ORIGINAL ARTICLE

Tetrahydrocurcumin alleviates hypertension, aortic stiffening and oxidative stress in rats with nitric oxide deficiency

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Tetrahydrocurcumin (THC), a major metabolite of curcumin, possesses strong antioxidant and cardioprotective properties. However, the activities of THC in hypertension and its associated complications remain unknown. The aim of this study was to investigate the effect of THC on hemodynamic status, aortic elasticity and oxidative stress in rats with N-nitro-L-arginine methyl ester (L-NAME)-induced hypertension. Hypertension was induced in male Sprague-Dawley rats by administration of L-NAME (50 mg kg^{-1} body weight) in drinking water for 5 weeks. THC at a dose of 50 or 100 mg kg^{-1} per day was administered daily during the fourth and fifth weeks when the hypertensive state had been established. The effects of THC on hemodynamics, aortic elasticity, endothelial nitric oxide synthase (eNOS) protein expression and oxidative stress markers were assessed. Marked increases in blood pressure, peripheral vascular resistance, aortic stiffness and oxidative stress were found in rats after L-NAME administration. THC significantly reversed these deleterious effects by reducing aortic wall thickness and stiffness. These effects were associated with increased aortic eNOS expression, elevated plasma nitrate/nitrite, decreased oxidative stress with reduced superoxide production and enhanced blood glutathione. Our results provide the first evidence that THC attenuates the detrimental effect of L-NAME by improving the hemodynamic status and aortic elasticity concomitant with reduction of oxidative stress. The present study suggests that THC might be used as a dietary supplement to protect against cardiovascular alterations under nitric oxide-deficient conditions.

INTRODUCTION

Hypertension is associated with changes in endothelial and vascular smooth muscle cell structure and function. It is widely accepted that nitric oxide (NO) plays a central role in protecting the cardiovascular system against hypertension and injury. N-nitro-L-arginine methyl ester (L-NAME), an L-arginine analogue, is the most frequently used NO synthase inhibitor in experimental animals. Chronic administration of L-NAME results in systemic arterial hypertension accompanied by an increased vascular resistance and decreased blood flow in various organs.[1] Moreover, it has been reported that NO synthase inhibitors have an effect on large arterial stiffness resembling that of chronic hypertension.[2] Therefore, chronic NO deprivation causes not only hypertension but also vascular remodeling, which is an adaptive response to the increased hemodynamic load within the vascular wall.

It has been well established that reduction in NO synthesis increases endothelial intracellular oxidative stress in animals. Several lines of evidence suggest that enhanced reactive oxygen species production is involved in the pathogenesis of the NO-deficient hypertensive model. Therefore, a unifying characteristic of L-NAME-induced hypertension is the presence of oxidative stress that participates in the maintenance of elevated arterial pressure and seems to be a common denominator underlying endothelial dysfunction in this model of experimental hypertension.[3] It has been demonstrated that nitric oxide synthase inhibition leads to increased accumulation of superoxide ($O_2^{\bullet-}$) generation,[4] activation of NADPH oxidase,[5] increased malondialdehyde concentrations in the heart and aorta[6] and enhanced vascular thromboxane B2 synthesis,[7] whereas the activities of antioxidant enzymes such as plasma superoxide dismutase[8] and hepatic glutathione peroxidase are reduced.[7] Moreover, it is found that overproduction of reactive oxygen species modulates vascular structure and function in several ways, including damaged endothelial and vascular smooth muscle cells, altered redox state and increased intracellular free calcium concentration.[9] Therefore, increased oxidative stress caused by NO deficiency may significantly contribute to the development of hypertension.

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Curcumin is a phenolic compound extracted from the powdered dry rhizome of *Curcuma longa* Linn. Several pharmacological properties of curcumin have been reported, including neuroprotective, hepatoprotective, anticarcinogenic, anti-inflammatory and antioxidant effects.[10] Tetrahydrocurcumin (THC) is one of the major metabolites of curcumin, which presents the same phenolic and β-diketo moieties as curcumin. THC has been reported to exhibit similar pharmacological properties as to curcumin. Previous studies have demonstrated that THC exhibits antioxidant activity by inhibiting the peroxidation of membrane lipid.[11] Additionally, an *in vitro* study has reported that THC possesses stronger antioxidant properties than curcuminoids, including curcumin, demethoxycurcumin and bisdemethoxycurcumin, suggesting that hydrogenation at the conjugated double bonds of the central seven-carbon chains is important for this activity.[12] Supplementation with THC reduces thiobarbituric acid reactive substance concentration in the liver and kidney of rabbits fed with a high cholesterol diet.[13] THC showed protective effects against oxidative stress by enhancing activities of key endogenous antioxidant enzymes, including superoxide dismutase, catalase and glutathione peroxidase, whereas it decreased thiobarbituric acid reactive substance and hydroperoxide formation.[14,15] Moreover, THC has shown cardioprotective effects by reducing the infarction size in an ischemic–reperfusion model of myocardial infarction.[16] This effect was associated with reduced lipid peroxidation and improved antioxidant status. Therefore, antioxidants may be beneficial for cardiovascular protection, especially against hypertension by a decrease in oxidative load in the vascular system.

Although THC has been reported to possess strong antioxidant activity, both *in vivo* and *in vitro*, information about its beneficial effects on the cardiovascular system is scarce. L-NAME-induced hypertension is a common model of experimental hypertension in animals and the model also demonstrates aspects of the chronic hypertensive state including changes in vascular structure. The present investigation was thus carried out to investigate whether THC could ameliorate the detrimental effect of L-NAME on hemodynamic disturbance and aortic stiffness in association with oxidative stress in rats by L-NAME-induced hypertension.

METHODS

Chemicals
THC (purity >99% w/w by high-performance liquid chromatography) was synthesized and provided by the Government Pharmaceutical Organization, Bangkok, Thailand. L-NAME, 5,5 dithio-bis-2-nitrobenzoic acid, EDTA, thiobarbituric acid, sodium dodecyl sulfate, glutathione (GSH), butylated hydroxytoluene, sulfanilamide dinitrophenylephrinenylhydrazine, N-1-nephtylethylenediamine dihydrochloride, 1,1,3,3-tetraethoxypropane, bromophenol blue, 2-mercaptoetanol and guanidine were purchased from Sigma-Aldrich Corp. (St Louis, MO, USA). Nitrate reductase, nicotinamide adenine dinucleotide phosphate (NADPH) and glucose-6-phosphate dehydrogenase were obtained from Roche Applied Sciences (Mannheim, Germany). Trichloroacetic acid, metaphosphoric acid, 1-methyl-2 vinyl-pyridinum trifate, lucigenin, Tween and skimmed milk were obtained from Fluka Chemika (Buch, Switzerland). Mouse monoclonal anti-endothelial nitric oxide synthase (eNOS) was obtained from BD Transduction Laboratory (San Jose, CA, USA). Anti-mouse IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals used were of analytical grade quality.

Animals
The procedures and experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee of Khon Kaen University (AEKKU01/2551). Adult male Sprague-Dawley rats weighing 200–220 g were obtained from the Animal Care Unit of The Faculty of Medicine, Khon Kaen University (Khon Kaen, Thailand). All rats were housed under constant temperature in a room with a 12-h light–dark cycle. They were fed with a standard chow diet (Chareon Pokapan Co. Ltd., Thailand).

After 1 week of acclimatization, hypertension was induced in the rats by administering L-NAME (50 mg kg^{-1} per day) in their drinking water for 5 weeks. After 3 weeks, hypertension had been established and was sustained in all animals. During the last 2 weeks, L-NAME hypertensive rats were randomly divided into three treatment groups ($n=8$–10 per group). Group 1 was intragastrically administered with polyethylene glycol, a THC vehicle, for 2 weeks. Group 2 and 3 were intragastrically administered with THC for 2 weeks at doses of 50 and 100 mg kg^{-1} per day, respectively. The age-matched rats received tap water *ad libitum* and the intragastrically administered polyethylene glycol group served as the normotensive control group. Systolic blood pressure (BP) was measured weekly in conscious rats using the noninvasive method of tail cuff plethysmography (BP analyzer, model 129, IITC, Woodlandhills, CA, USA).

Measurement of the hemodynamic status
To determine resting baseline BP and heart rate, the animals were anesthetized with an i.p. injection of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}). Body temperature was maintained at $37 \pm 2\,^\circ$C throughout the measurement period with a heating pad. A tracheotomy was performed and the animals were allowed to breathe spontaneously. The femoral artery was catheterized with polyethylene catheters filled with heparinized saline and connected to a pressure transducer for continuous monitoring of BP and heart rate using *Acqknowledge* data acquisition analysis software (BIOPAC Systems Inc., Goleta, CA, USA). Baseline values of BP and heart rate were monitored for 20 min, and a second polyethylene catheter was inserted into the femoral vein to allow i.v. drug delivery. Subsequently, hindlimb blood flow was continuously measured by opening the abdominal cavity below the kidneys and placing an electromagnetic flow probe around the abdominal aorta connected to an electromagnetic flowmeter (Carolina Medical Electronics Inc., East Bend, NC, USA). Hindlimb weight was obtained by cutting the hindlimb of the rat and weighing. Hindlimb vascular resistance was calculated from mean arterial pressure and hindlimb blood flow in 100 mg tissue units. At the end of the experimental period, rats were killed by overdose of an anesthetic drug. Blood samples were withdrawn from the abdominal aorta into EDTA tubes for the assay of oxidative stress and antioxidant markers. The thoracic aorta and carotid arteries were rapidly excised from the animal and used for analysis of eNOS protein expression and O_2^- production, respectively. In a separate set of experiments, the static elastic properties of animals in all groups were measured *in situ* after the animals were killed by overdose of the anesthetic drug.

Assessment of aortic elasticity
The aortic elasticity was determined as previously described.[17] Briefly, rats were dissected to expose the thoracic aorta. Two polyethylene catheters were inserted into the thoracic aorta, one distal to the arch and the other just below the diaphragm. The aorta was flushed with normal saline to remove any thrombi, and then a barium sulphate perfusion apparatus was attached to the thoracic cannula. The animals and the apparatus were placed underneath a video camera fitted with a macro lens. Preconditioning of the vessel was performed in order to ensure repeatability by inflating the aorta to 200 mm Hg for 30 s. After deflation, the aorta was inflated to pressures of 0, 10, 20, 30, 50, 70, 90, 110, 120, 140, 160, 180 and 200 mm Hg. Each pressure was maintained for 30 s to ensure equilibration of pressure between the perfusion apparatus and the aorta. The arterial images taken at each pressure were captured and processed with image analysis software (Image-Pro Plus, Silver Spring, MD, USA) for calculation of the external diameter of the vessel. This procedure was repeated for each inflation pressure and two diameter readings at each pressure were averaged. After completing the inflation experiment, the *in-situ* length of the thoracic aorta was measured and weighed.

To analyze the data obtained from the pressure–diameter experiment, Langewouters's equation[18] was used to fit a smooth curve to the data points. The aortic weight and the *in-situ* length were used to calculate the wall thickness (h) of the vessel from the equation $h=(D_e/2)-R_i$, where D_e is the external diameter and R_i is the internal radius. Midwall radius (R_m) was

calculated from the equation $R_m = R_e - (h/2)$, where R_e is the external radius. The wall thickness of the vessel at each pressure was normalized by dividing it by the R_m. R_m at each pressure was normalized by dividing it by the R_m at a pressure of 0 mm Hg. The functional stiffness as expressed by the functional elastic modulus (Ep) of the aorta was examined because of its importance as a determinant of large artery function and cardiac load. Ep is a measurement of the relative change in blood vessel diameter in response to a known change in pressure.[19]

Assay of vascular superoxide anion production

Vascular $O_2^{\bullet-}$ production was measured using the lucigenin-enhanced chemiluminescence method as previously described, with some modifications.[20] The carotid artery was rapidly excised, placed on ice and cleaned of the adherent fat and connective tissue. The vessel segments were cut into 3–5 mm length and incubated with Krebs-KCL buffer and allowed to equilibrate at 37 °C for 30 min. Lucigenin 100 µM was added to the sample tube and placed in a luminometer (Turner Biosystems, Sunnyvale, CA, USA). The photon counts were integrated every 15 s for 5 min, averaged and the respective background counts were subtracted. The vessels were dried for 24 h at 45 °C and weighed. The superoxide anion production in vascular tissue was expressed as relative light unit counts per min per mg dry weight.

Assay of plasma malondialdehyde

Plasma malondialdehyde concentration was quantified as thiobarbituric acid reactive substance by a spectophotometric method as previously described[21] with some modifications.[20] In brief, 150 µl plasma samples were reacted with 10% trichloroacetic acid, 5 mM EDTA, 8% sodium dodecyl sulfate and 0.5 µg ml^{-1} of butylated hydroxytoluene. The mixture was incubated for 10 min at room temperature, then 0.6% thiobarbituric acid was added, and the mixture was boiled in a water bath for 30 min. After cooling to room temperature, the mixture was centrifuged at 10 000 g for 5 min. The absorbance of the supernatant was measured at 532 nm by spectrophotometer. A standard curve was generated with appropriate concentrations of 1, 1, 3, 3-tetraethoxypropane (0.3–10 µmol l^{-1}).

Assay of plasma protein carbonyl

Oxidizing protein damage was assessed by the formation of carbonyl groups, following a previously described method[22] with some modifications.[23] This assay involves the derivatization of carbonyl groups with dinitrophenylhydrazine, which leads to formation of a stable dinitrophenyl hydrazone product. Briefly, plasma samples were incubated with and without dinitrophenylhydrazine in 3.6 M HCl for 1 h in the dark. Subsequently, protein was precipitated and then dissolved in 6 M guanidine. The carbonyl content was determined from the absorbance at 360 nm after subtraction of HCl-treated blanks by use of a molar absorption coefficient of 22 000 M^{-1} cm^{-1}. The amount of the plasma protein was analyzed by the Bradford dye-binding method.

Assay of plasma nitrate/nitrite

Accumulation of nitrate and nitrite in the blood is recognized as a measure of the oxidative products of NO. The assay of nitrate and nitrite levels was performed according to a previously described method,[24] with some modifications.[25] Plasma samples were deproteinized by ultrafiltration using centrifugal concentrations (Pall Corp., Ann Arbor, MI, USA). The nitrate in the supernatant was reduced to nitrite by nitrate reductase, and then the mixture was reacted with Griess solution (4% sulfanilamide in 0.3% napthylenediamine dihydrochloride) for 15 min. The absorbance of samples was measured on an enzyme-linked immunosorbent assay plate reader with a filter wavelength of 540 nm (Tecan GmbH, Grodig, Austria).

Western blot analysis

eNOS protein expression was determined in the aortic homogenates following a previously described method[26] with some modifications. In brief, the thoracic aorta was homogenized in lysis buffer. Protein samples were separated in sodium dodecyl sulfate-polyacrylamide gel by electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight with a monoclonal mouse antibody eNOS (1:3000 dilution; BD Transduction

Laboratory), followed by incubation with horseradish peroxidise goat anti-mouse IgG secondary antibody (1:2000 dilution; Santa Cruz Biotechnology) at room temperature for 2 h. The blots were incubated in ECL substrate solution. The intensity of specific eNOS and β-actin bands were visualized and captured by Imagequant (GE Healthcare, Pittsburgh, PA, USA). The expression of eNOS protein was normalized to β-actin expression from the same sample. The data are shown as percent of normal controls.

Assay of glutathione

Assay of total glutathione in the whole blood was performed as previously described[27] and glutathione disulfide was assayed by a previously described method with some modifications.[28] Briefly, 100 µl of whole blood was immediately reacted with 33 mM 1-methyl-2 vinyl-pyridinum trifate or distilled water and subsequently treated with 5% cold metaphosphoric acid. Optical density at 412 nm was read 10 times at 15 s intervals by using a spectrophotometer (Biochrom, Cambridge, UK). A standard curve was generated by using appropriate concentrations of standard GSH. The redox ratio was calculated as GSH/glutathione disulfide.

Statistical analysis

Results are presented as mean ± s.e.m., and n refers to the number of animals used. Statistical comparisons of the differences between data were performed using one-way analysis of variance followed by Newman–Keuls post-hoc test to show specific group differences. The relationship between quantitative variables was analyzed by regression analysis (Pearson's correlation). All analyses were performed using Sigmastat software version 3.1 (Systat Software, San Jose, CA, USA). Statistical significance was determined at a level of $P < 0.05$.

RESULTS

Effects of THC on hemodynamic changes in L-NAME-induced hypertension

At the beginning of the experiments, there were no significant differences in average baseline values of systolic pressure (SP) among all experimental groups (Figure 1). Administration of L-NAME caused a progressive increase in SP when compared with normal controls. The increased SP was already significant after the first week of L-NAME administration. Treatment with THC (50 and 100 mg kg^{-1} per day) at the fourth and fifth weeks reduced the increase in SP induced by L-NAME. It was noted that there was a trend of decreasing SP towards normal in the THC groups, whereas
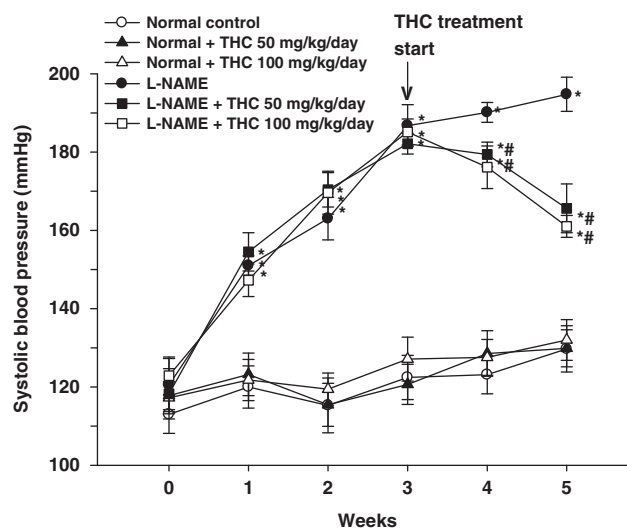

Figure 1 Effect of THC on systolic BP during L-NAME administration for 5 weeks. Results are expressed as mean ± s.e.m. ($n = 8$–10 per group). *$P < 0.05$ vs. normal control group, #$P < 0.05$ vs. L-NAME control group.

the L-NAME control was continuing to increase with time (Figure 1). Moreover, the data showed that THC had no effect on SP in normal control rats.

Chronic L-NAME administration for 5 weeks caused persistent severe hypertension as shown by a significant increase in SP, diastolic pressure and mean arterial pressure when compared with normal controls ($P < 0.05$; Table 1). It is interesting that treatment with THC at 50 and 100 mg kg^{-1} per day during the last 2 weeks significantly decreased the arterial pressure in a dose-dependent manner. Although THC therapy significantly decreased the BP of L-NAME-treated rats, the BP of these animals was still higher than that of normotensive rats, as L-NAME induced very severe hypertension. THC at either low or high dose had no effect on the BP of the normotensive groups,

suggesting that THC had no hypotensive effect on normotensive animals (Table 1). Hindlimb blood flow in L-NAME hypertensive rats was markedly lower than normal values; consequently, the calculated hindlimb vascular resistance was increased in comparison with normotensive controls. Interestingly, THC markedly decreased the hindlimb vascular resistance of L-NAME hypertensive rats ($P < 0.05$; Table 1). During 5 weeks of treatment, there were no significant changes in heart rates among all experimental groups (Table 1).

Effects of THC on aortic elasticity

Figures 2a and c show the relationship between pressure and aortic wall thickness. In all groups, the wall thickness of the thoracic aorta

Table 1 Effect of THC on hemodynamic status of rats in all experimental groups

Group	SBP (mm Hg)	DBP (mm Hg)	MAP (mm Hg)	HR (beats per min)	HBF (ml min^{-1} per 100 g tissue)	HVR (mm Hg min per 100 g tissue per ml)
Normotensive						
Vehicle	129.7 ± 5.3	89.3 ± 3.6	101.8 ± 2.7	304.9 ± 15.3	8.6 ± 0.9	12.5 ± 0.9
THC 50 mg kg^{-1} per day	126.2 ± 2.4	77.6 ± 3.2	98.5 ± 3.0	308.4 ± 16.0	8.9 ± 0.8	11.8 ± 1.1
THC 100 mg kg^{-1} per day	124.9 ± 3.6	79.7 ± 3.9	97.8 ± 2.8	324.3 ± 17.6	8.9 ± 0.6	11.4 ± 1.0
L-NAME-induced hypertension						
Vehicle	218.7 ± 3.3*	167.0 ± 1.5*	189.8 ± 1.9*	338.2 ± 12.0	2.7 ± 0.5*	83.2 ± 11.2*
THC 50 mg kg^{-1} per day	204.0 ± 4.0*,†	145.8 ± 4.0*,†	174.1 ± 1.7*,†	333.6 ± 9.8	3.4 ± 0.4*	55.2 ± 8.4*,†
THC 100 mg kg^{-1} per day	194.3 ± 1.6*,†	145.2 ± 1.5*,†	165.5 ± 1.8*,†,‡	303.3 ± 14.3	3.4 ± 0.5*	52.2 ± 7.8*,†

Abbreviations: DBP, diastolic blood pressure; HBF, hindlimb blood flow; HR, heart rate; HVR, hindlimb vascular resistance; MAP, mean arterial pressure; SBP, systolic blood pressure.
Values are expressed as mean ± s.e.m., n=8–10 per group.
*$P < 0.05$ vs. normal control group. †$P < 0.05$ vs. L-NAME control group. ‡$P < 0.05$ vs. L-NAME treated with THU 50 mg kg^{-1} per day.
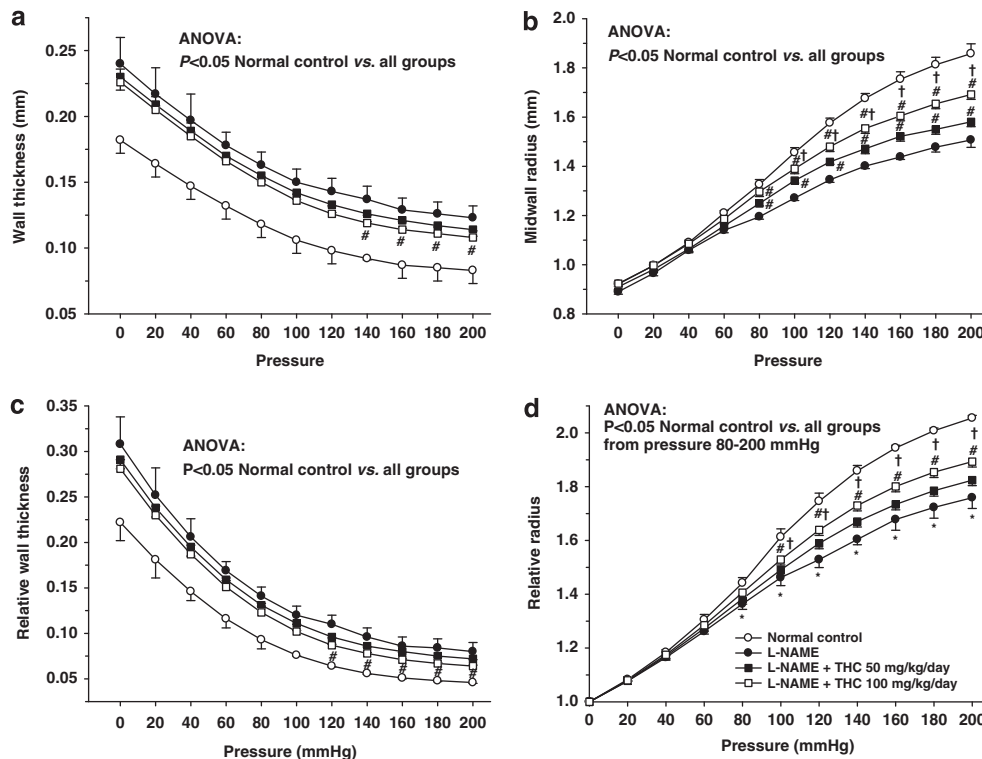

Figure 2 Effect of THC on wall thickness (a), aortic R_m (b), relative wall thickness (c) and relative radius (d) of the thoracic aorta in all experimental groups. Results are expressed as mean ± s.e.m. (n=6 per group). #$P < 0.05$ vs. L-NAME control group, †$P < 0.05$ vs. L-NAME treated with THC 50 mg kg^{-1} per day.

decreased with increasing pressure up to approximately 100 mm Hg after which there was little change. The value of wall thickness which was obtained at a pressure of 0 mm Hg was unaffected by the pressure–radius relationship and therefore, determined only by the amount of material in the vessel wall. It was found that chronic L-NAME administration produced the development of vascular structural changes as indicated by a significant increase in wall thickness of the thoracic aorta (Figure 2a), corresponding to an increase in the relative wall thickness (the ratio of the wall thickness to radius) compared with normal controls at all pressures (Figure 2c). Treatment with THC 100 mg kg^{-1} per day was associated with a significant reduction in wall thickness (Figure 2a) and also the relative wall thickness (Figure 2c). The R_m and relative radius of the thoracic aorta of L-NAME-treated rats were significantly diminished at pressures within the in-vivo range when compared with normal controls, indicating a decrease in aortic compliance after L-NAME administration ($P < 0.001$; Figures 2b and d). Treatment with THC ameliorated the adverse structural remodeling of the aortic wall induced by L-NAME in a dose-dependent manner, as shown by the increase in aortic compliance ($P < 0.001$; Figures 2b and d).

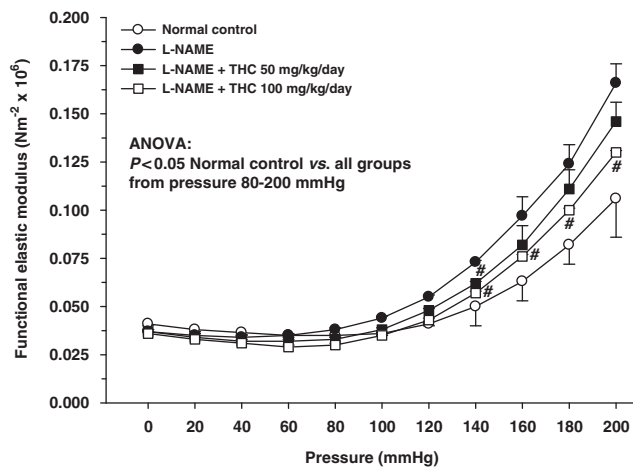Figure 3 shows changes in the functional elastic modulus as a function of pressure among all groups. In L-NAME hypertensive rats, the pressure-functional modulus curve was shifted towards the left when compared with those of normotensive controls (Figure 3), showing that the aortic wall becomes stiffer after L-NAME administration. Interestingly, THC at dose of 100 mg kg^{-1} per day significantly reduced the aortic stiffness of L-NAME hypertensive rats ($P < 0.05$, Figure 3). We found that administration of THC did not alter the aortic elastic properties of the normotensive rats (data not shown).

Effect of THC on oxidative stress and antioxidant status

Rats receiving L-NAME for 5 weeks exhibited high oxidative stress, which was indicated by a five fold increase in $O_2^{\bullet-}$ production in the vascular tissues over the normotensive control rats ($P < 0.05$, Table 2). Treatment with THC at a dose of 50 or 100 mg kg^{-1} significantly decreased the vascular $O_2^{\bullet-}$ production by 18% and 23%, respectively (Table 2). The rate of $O_2^{\bullet-}$ generation from vascular segments remained unchanged in normotensive rats treated with THC.

With regard to the lipid peroxidation and protein oxidation markers, we found a marked increase in plasma malondialdehyde and protein carbonyl levels in L-NAME-treated rats, and THC significantly reduced these two oxidative stress markers ($P < 0.05$, Table 2). Additionally, it appeared that the antioxidant defense system was depleted in NO-deficient rats, as GSH and the ratio of GSH/glutathione disulfide in the blood were dramatically reduced ($P < 0.05$, Table 2). THC, especially at high dose, partially prevented the depletion of blood GSH and restored the redox status of L-NAME-treated rats (Table 2). Interestingly, the alleviation of oxidative stress by THC was well correlated with the improvement in hemodynamic status, as we found a strong positive correlation between systolic BP and vascular $O_2^{\bullet-}$ production ($r = 0.925$, $P < 0.001$, Figure 4).

Effect of THC on nitrate/nitrite production and eNOS protein expression

We studied the eNOS protein expression which is known to regulate NO production. It is apparent that eNOS protein expression in the thoracic aorta was decreased by 70% after L-NAME administration when compared with age-matched controls. Concomitant treatment with THC for 2 weeks, with either the low or high dose, significantly restored the eNOS protein expression ($P < 0.05$, Figure 5a). Treatment with THC did not modify the level of eNOS expression in normotensive rats. Figure 5b shows a significant reduction in plasma nitrate/nitrite level of L-NAME-treated rats, confirming a reduction of NO production after L-NAME administration. Plasma nitrate/nitrite level significantly increased after THC treatment (Figure 5b).

Figure 3 Effect of THC on functional stiffness of the thoracic aorta in all experimental groups. Results are expressed as mean ± s.e.m. ($n = 6$ per group). $^{\#}P < 0.05$ vs. L-NAME control group.

Table 2 Effect of THC on oxidative stress and antioxidants status of rats in all experimental groups

Group	Superoxide production (counts per mg dry weight per min)	Plasma MDA (μM)	Plasma protein carbonyl (nmol per mg protein)	GSH level (μM)	GSH/GSSG
Normotensive					
Vehicle	41.6 ± 5.8	5.7 ± 0.5	1.4 ± 0.1	614.4 ± 26.7	119.6 ± 11.5
THC 50 mg kg^{-1} per day	44.3 ± 3.2	5.1 ± 0.4	1.1 ± 0.1	648.6 ± 35.9	116.8 ± 10.8
THC 100 mg kg^{-1} per day	42.1 ± 2.1	5.0 ± 0.3	1.2 ± 0.1	639.3 ± 18.8	125.5 ± 7.9
L-NAME-induced hypertension					
Vehicle	224.9 ± 10.3*	26.1 ± 2.1 *	2.6 ± 1.6 *	364.3 ± 23.5	54.5 ± 5.2 *
THC 50 mg kg^{-1} per day	184.6 ± 5.6*,†	13.7 ± 1.6*,†	2.1 ± 0.1*,†	393.4 ± 25.8*	67.5 ± 6.6 *
THC 100 mg kg^{-1} per day	173.5 ± 9.7*,†	11.8 ± 0.5*,†,‡	1.9 ± 0.1*,†,‡	449.6 ± 14.3*,†	80.5 ± 4.8 *,†

Abbreviations: GSH, glutathione; GSSG, glutathione disulfide; MDA, malondialdehyde.
Values are expressed as mean ± s.e.m., n = 8–10 per group.
*$P < 0.05$ vs. normal control group. †$P < 0.05$ vs. L-NAME control group. ‡$P < 0.05$ vs. L-NAME treated with THC 50 mg kg^{-1} per day.

DISCUSSION

We have found that treatment with THC led to a significant reduction in the detrimental effect of L-NAME-induced hypertension in rats. THC was shown to reduce arterial blood pressure, improved hemodynamic status and aortic elasticity following chronic inhibition of NO synthesis. The ameliorating effects of THC might be mediated through its strong antioxidant property and its ability to partially induce the endogenous antioxidant system.

This study has confirmed that chronic L-NAME administration induces systemic hypertension and increases total peripheral resistance as a consequence of diminished NO production.[29] The thoracic aorta from L-NAME hypertensive rats was shown to have been remodelled by hypertrophy and to have increased its functional stiffness[30] and moderated its compliance.[31] In addition, our present study revealed that arterial BP was elevated, whereas the aortic wall thickness was increased. These results indicate that the elevated BP may be causally related to the changes in vascular morphology and mechanical properties as a vascular adaptation to keep wall hoop stress at a

normal level.[32] Vascular remodeling has been reported to be a consequence of a blockade of NO that leads to an abolition of the ability of the endothelium to protect against arteriosclerosis and to inhibit inflammation, smooth muscle cell proliferation and migration.[33,34] Alternatively, the vascular remodeling might be influenced by an imbalance between oxidative stress and antioxidant. Excessive $O_2^{\bullet-}$ production promotes vascular cell growth, inflammation and increased collagen deposition.[35] Vascular remodeling is associated with altered mechanical properties, which might affect lumen diameter and consequently peripheral vascular resistance.[36] We observed increased vascular stiffness accompanied by decreased blood flow and increased vascular resistance in rats treated with L-NAME.

Our data show that THC attenuated the elevation of BP in the hypertensive state without a hypotensive effect on normotensive animals. Overall, the results suggest that the effect of THC in moderating hypertension involved a reduction of total peripheral resistance. Moreover, THC treatment, especially at $100 \, mg \, kg^{-1}$, ameliorated vascular remodeling and the adverse hemodynamic status produced by chronic L-NAME administration. It seems likely that treatment with THC for a longer period of time than in the present study (2 weeks) may be able to further reduce high pressure and its deleterious consequences, as the fall in BP had shown no signs of plateauing at the end of the treatment period, (Figure 1). We did not continue the experiment for a longer period, because the L-NAME control rats were showing signs of severe morbidity.

We have demonstrated that L-NAME–induced high BP is associated with the overproduction of vascular $O_2^{\bullet-}$. This overproduction may be due to an increase in NADPH oxidase activity, which is a major source of vascular $O_2^{\bullet-}$ production.[37] It has been suggested that a large amount of $O_2^{\bullet-}$ production impairs NO bioavailability.[3] The present results show decreased plasma nitrate/nitrite levels as well as a significant decrease of aortic eNOS expression in NO deficient rats. This is in agreement with previous studies that found decreased aortic eNOS mRNA and protein expression in rats receiving L-NAME.[5,38–40] Decreased eNOS protein expression in this study might be due to the downregulation of eNOS gene expression. Conversely, other reports have shown that L-NAME induces an increased eNOS mRNA expression.[41] It is therefore not easy to explain the discrepancy among these results. In any case, the decreased mRNA expression or decreased
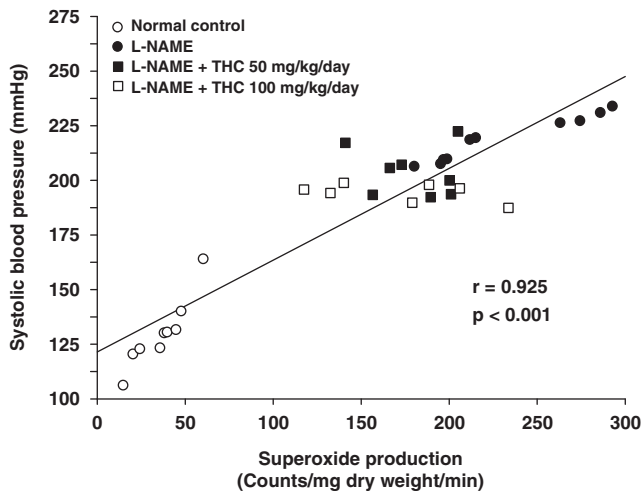

Figure 4 The relationship between systolic BP and superoxide anion production in the vascular tissue of all study animals.
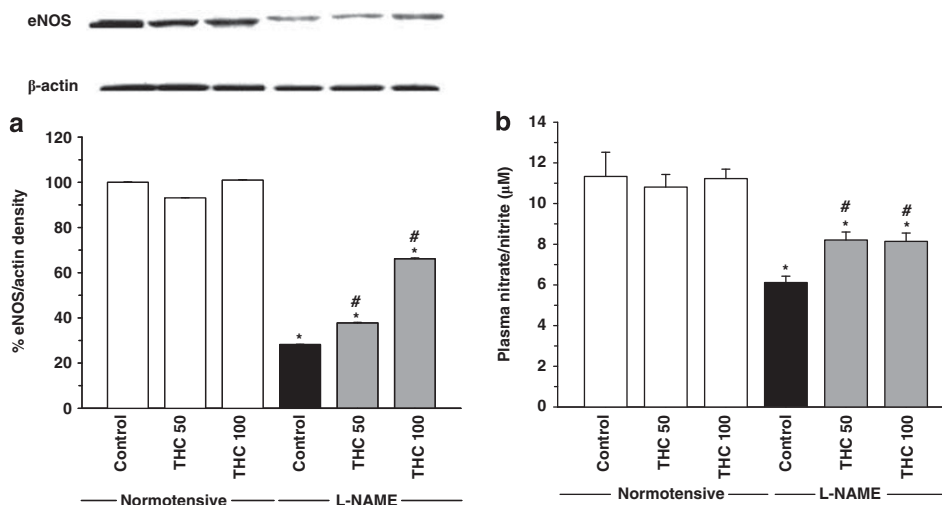

Figure 5 Effect of THC on a densitometric analysis and representative blot of eNOS protein expression in the thoracic aorta (**a**) and plasma nitrate/nitrite level (**b**) in all experimental groups. Results are expressed as mean ± s.e.m. (*n*=6 per group). *$P<0.05$ *vs.* normal control group, #$P<0.05$ *vs.* L-NAME control group.

nitric oxide synthase activity is consistent with our finding that there was a decreased eNOS protein expression in aortic tissues of L-NAME-treated rats.

Our study showed that THC significantly suppressed the over-production of $O_2^{\bullet-}$ and increased eNOS protein expression in the arterial tissues of L-NAME hypertensive rats. It has been reported that THC exerts its anti-inflammatory property by suppressing the activation of NF-kB through inhibition of IKK activity,[42] although we did not examine NF-kB activation. Kitamoto *et al.*[43] have demonstrated that long-term inhibition of nitric oxide synthase increased the activity of NF-kB and AP-1 in the rat aortic wall. It is, therefore likely that the inhibition of NF-kB activation may lead to suppression of its down-stream gene expression, particularly NADPH-oxidase.[44–45] A down-regulation of NADPH-oxidase leads to decreased $O_2^{\bullet-}$ production, and consequently, an increase in NO bioavailability decreased oxidative stress and inhibition of vascular remodelling. Moreover, THC may decrease reactive oxygen species by directly scavenging them and inducing the activity of endogenous antioxidant enzymes, including superoxide dismutase, catalase, GP× and GST.[14,15] Previous studies have demonstrated that polyphenol could upregulate the eNOS expression of vascular cells by the activation of transcriptional factors of the proximal portions of the eNOS promoter, such as Sp1, GATA.[46] This, therefore, might be a plausible mechanism for the increase in eNOS expression in vascular tissues after THC treatment.

The increased oxidative stress as demonstrated by a marked elevation of lipid peroxidation and protein oxidation in L-NAME-treated rats resulted in a reduction of GSH and the cellular redox status of GSH. THC may increase antioxidant enzyme expression and also decrease reactive oxygen species formation, as mentioned above. As a result, THC could substantially restore GSH to about 73% of control levels, whereas in the L-NAME-treated group the GSH level was down to 59% of control values. We note that GSH levels are still below the control level, as L-NAME was still administered to rats and oxidative stress was continued.

In conclusion, the present results demonstrate that THC partially reduces high BP in L-NAME-induced hypertensive rats. This was associated with an improvement of peripheral vascular resistance and aortic elasticity. The underlying mechanism might be partly due to the strong antioxidant properties of THC, including the suppression of $O_2^{\bullet-}$ formation and enhanced antioxidant GSH, thereby increasing the NO bioactivity. Further studies are needed to elucidate the molecular and cellular mechanisms by which THC increases eNOS expression, whereas L-NAME is continuously inhibiting NO synthesis. The overall findings of this study support the idea of using THC as a dietary supplement to protect against adverse cardiovascular effects due to NO deficiency.

ACKNOWLEDGEMENTS

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