clipping for five days and continued to increase throughout the study period ($P < 0.05$). When compared to untreated controls, CUR at the two doses used moderates the SBP in 2K-1C rats in a dose dependent manner ($P < 0.05$, Fig. 1). With regard to the hemodynamic data, increased arterial blood pressure (systolic, diastolic and mean arterial blood pressure), decreased HBF and increased HVR were found in 2K-1C hypertensive rats, and this hemodynamic disturbance was

significantly alleviated after CUR treatment ($P < 0.05$, Table 1). The hemodynamic statuses of the sham groups with or without CUR treatment were not different (Table 1), suggesting that CUR had no hypotensive effect on normotensive animals. Moreover, heart rates and body weight were not different among all groups throughout the duration of experiment (Table 1).

CUR Improves Hypertension-Induced Endothelial Dysfunction

To examine the effect of CUR on 2K-1C hypertension-induced endothelial dysfunction, aortic rings were isolated and their vascular reactivity was assessed in organ bath experiments. Fig. 2 shows endothelially-dependent and -independent vasorelaxation induced by ACh and SNP (dose range: 10^{-9} - 10^{-5} M). A significant impairment of the vascular response to ACh was found in the aortic rings of 2K-1C rats when compared with those isolated from the sham group ($P < 0.05$, Fig. 2A). In contrast, the vasorelaxant responses to SNP were not different among the groups (Fig. 2B). These results reflect endothelial dysfunction in 2K-1C hypertension. The impairment of endothelial vasorelaxation is confirmed by a reduction of plasma nitrate/nitrite concentration ($P < 0.05$, Table 2) and a downregulation of eNOS protein expression in the aortas of 2K-1C hypertensive rats ($P < 0.05$, Fig. 3). Treatment with CUR in a dose-dependent manner significantly enhanced endothelial-dependent vasorelaxation induced by ACh ($P < 0.05$, Fig. 2A), but had no effect on the endothelial-independent response induced by SNP (Fig. 2B). The improvement of endothelial dysfunction in 2K-1C rats-treated with CUR was associated with increased nitrate/nitrite levels ($P < 0.05$, Table 2) and also upregulated eNOS expressions ($P < 0.05$, Fig. 3).

CUR Improves Hypertension-Induced Vascular Structural Changes and Remodeling

2K-1C renovascular hypertension was associated with arterial wall hypertrophy, with significant increases in wall thickness, aortic medial CSA and M/L ratio after 6 weeks of

hypertension compared to sham operated animals ($P < 0.001$, Fig. 4A, B and C), whereas there were no significant differences in the lumen areas between the 2K-1C and the sham groups (Fig. 4D). Cell counting showed increased an increased number of VSMCs per medial CSA, suggesting medial hyperplasia in the 2K-1C group compared with the sham group ($P < 0.05$, Fig. 5A). In addition, a significant increase in the number of VSMCs and the relative amounts of SMA, collagen and elastin in the aortic wall of 2K-1C rats were also seen ($P < 0.05$, Fig. 5B, C and D). Treatment with CUR prevented the morphological changes of the aortic wall seen in the 2K-1C hypertensive rats ($P < 0.05$, Fig. 4 and 5) although only at the high dose did it significantly moderate the increase in SMA, elastin and collagen associated with the 2K-1C treatment. It was found that the contents of SMA, collagen and elastin in the aortic media of sham controls were unaltered by vehicle or CUR treatment.

Representative immunohistochemistry photomicrographs showing MMP-2 and MMP-9 staining in the aortas are seen in Figure 6A and B. We found higher MMP-2 (Fig. 6A) and MMP-9 (Fig. 6B) levels in the aortas of 2K-1C hypertensive rats compared with sham controls ($P < 0.05$). CUR, especially at high dose, significantly attenuated the 2K-1C hypertension-induced increase in MMP-2 and MMP-9 levels in the aortic walls, whereas no changes in the MMP levels were observed in the sham + CUR group ($P < 0.05$, Figs. 6A and B).

CUR Reduces ACE Activity and Oxidative Stress

Plasma ACE levels were increased in 2K-1C rats compared to sham-operated animals ($P < 0.05$, Table 2). Treatment with CUR 100 mg/kg significantly reduced the plasma ACE level ($P = 0.05$, Table 2). CUR did not affect plasma ACE activity in sham-operated controls.

Increased superoxide level was found in the arteries of 2K-1C rats when compared with sham groups ($P < 0.05$, Table 2). In parallel with these results, we found increased p47^{phox}

NADPH oxidase subunit in the aortas from the clipped animals ($P < 0.05$, Fig. 7), indicating increased ROS production in renovascular hypertension. Further confirming increased oxidative stress associated with hypertension, we found that 2K-1C rats had higher levels of plasma MDA and protein carbonyl than respective sham controls ($P < 0.05$, Table 2). Treatment with CUR significantly attenuated superoxide production, MDA and protein carbonyl levels in 2K-1C rats ($P < 0.05$, Table 2), and these deleterious effects were associated with a downregulation of p47^{phox} NADPH oxidase subunit (Fig. 7).

Discussion

The main findings of this study are that treatment with CUR blunts the increase in blood pressure and improves endothelial dysfunction and vascular remodeling in 2K-1C renovascular hypertension. These beneficial effects of CUR may contribute to the improvement of hemodynamic status, attenuation of vascular structural alterations, reduction in ACE and MMP levels, alleviation of oxidative stress, and increase in NO bioavailability.

In agreement with previous observations [16,35], we found increased peripheral vascular resistance and reduced endothelium-dependent vasorelaxation-induced by ACh in 2K-1C hypertensive rats. These effects were evidently associated with a reduction in blood flow and suppression of eNOS expression, confirming the presence of endothelial dysfunction and reduced NO bioavailability in this hypertensive model.

Endothelial dysfunction is often associated with pronounced oxidative stress that is due to, at least in part, to increase $O_2^{\bullet-}$ from NADPH oxidase and increased degradation of NO by reaction with $O_2^{\bullet-}$, thereby, reducing its bioavailability [15,18]. Thus, eNOS becomes uncoupled, causing $O_2^{\bullet-}$ generation rather than NO production. NADPH oxidase is a multi-subunit enzymatic complex which has been shown to be one of the main sources of $O_2^{\bullet-}$ in the vascular wall [36]. Upregulation of this oxidase, in particular p47^{phox} contributes to the pathogenesis of

oxidative stress in several animal models of hypertension [15,33,37]. In this study, increased $O_2^{\bullet-}$ production was associated with increased p47^{phox} expression in the vascular wall of 2K-1C rats. Moreover, increased oxidative stress as indicated by enhanced MDA and protein carbonyl levels was also found in 2K-1C hypertension. Therefore, reduced NO bioavailability and enhanced oxidative stress in 2K-1C hypertension lead to less vasodilation, thereby contributing to endothelial dysfunction and increased vascular resistance in hypertension. It has been reported that polyphenols present in fruits and vegetables are able to modulate the production of NO in vascular endothelium, contributing to the prevention of endothelial dysfunction [20]. We found that CUR, a strongly phenolic compound, restored hemodynamic status, enhanced ACh-induced vasodilation, upregulated eNOS expression and increased nitrate/nitrite concentration in 2K-1C hypertensive rats. Therefore, our findings suggest the beneficial effects of CUR on the improvement of vascular dysfunction and alleviation of oxidative stress in 2K-1C hypertensive rats. Further support for this suggestion comes from the observation that CUR treatment inhibits the increases in MDA and protein carbonyl levels, enhanced $O_2^{\bullet-}$ production and p47^{phox} expression in the arteries of 2K-1C hypertensive rats.

Hypertension is strongly implicated in the progression of functional and structural alterations in the vascular system and vascular remodeling is an adaptive response to elevation of BP. As we have found in this study, the process of vascular remodeling leads to increased aortic wall thickness which appears to be due to changes in VSMCs and ECM components [9,38]. Consistent with this idea, we observed arterial wall hypertrophy, increased collagen and elastin deposition, VSMC hyperplasia and hypertrophy (as suggested by the increased area x staining-intensity product) in the aortas of 2K-1C rats, and that these alterations are reversed by dietary supplementation with CUR. Since the lumen area of the aortas of 2K-1C rats did not alter, this

suggests that the aortic remodeling is eccentric, thus leaving the resistance of the aorta unchanged.

The main function of MMPs, a family of structurally related, zinc-containing enzymes, has been reported to be the degradation and removal of ECM components from the tissue, encouraging VSMC migration and proliferation and the production of adhesion molecules [11,39]. The gelatinases MMP-2 and MMP-9, which cleave the basement membrane, type IV collagen, laminin and elastin, are the most studied MMPs in the vasculature [5]. Increased expression and activity of MMPs, especially MMP-2 and MMP-9 have been reported to induce vascular changes in animal models of hypertension [8-9,12], suggesting that MMP activation contributes to vascular remodeling associated with hypertension. The apparently paradoxical result that the scleroprotein content is raised in the hypertensive animals is associated with raised levels of the proteolytic enzymes MMP-2 and MMP-9 may be explained by the concept that hypertension-induced vascular remodeling is associated with increased MMP activity [8-9,12,40]. Furthermore, there is evidence that MMPs are actually directly involved in the blood pressure increase [41-42]. Consistent with our results, increased MMP-2 expression and excessive collagen deposition have been found in the arterial wall of 2K-1C hypertensive rats, and the antioxidant therapy inhibited hypertension-induced upregulation of MMP-2 expression [9]. Moreover, it has been suggested that increased ROS levels in 2K-1C rats may produce vascular changes that are mediated by MMPs [43]. Our results support the interplay between MMP-2, MMP-9 and the extracellular matrix proteins during the remodeling process. However, more work is needed to identify their exact role.

The expression of MMPs can be activated by Ang II [13] and ROS involved in preservation of MMP latency by reaction with thiol groups [44]. In fact, it has been shown that,

either increased Ang II or excessive vascular $O_2^{\cdot-}$ production are implicated in vascular cell growth, inflammation, increased ECM deposition, vascular remodeling, promotion of MMP-2 expression and activity, and also reduced tissue inhibitor of MMP levels in 2K-1C rats [12,45]. This study showed an increase in MMP-2 and MMP-9 expressions in the arterial wall of 2K-1C rats and these alterations are associated with increased ACE activity, thus indicating an important activation of the RAAS in the 2K-1C model [46]. Interestingly, we found that CUR significantly attenuated MMP-2 and MMP-9 expressions and reduced ACE activity in the clipped rats. Therefore, one of the possible mechanisms to explain the antihypertensive effect of CUR may be related to ACE- inhibitory activity since many ACE inhibitors inhibit MMPs. In addition, antioxidants can downregulate MMPs as previously shown in the 2K-1C hypertensive model [12], suggesting a major role of ROS in MMP activity.

Previous studies have demonstrated that CUR possesses antioxidant activity by directly scavenging free radicals and hydrogen donors and that it also exhibits anti-inflammatory properties [12,22,26]. Our previous results on L-NAME hypertensive rats demonstrated that CUR effectively restored NO production in association with a reduction of the over production of $O_2^{\cdot-}$ in vascular tissue [23]. In addition, treatment with the antioxidant tempol and the polyphenol-rich plant *Euterpe oleracea Mart*, was associated with a downregulation of vascular MMP-2 expression and activity in 2K-1C hypertensive rat model [12,19]. The antioxidant effect of CUR is probably mediated by phenolic compounds in turmeric since recent evidence has demonstrated that polyphenols inhibit expression and activation of MMP-2 in VSMCs [7]. Increased MMP-2 expression and activity lead to less vasodilation or increased vasoconstriction, contributing to endothelial dysfunction and increased vascular resistance in hypertension [16,47-48].

Some limitations of this study should be taken into consideration. Firstly, histomorphometric analysis of scleroprotein can suffer from poor reproducibility unless suitable precautions are taken. With regard to the immunohistochemistry, we have optimized the experimental conditions to generate a strong and specific signal for the antigen of interest. The measurements were performed in the same batch and stained together with a positive control at the same time. Secondly, we note that for untreated 2K-1C animals the total area fraction of stained materials (elastin, collagen and the metalloproteinases) is slightly greater than 100%. We conclude, as others have done, that some of the components might be doubly stained. However, we believe that the results of the SMA staining together with the elastin and collagen data, while not providing absolute values of aortic composition, allow inter-group comparisons and clearly show how the changes in ECM proteins, VSMC and MMP content associated with hypertension are reduced in the animals treated with curcumin.

In conclusion, the results of this study suggest that CUR moderates development of hypertension, endothelial dysfunction, vascular remodeling and oxidative stress in 2K-1C renovascular hypertension. The mechanisms of these effects might involve an increase in antioxidant activity and NO bioavailability, together with a decrease in ACE, MMP-2 and MMP-9 levels after CUR treatment. Our findings suggest that supplementation of CUR in the daily diet may be useful for the prevention of hypertension. Epidemiological and or clinical studies are needed to support this contention. Although the effects of dietary CUR on acute myocardial infarction after coronary artery bypass grafting, rheumatoid arthritis and cancer have been investigated [21,49-50], to our knowledge no such study into its antihypertensive effects have been performed to date.

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Figure legends

Fig. 1. Changes in systolic blood pressure before and after renal artery clipping measured by tail-cuff plethysmography in all experimental groups. Curcumin, CUR; Systolic blood pressure, SBP. In this and all subsequent plots, data are means \pm SEM. (n=10/group). * $P < 0.05$ versus sham-operated group, † $P < 0.05$ versus 2K-1C group and ‡ $P < 0.05$ versus 2K-1C+CUR50 group.

Fig. 2. Endothelial-dependent vasorelaxation induced by acetylcholine (A) and endothelial-independent relaxation induced by sodium nitroprusside (B) in aortic rings pre-contracted with phenylephrine (1 μ M). Curcumin, CUR. * $P < 0.05$ versus sham-operated group, † $P < 0.05$ versus 2K-1C group and ‡ $P < 0.05$ versus 2K-1C+CUR50 group.

Fig. 3. Effect of curcumin on eNOS protein expression in the aortas of all experimental groups. Height of bars represents densitometric values normalized to the corresponding β -actin. Curcumin, CUR. * $P < 0.05$ versus sham-operated group, † $P < 0.05$ versus 2K-1C group.

Fig. 4. Effect of curcumin on structural modifications induced in the aortas of all experimental groups. Representative photomicrographs of the aortic samples ($\times 40$) stained with H&E are shown together with histomorphometric data: the wall thickness (A), cross-sectional area (CSA) of the medial layer (B), media to lumen ratio (M/L) (C), and the lumen area (D) of the aortas. Curcumin, CUR. n=6/group. * $P < 0.05$ versus sham-operated group, † $P < 0.05$ versus 2K-1C group and ‡ $P < 0.05$ versus 2K-1C+CUR50 group.

Fig. 5. Effect of curcumin on vascular smooth muscle cells, smooth muscle actin, collagen and elastin content in the aortas of all experimental groups. The representative photomicrographs of the aortic samples ($\times 200$) were stained with H&E, Picrosirius Red and Miller's elastic stain for assessment of the number of vascular smooth muscle cells per cross-sectional area (panel A) and the area fractions of collagen (panel B), elastin (panel C) and smooth muscle actin (panel D) in

the aortic medial layer. Curcumin, CUR; cross-sectional area, CSA; smooth muscle actin (SMA). n=6/group. * $P < 0.05$ versus sham-operated group, † $P < 0.05$ versus 2K-1C group and ‡ $P < 0.05$ versus 2K-1C+CUR50 group.

Fig. 6. Effect of curcumin on MMP-2 (panel A) and MMP-9 (panel B) localization in the aortas of all experimental groups. Curcumin, CUR. n=6/group. * $P < 0.05$ versus sham-operated group, † $P < 0.05$ versus 2K-1C group and ‡ $P < 0.05$ versus 2K-1C+CUR50 group.

Fig. 7. Effect of curcumin on p47^{phox} NADPH oxidase subunit in the rat aortas. Height of bars represents densitometric values normalized to the corresponding β -actin. Curcumin, CUR. * $P < 0.05$ versus sham-operated group, † $P < 0.05$ versus 2K-1C group.

Table 1 Effect of CUR on hemodynamic status in all experimental groups.

Variable	Sham	Sham+CUR100	2K-1C	2K-1C+CUR50	2K-1C+CUR100
BW (g)	400.1±2.6	404.4±2.1	391.4±10.0	391.8±7.5	391.5±4.9
SBP (mmHg)	127.7±3.2	125.3±2.8	203.9±2.9 ^a	177.2±7.0 ^{a,b}	165.5±4.8 ^{a,b}
DBP (mmHg)	86.8±2.5	84.5±2.4	137.7±3.6 ^a	119.9±6.8 ^{a,b}	111.6±4.4 ^{a,b}
MAP (mmHg)	103.4±2.8	101.3±2.5	165.6±2.8 ^a	144.7±7.3 ^{a,b}	134.3±4.6 ^{a,b}
HR (beats/min)	397.7±8.0	388.1±6.6	407.1±9.2	416.3±13.3	411.0±13.5
HBF (ml/min/100 g tissue)	6.0±0.4	6.1±0.4	3.5±0.4 ^a	4.8±0.4 ^a	5.6±0.5 ^{a,b}
HVR (mmHg/mL/min/	18.2±0.4	16.9±1.1	44.3±6.6 ^a	31.8±2.4 ^{a,b}	28.1±2.6 ^{a,b}

100 g tissue)

Data are means \pm SEM. (n=10/group), ^a $P < 0.05$ versus sham-operated group, ^b $P < 0.05$ versus 2K-1C group. CUR, curcumin; BW, body weight; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure; HR, heart rate; HBF, hindlimb blood flow; HVR, hindlimb vascular resistance. All hemodynamic variables refer to measurements made on anaesthetized animals 6 weeks after the start of the treatment period.

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Table 2 Effect of CUR on the levels of oxidative stress markers, nitrate/nitrite and ACE activity in all experimental groups.

Parameter	Sham	Sham+CUR100	2K-1C	2K-1C+CUR50	2K-1C+CUR100
Vascular O ₂ ⁻ production (Count/min/mg dry weight)	53.2±5.6	55.2±9.6	127.4±10.0 ^a	91.6±6.1 ^{a,b}	84.3±6.9 ^{a,b}
Plasma MDA (μM)	4.80±0.30	4.82±0.43	8.98±0.57 ^a	6.44±0.46 ^{a,b}	5.74±0.18 ^{a,b,c}
Plasma protein carbonyl (nmol/mg protein)	1.18±0.04	1.16±0.12	2.33±0.24 ^a	1.71±0.23 ^{a,b}	1.51±0.11 ^{a,b}
Plasma nitrate/nitrite (μM)	18.6±1.8	18.7±1.5	9.3±0.9 ^a	13.7±1.1 ^{a,b}	15.5±1.7 ^{a,b}
Plasma ACE-activity (mU/mL)	79.8±4.8	81.5±2.3	132.0±5.6 ^a	121.9±7.6 ^a	101.0±6.3 ^{a,b,c}

Curcumin, CUR; Malondialdehyde, MDA; Angiotensin converting enzyme, ACE. Data are means ± SEM. (n=10/group), ^a*P* < 0.05 versus sham-operated group, ^b*P* < 0.05 versus 2K-1C group, and ^c*P* < 0.05 versus 2K-1C+CUR50 group.

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