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Chronic (−)-epicatechin improves vascular oxidative and inflammatory status but not hypertension in chronic nitric oxide deficient rats

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Abstract

The present study analysed the effects of the flavanol (−)-epicatechin in rats after chronic inhibition of NO synthesis with N^{G}-nitro-L-arginine methyl ester (L-NAME), at doses equivalent to those achieved in the studies involving human subjects. Wistar rats were randomly divided into four groups: (1) control-vehicle, (2) L-NAME, (3) L-NAME-epicatechin 2 (L-NAME-Epi 2) and (4) L-NAME-epicatechin 10 (L-NAME-Epi 10). Rats were daily administered by gavage for four weeks: vehicle, (−)-epicatechin 2 or 10 mg/kg. Animals in the L-NAME groups daily received L-NAME 75 mg/100 ml in drinking-water. The evolution in systolic blood pressure and heart rate, and morphological and plasma variables, proteinuria, vascular superoxide, reactivity and protein expression at the end of the experiment were analysed. Chronic (−)-epicatechin treatment did not modify the development of hypertension and only weakly affected the endothelial dysfunction induced by L-NAME but prevented the cardiac hypertrophy, the renal parenchyma and vascular lesions and proteinuria, and blunted the prostanoid-mediated enhanced endothelium-dependent vasoconstrictor responses and the cyclooxygenase-2 and endothelial NO synthase (eNOS) up-regulation. Furthermore, (−)-epicatechin also increased Akt and eNOS phosphorylation and prevented the L-NAME-induced increase in systemic (plasma malonyldialdehyde and urinary 8-iso-$PGF_{2\alpha}$) and vascular (dihydroethidium staining, NADPH oxidase activity and $p22^{phox}$ up-regulation) oxidative stress, proinflammatory status (intercellular adhesion molecule-1, IL-1β and TNFα up-regulation) and extracellular-signal-regulated kinase 1/2 phosphorylation. The present study shows for the first time that chronic oral administration of (−)-epicatechin does not improve hypertension but reduced pro-atherogenic pathways such as oxidative stress and proinflammatory status of the vascular wall induced by blockade of NO production.

Key words: **(−)-Epicatechin: N^{G}-nitro-L-arginine methyl ester: Hypertension: Superoxide: Inflammation**

Flavanols, such as (−)-epicatechin, catechin and their oligomers, represent a major class of flavonoids that are commonly present in most higher plants, and with high content in certain foods, such as grapes, tea and cocoa. Several epidemiological investigations and dietary interventions in human subjects using flavanols-containing foods indicate an inverse relationship between flavanol intake and the risk of CVD[1–5]. A very wide range of biological actions of flavanols-rich diet support these potential cardiovascular protective effects including the improvement of vasodilation[6–8], blood pressure[9,10], insulin resistance[11], the attenuation of platelet reactivity[12], and the improvement of immune responses and antioxidant defence system[13]. However, little is known about the molecular mechanisms of flavanol-mediated bioactivities in both humans and animals. The reasons for these shortcomings are, at least in part, based on the fact that food matrices contain a multitude of phytochemical constituents, of which an unknown number may exert physiological effects. The effect of high-flavanol cocoa was mimicked by oral intake of pure (−)-epicatechin isolated from cocoa, and the maximum effect on endothelial function coincided with the peak of the plasma level of (−)-epicatechin metabolites[8].

Abbreviations: eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular-signal-regulated kinase 1/2; ICAM-1, intercellular adhesion molecule-1; iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$; L-NAME, N^{G}-nitro-L-arginine methyl ester; MDA, malondialdehyde; NOS, nitric oxide synthase; ROS, reactive oxygen species; SBP, systolic blood pressure.

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(−)-Epicatechin controls vascular tone *in vitro* inducing endothelium-dependent NO-mediated vasodilation[14,15] and improving endothelial dysfunction in diabetes[16]. (−)-Epicatechin *in vitro* induced activation of endothelial NO synthase (eNOS) through two mechanisms: (i) eNOS phosphorylation by the participation of the phosphatidylinositol 3-kinase pathway and (ii) activation of the Ca^{2+}/calmodulin-dependent kinase II pathway[17]. Moreover, (−)-epicatechin elevates NO in endothelial cells via inhibition of the main superoxide generating system NADPH oxidase[18]. Furthermore, a number of endothelial cell-protective actions of (−)-epicatechin were described, including protection against cytotoxicity, oxidative stress-related modifications of proteins and DNA, and proteasomal breakdown of eNOS[18].

We hypothesised that chronic (−)-epicatechin treatment might prevent the development of pathological changes associated with hypertension independently on its protective effects on NO. Therefore, we analysed the effects of (−)-epicatechin, at doses equivalent to those achieved in the human diet, in a model of chronic inhibition of NO synthase (NOS) using the NOS inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME). This model develops arterial hypertension and is associated with a vascular pro-oxidant, pro-atherogenic and proinflammatory phenotype[19,20].

Methods

Experimental protocols

The experimental protocol followed the European Union guidelines for animal care and protection. Male Wistar rats (315–400 g) were used in the study after an adaptation period of 2 weeks for vehicle administration and blood pressure measurements. Rats were randomly assigned to four different treatment groups for 4 weeks: (a) vehicle (control, 1 ml of 1 % methylcellulosa once daily, *n* 8), (b) vehicle plus L-NAME (75 mg/100 ml in drinking-water, approximately 75 mg/kg per d, *n* 10), (c) (−)-epicatechin (2 mg/kg oral inoculation by gavage, mixed in 1 ml of 1 % methylcellulosa once daily, *n* 10) plus L-NAME and (d) (−)-epicatechin (10 mg/kg oral inoculation, mixed in 1 ml of 1 % methylcellulosa once daily, *n* 10) plus L-NAME. The administration doses of (−)-epicatechin in this study correspond to approximately 120–600 mg in humans (at a body weight of 60 kg), which are in the range than can be achieved in the human diet (e.g. flavanol content in dark chocolate used in intervention studies) and approximately two to ten times the average daily catechin intake in humans. The treatment with (−)-epicatechin was stopped two days before the end of the study in order to analyse the long-term effects of (−)-epicatechin without the involvement of the effects of acute administration; and on the last day, the animals were placed in metabolic cages to collect urine for 24 h.

Blood pressure measurements

Systolic blood pressure (SBP) was measured every week in conscious rats by tail-cuff plethysmography[21]. At least seven determinations were made in every session and the mean of the lowest three values within 5 mmHg was taken as the SBP.

Plasma and urinary determinations

Proteinuria was determined according to Bradford method and the results were expressed as mg of protein excreted per 100 g of rat during 24 h. Plasma levels of malondialdehyde (MDA) were assessed following the method described by Esterbauer & Cheeseman[22]. Total 8-iso-$PGF_{2\alpha}$ (iso-$PGF_{2\alpha}$) and TNFα were measured by an enzyme-immunoassay kit (Cayman Chemical and Diaclone, Inc., Besancon, France, specific for rat TNFα, respectively).

Histological techniques

Formaldehyde-fixed, paraffin-embedded longitudinal kidney Wistar rat sections in sagittal plane were stained with haematoxylin and eosin, and periodic acid-Schiff stain. The extent of vascular injury (stenosis, hyaline arteriopathy and myointimal proliferative hyperplasia) was assessed by examining profiles of arteries and arterioles in a single kidney section and counting the number of vessels affected. The presence of glomerular lesions (glomerulosclerosis and capsular fibrosis) was evaluated in at least 200 glomeruli. Tubular atrophy and tubular casts were also evaluated. The morphological study was done in blinded fashion on 4-μm sections with light microscopy, using the most appropriate stain for each lesion. The values were expressed as the percentage of rats with lesions in each group, and the severity of lesions was calculated semiquantitatively using a 0-to-3 scale (0, absence; 1, mild (< 10 % of vessel, tubules or glomeruli involved); 2, moderate (10–25 %); 3, severe (> 25 %)).

Vascular contractility in vitro

Descending thoracic aortic rings (3 mm) and the fourth branch of the mesenteric artery (1·7–2 mm) were dissected from animals and were mounted in organ chambers and in a wire myograph (model 610M; Danish Myo Technology, Aarhus, Denmark), respectively, filled with Krebs solution as previously described[23]. In endothelium-denuded aorta, the concentration–relaxation response curves to nitroprusside (10^{-9}–10^{-5} M) were performed in rings pre-contracted by 10^{-6} M-phenylephrine. The relaxant responses to acetylcholine were also studied in both intact-small mesenteric artery and -aorta pre-contracted by phenylephrine (5 μM in mesenteric artery and 1 or 0·1 μM in control or L-NAME treated aortic rings, respectively, to obtain a similar level of pre-contraction). Contractions evoked by acetylcholine were tested in aortic rings with endothelium incubated for 30 min with L-NAME (10^{-4} M); responses were expressed as a percentage of a previous response to 80 mM-KCl.

In situ *detection of vascular superoxide anion levels*

Unfixed thoracic aortic rings were cryopreserved (PBS 0·1 mol/l, plus 30 % sucrose for 1–2 h), included in optimum

cutting temperature, frozen (−80°C), and 10 μm cross-sections were obtained in a cryostat. Sections were incubated for 30 min in HEPES-buffered solution containing dihydroethidium (10^{-5} M), counterstained with the nuclear stain 4′-6-diamidino-2-phenylindole and photographed on a fluorescence microscope. Superoxide anion (O_2^-) level was estimated from the ratio of ethidium:4′-6-diamidino-2-phenylindole fluorescence[24]. In preliminary experiments, dihydroethidium fluorescence was almost abolished by the O_2^- scavenger tiron, indicating the specificity of this reaction.

NADPH oxidase activity

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings, as previously described[25]. Aortic production of O_2^- was stimulated by the addition of NADPH (100 μM). Rings were then placed with or without NADPH, and lucigenin was injected automatically at a final concentration of 5 μM to avoid known artifacts when used at higher concentrations in a scintillation counter (Lumat LB 9507; Berthold, Germany). Vessels were then dried, and dry weight was determined. NADPH oxidase activity is expressed as relative luminescence units/min per mg dry aortic tissue.

Western blotting analysis

Aortic homogenates were run on a SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes, incubated with primary monoclonal mouse anti-eNOS (Cell Signalling Technology, MA, USA), anti-phospho-eNOS (Ser-1177) (Cell Signalling Technology), rabbit polyclonal anti-cyclooxygenase 2 (COX-2; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-phospho-Akt-Ser-473, rabbit anti-Akt, rabbit anti-extracellular-signal-regulated kinase 1/2 (ERK1/2; Cell Signalling Technology) or mouse anti-phospho-ERK1/2-Thr183 and Tyr185 (Sigma-Aldrich), polyclonal goat anti-$p22^{phox}$, or polyclonal rabbit anti-$p47^{phox}$ (SantaCruz Biotechnology) antibodies overnight and with the correspondent secondary peroxidase conjugated antibodies. Antibody binding was detected by an enhanced chemiluminescence system (Amersham Pharmacia Biotech, Amersham, UK) and densitometric analysis was performed using Scion Image-Release Beta 4.02 software. Samples were re-probed for expression of smooth muscle α-actin or ERK1/2. Protein abundance:α-actin ratio or phospho-Akt/Akt, phospho-ERK1/2/ERK1/2 and phospho-eNOS/eNOS were calculated and data are expressed as a percentage of the values in control aorta from the same gel.

RT-PCR analysis

For RT-PCR analysis, total RNA was extracted and converted to complementary DNA by standard methods. PCR was performed with a Techne Techgene thermocycler (Techne, Cambridge, UK). Initial denaturation was done at 95°C for 3 min and followed by thirty-two to thirty-five (thirty-two for $p22^{phox}$, eNOS, intercellular adhesion molecule-1 (ICAM-1), TNFα and IL-1β, and thirty-five for $p47^{phox}$) cycles of amplification. Each cycle consisted of 1 min of denaturation at 94°C, 45 s of annealing at 55°C for $p22^{phox}$, ICAM-1 and IL-1β, 57°C for TNFα and 58°C for eNOS and 57°C for $p47^{phox}$, and 1 min for enzymatic primer extension at 72°C. After the final cycle, the temperature was held at 72°C for 10 min to allow reannealing of amplified products. RT-PCR products were then size-fractionated through a 1 % agarose gel, and the bands were visualised with ethidium bromide and quantified by densitometric analysis performed on the scanned images using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com). The sequences for primers were as follows: $p47^{phox}$ (100 bp) sense, 5′-ATGACAGCCAGGTGAAGAAGC-3′ and antisense, 5′-CGATAGGTCTGAAGGCTGATGG-3′; $p22^{phox}$ (220 bp) sense 5′-GCGGTGTGGACAGAAGTACC-3′ and antisense, 5′-CTTGGGTTTAGGCTCAATGG-3′; eNOS (161 bp) sense 5′-ATGGATGAGCCAACTCAAGG-3′ and antisense, 5′-TGTCGTGTAATCGGTCTTGC-3′; ICAM-1 (386 bp) sense 5′-AGGTATCCATCCATCCCACA-3′ and antisense, 5′-AGTGTCTCATTGCCACGGAG-3′; TNFα (468 bp) sense 5′-ATGTGGAACTGGCAGAGGAG-3′ and antisense, 5′- GGCCATGGAACTGATGAGAG-3′; IL-1β (497 bp) sense 5′- AGGCAGTGTCACTCATTGTG-3′ and antisense, 5′-GGAGAGCTTTCAGCTCACAT-3′. Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control for the co-amplification. The signals were expressed relative to the intensity of glyceraldehyde 3-phosphate dehydrogenase in each sample.

Drugs

All drugs and chemicals were obtained from Sigma (Alcobendas, Spain), and dissolved in distilled deionised water, except for (−)-epicatechin, which was mixed with 1 % methylcellulosa.

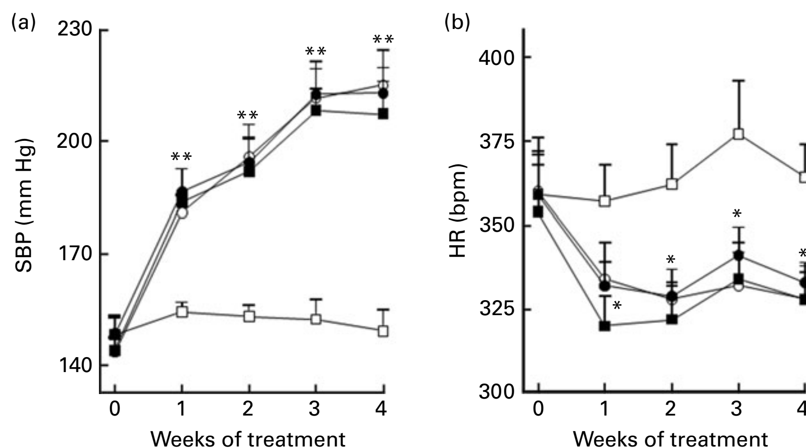
Statistical analysis

Results are expressed as means with their standard errors. Statistically significant differences were calculated by one-way ANOVA analysis followed by Bonferroni's *post hoc* test. $P<0·05$ was considered statistically significant. Renal lesion severity was analysed by a Mann–Whitney *U* test.

Results

Effects in blood pressure and left ventricular and kidney weight indices

Rats receiving chronic L-NAME treatment showed a progressive increase in SBP and decrease in heart rate (Fig. 1), which were already significant after the first week. Concomitant treatment with (−)-epicatechin (2 and 10 mg/kg) did not prevent the changes in either SBP or heart rate. At the end of the study period, L-NAME significantly increased the heart (13 %) and left ventricular (22 %) weight indices as compared to the control group. In rats receiving L-NAME plus (−)-epicatechin at 10 mg/kg, these parameters were significantly reduced as compared to the L-NAME group (Table 1).



Q14 **Fig. 1.** Effects in (a) systolic blood pressure (SBP) and (b) heart rate (HR) as measured by tail-cuff plethysmography in control (□), N^G-nitro-L-arginine methyl ester (L-NAME, ■), L-NAME + 2 mg/kg epicatechin (Epi 2, ○) and L-NAME + 10 mg/kg epicatechin (Epi 10, ●) groups. Values are means, with their standard Q3 errors represented by vertical bars. Mean values were significantly different between L-NAME and control group: * $P<0·05$, ** $P<0·01$. bpm, beats per minute.

257 *Effects in renal histology and proteinuria*

258 L-NAME induced moderate/severe renal injury which affected 259 40 % of the animals. Hyaline and proliferative (myointimal 260 proliferative hyperplasia) arteriopathy were the main and 261 most intense lesions associated with thickening of the 262 Q10 vascular wall and decrease of lumen (Fig. 2(a) and Table S2, 263 supplementary material for this article can be found at 264 http://www.journals.cambridge.org/bjn). The intensity, num- 265 ber and size of the vessel affected with hyaline arteriopathy 266 and myointimal proliferative hyperplasia were significantly 267 decreased by treatment with (−)-epicatechin 10 mg/kg. Glo- 268 merular and tubulointerstitial lesions were not present, 269 except in L-NAME and L-NAME-plus epicatechin 2 groups 270 which present only weak and scattered tubular cast and tubu- 271 lar atrophy. Proteinuria was markedly increased in the 272 L-NAME group and this effect was reduced only by 10 mg/kg 273 (−)-epicatechin (Fig. 2(b)).

274 *Effects in systemic reactive oxygen species*

275 Plasma MDA level, a marker of lipid peroxidation induced by 276 reactive oxygen species (ROS), in L-NAME-treated animals was 277 increased (89 %) as compared to the control group. In L-NAME 278 plus (−)-epicatechin-treated rats, MDA concentration was 279 reduced only in rats treated with 10 mg/kg (−)-epicatechin 280 (Fig. 3(a)). The 24 h urinary iso-PGF$_{2\alpha}$ excretion, a more 281 specific marker of oxidative stress, was also increased in the 282 L-NAME group. In both groups of (−)-epicatechin-treated L- 283 NAME rats, iso-PGF$_{2\alpha}$ excretion showed similar values to 284 those of control rats (Fig. 3(b)).

285 *Effects in vascular NO pathway*

286 Aortae from L-NAME-treated rats showed strongly reduced 287 endothelium-dependent vasodilator responses to acetyl- 288 choline (Fig. 4(a)). The aortae from L-NAME plus (−)-epicate- 289 chin at 10 mg/kg treated animals showed a small increase 290 in the vasodilation induced by acetylcholine as compared to 291 animals from the L-NAME group. In aortic rings, no differences 292 were observed among groups in the endothelium-indepen- 293 dent vasodilator responses to the NO donor sodium nitro- 294 prusside (Fig. 4(b)). In intact-small mesenteric arteries, 295 acetylcholine elicited concentration-dependent relaxations 296 that were inhibited by chronic L-NAME treatment (Fig. 4(c)). 297 (−)-Epicatechin 2 mg/kg did not prevent this effect of 298 L-NAME and the dose of 10 mg/kg marginally but significantly 299 increased the relaxant response induced by the highest 300 concentration of acetylcholine.

301 eNOS mRNA in the aorta from the L-NAME group was 302 increased as compared to the control group and both

Table 1. Body weight (BW) and cardiac and renal indices
(Mean values with their standard errors)

		BW (g)		HW (mg)		LVW (mg)		KW (mg)		HW:BW ratio		LVW:BW ratio		KW:BW ratio	
Group	*n*	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	8	384	11	972	46	670	27	1030	39	2·52	0·07	1·74	0·02	2·68	0·07
L-NAME	10	363	11	1041	47	770*	31	953	37	2·86**	0·07	2·12**	0·06	2·62	0·14
L-NAME + epi 2	10	339	12	944	39	691	31	864	42	2·79	0·07	2·04	0·07	2·55	0·08
L-NAME + epi 10	10	355	13	951	45	698	30	936	42	2·67†	0·07	1·96†	0·04	2·63	0·08

HW, heart weight; LVW, left ventricular weight; KW, kidney weight; L-NAME, N^G-nitro-L-arginine methyl ester; epi, epicatechin.
Q15 Mean values were values significantly different from those of control group: * $P<0·01$, ** $P<0·01$.
† Mean values were values significantly different from those L-NAME group ($P<0·05$).

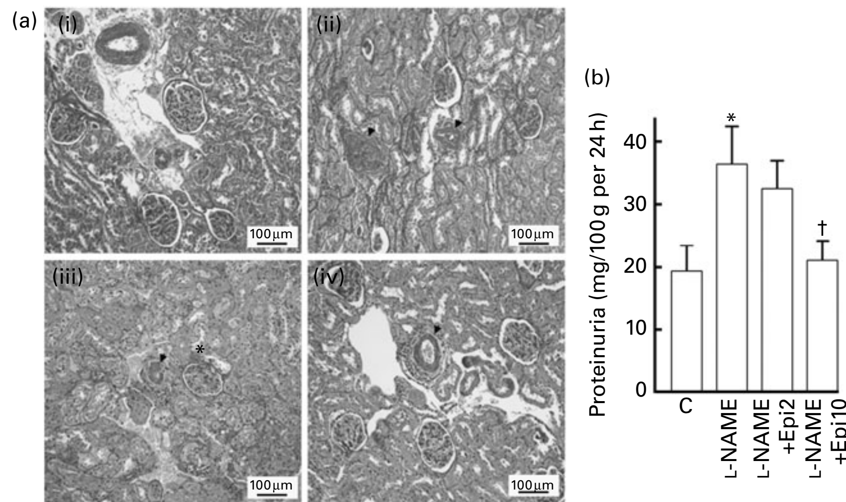


Fig. 2. Effects of epicatechin (Epi) in renal injury. (a) Renal parenchyma in N^G-nitro-L-arginine methyl ester (L-NAME) hypertension model. (i) Absence of vascular, glomerular or tubulointerstitical lesions in control (C) group; (ii) vessels with hyaline arteriopathy and myointimal proliferative hyperplasia (arrows) in L-NAME group; (iii) moderate/severe hyaline arteriopathy in afferent arteriole of glomerulus (*) and interlobular arteria with lumen reduction (arrow) in L-NAME + Epi 2 group; (iv) circumferential hyalin arteriopathy without lumen reduction in L-NAME + Epi 10 group (arrow). (b) Proteinuria in all experimental groups. Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different between L-NAME and C group ($P<0.05$). † Mean values were significantly different between L-NAME-Epi and C group ($P<0.05$).

groups of rats treated with L-NAME plus (−)-epicatechin showed reduced expression of eNOS as compared to the L-NAME group alone (Fig. 5(b)). We also observed that (−)-epicatechin treatment significantly increased eNOS phosphorylation at Ser-1177, as compared to the control and the L-NAME groups (Fig. 5(c)).

Effects on vascular cyclo-oxygenase pathway

Aortae from L-NAME-treated rats showed strongly increased endothelium-dependent vasoconstrictor responses to acetylcholine (Fig. 6(a)), which are related to COX-derived metabolites. This effect was accompanied by an increased COX-2 mRNA (Fig. 6(b)) and protein expression (Fig. 6(c)). The aortae from L-NAME plus (−)-epicatechin at 10 mg/kg treated animals showed reduced vasoconstrictor responses to acetylcholine and COX-2 expression, as compared to the L-NAME group.

Effects in vascular superoxide anion production and NADPH oxidase pathway

To characterise and localise vascular ROS production, sections of aorta were incubated with dihydroethidium which is

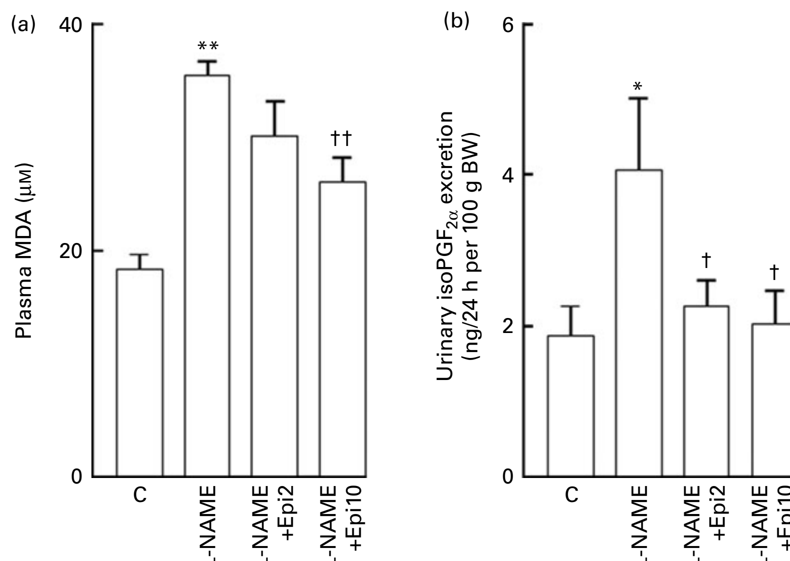
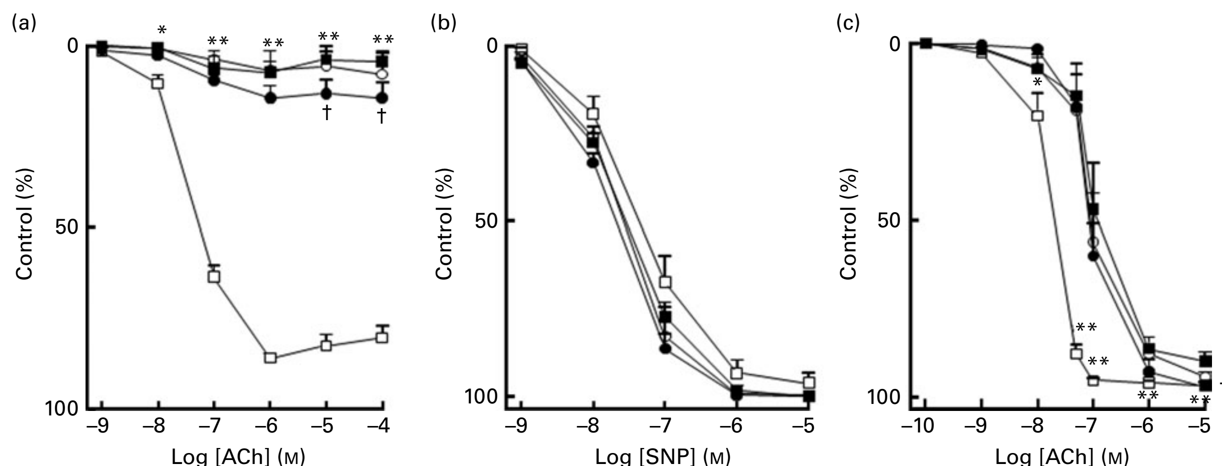


Fig. 3. Effects of epicatechin (Epi) in systemic oxidative markers. (a) Plasma malondialdehyde (MDA) content and (b) urinary excretion of 8-iso-$PGF_{2\alpha}$ in control (C), N^G-nitro-L-arginine methyl ester (L-NAME), L-NAME + 2 mg/kg Epi (Epi 2) and L-NAME + 10 mg/kg Epi (Epi 10) groups. Values are means, with their standard errors represented by vertical bars. Mean values were significantly different between L-NAME and C group: *$P<0.05$, **$P<0.01$. Mean values were significantly different between L-NAME-Epi and L-NAME group: †$P<0.05$; ††$P<0.01$. BW, body weight.



Q14 **Fig. 4.** Effects of epicatechin (Epi) on endothelial function. Vascular relaxant responses induced by (a) acetylcholine (ACh) and (b) sodium nitroprusside (SNP) in aortae pre-contracted by 1 µM phenylephrine (Phe), and by ACh in small mesenteric arteries contracted by 5 µM-Phe (c) in aortae from control (□), N^G-nitro-L-arginine methyl ester (L-NAME, ■), L-NAME + 2 mg/kg Epi (Epi 2, ○) and L-NAME + 10 mg/kg Epi (Epi 10, ●) groups. Values are means, with their standard errors represented by vertical bars. Mean values were significantly different between L-NAME and control group: * $P<0·05$, ** $P<0·01$. † Mean values were significantly different between L-NAME-Epi and L-NAME group ($P<0·05$).

323 oxidised by O_2^- to yield the red fluorescent DNA stain ethidium. Fluorescence was almost suppressed by the O_2^- scavenger tiron (10 mM, data not shown). Rings from L-NAME rats showed marked increased staining in adventitial, medial and endothelial cells (Fig. 7(a)). Red fluorescence, normalised to the blue fluorescence of the nuclear stain 4′-6-diamidino-2-phenylindole, was reduced after 10 mg/kg (−)-epicatechin treatment (Fig. 7(b)). 330

331 NADPH increases lucigenin luminescence in normal aortic rings, which was almost abolished by the flavoprotein inhibitor diphenyliodonium (10 µM, not shown), Q3 showing that external NADPH increased NADPH oxidase activity in vascular tissue. NADPH oxidase activity was increased in aortic rings from L-NAME rats as compared to control rats. Chronic treatments with (−)-epicatechin, at both doses, abolished this increased NADPH oxidase activity in L-NAME-treated rats 338

339 (Fig. 8(a)). Significant p22^{phox} mRNA (Fig. 8(b)) and protein (Fig. 8(c)) up-regulation, without changes in p47^{phox} (Fig. 8(d) and (e)), was observed in aortic tissue from L-NAME rats. (−)-Epicatechin treatment, at both doses, inhibited the p22^{phox} gene overexpression in L-NAME-treated animals. 344

Effects on inflammatory status 345

346 The mRNA expression of ICAM-1 (Fig. 9(a)) and proinflammatory cytokines IL-1β (Fig. 9(b)), or TNFα (Fig. 9(c)) in aortic homogenates was higher in aortae from L-NAME groups as compared to control rats. Plasma TNFα levels (Fig. 9(d)) were also increased in L-NAME-treated rats. (−)-Epicatechin treatment, at both doses, significantly down-regulated these genes, and reduced plasma levels of TNFα. 352

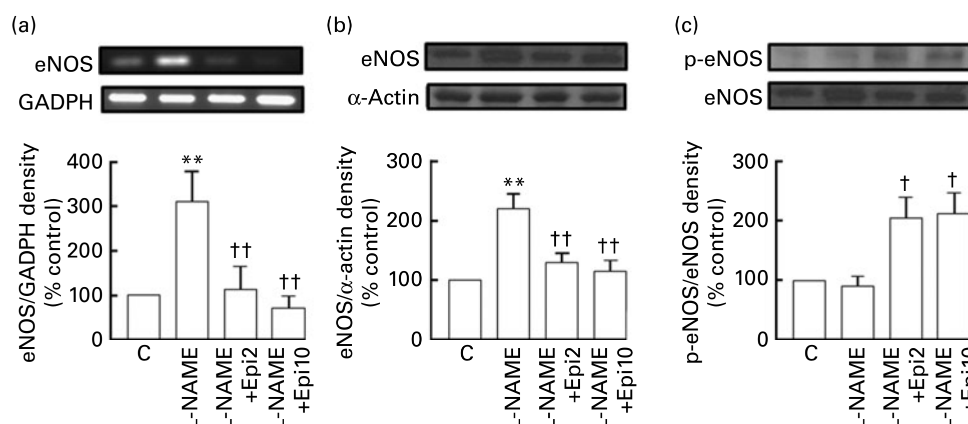


Fig. 5. Effects of epicatechin (Epi) on endothelial nitric oxide synthase (eNOS). Gene expression of eNOS by (a) RT-PCR, (b) Western blot and (c) Ser-1177-phospho-eNOS (p-eNOS) in control (C), N^G-nitro-L-arginine methyl ester (L-NAME), L-NAME + 2 mg/kg Epi (Epi 2) and L-NAME + 10 mg/kg Epi (Epi 10) groups. Q14 Panels show representative bands and histograms represent densitometric values normalised to the corresponding RT-PCR products of (a) glyceraldehyde 3-phosphate dehydrogenase (GADPH) or normalised to the corresponding (b) α-actin or (c) eNOS. Values are means, with their standard errors represented by vertical bars (*n* 3–5). **Mean values were significantly different between L-NAME and C group ($P<0·01$). Mean values were significantly different between L-NAME-Epi and L-NAME group: † $P<0·05$; †† $P<0·01$.

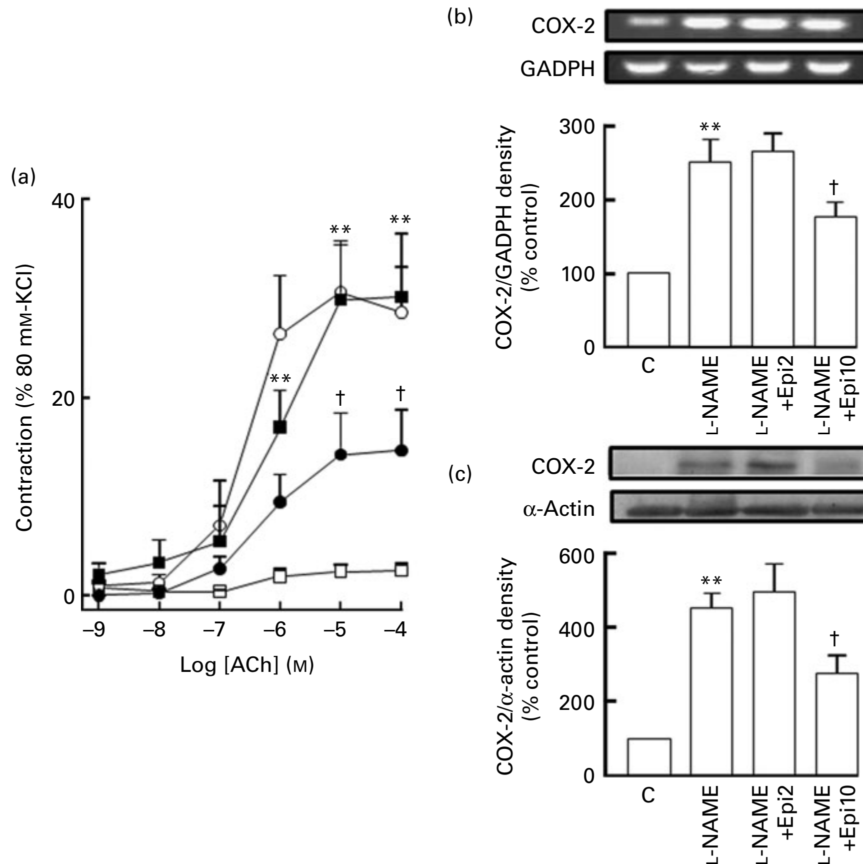


Fig. 6. Effects of epicatechin (Epi) on the cyclooxygenase (COX) pathway. (a) Endothelium-dependent contractions induced by acetylcholine (ACh) in aortae from control (C), N^G-nitro-L-arginine methyl ester (L-NAME), L-NAME + 2 mg/kg Epi (Epi 2) and L-NAME + 10 mg/kg Epi (Epi 10) groups. ACh-induced contractions were induced in arteries treated with L-NAME (10^{-4} M) in the organ bath and expressed as a percentage of the response to 80 mM-KCl. Expression of (b) COX-2 at the level of mRNA by RT-PCR and (c) protein by Western blot in all experimental groups. Panels show representative bands and histograms represent densitometric values normalised to the corresponding RT-PCR products of (b) glyceraldehyde 3-phosphate dehydrogenase (GADPH) or normalised to the corresponding (c) α-actin. Values are means, with their standard errors represented by vertical bars (*n* 3–5). **Mean values were significantly different between L-NAME and C group ($P<0·01$). † Mean values were significantly different between L-NAME-Epi and L-NAME group ($P<0·05$).

Q14

Effects on vascular extracellular-signal-regulated kinase 1/2 and Akt pathways

Q11 The expression of phospho-Akt (Fig. 10(a)) and phospho-ERK1/2 (Fig. 10(b)) proteins was increased in aorta by L-NAME. Chronic (−)-epicatechin treatment increased Akt phosphorylation and reduced ERK1/2 phosphorylation in rats with chronic NO-deficient hypertension.

Discussion

Administration of L-NAME in drinking-water induces a progressive increase in arterial blood pressure which is attributed to a reduced synthesis of the vasodilator NO and has been widely used as a model of chronic hypertension[20]. The present study shows for the first time that a single oral daily dose of (−)-epicatechin (2 or 10 mg/kg) partially or fully prevented most of the effects induced by L-NAME (Fig. 11) such as (a) increases in the left ventricular hypertrophy, (b) proteinuria, (c) renal histological lesions, (d) increased plasma MDA concentrations and urinary iso-$PGF_{2\alpha}$ excretion, (e) increased endothelium-dependent contraction and COX-2 overexpression, (f) increased vascular production of O_2^- and NADPH oxidase activity and (g) increased vascular inflammatory status. In most cases, these effects were dose-dependent. However, it did not inhibit the development of hypertension and had only minor effects on the impaired endothelium- and NO-dependent relaxation. Interestingly, changes in several end-points were not dependent on the presence of (−)-epicatechin in blood, since they were obtained 48 h after the deprivation of the flavanol, indicating that it alters the course of the disease via permanent structural changes and/or alteration of gene expression.

(−)-Epicatechin, at concentrations >30 μM, exhibits vasodilator effects *in vitro,* which are partially endothelium- and NO-dependent[14,15]. (−)-Epicatechin activates eNOS in human coronary artery endothelial cells by (i) Ser-633 and Ser-1170 phosphorylation and Thr-495 dephosphorylation, and (ii) via Ca^{2+}/calmodulin-dependent kinase II pathways, leading to increased NO production[17]. Moreover, (−)-epicatechin and its two *in situ* O-methylated metabolites elevate NO in endothelial cells via inhibition of NADPH oxidase[18]. However, it is predictable that this acute effect was absent *in vivo* in animals

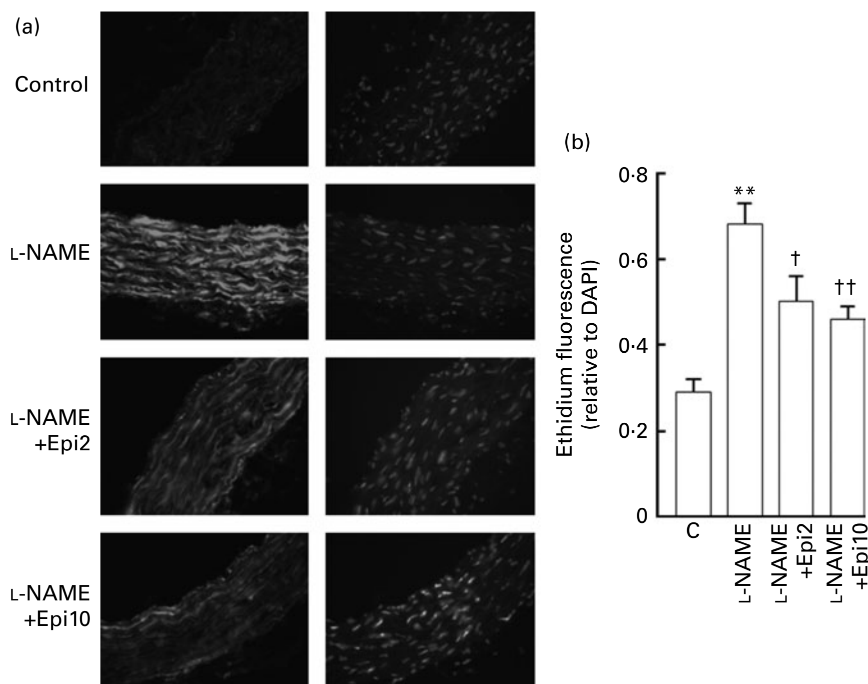


Fig. 7. Effects of epicatechin (Epi) in aortic superoxide anion (O_2^-) levels. (a) Left pictures show arteries incubated in the presence of dihydroethidium which produces a red fluorescence when oxidized to ethidium by O_2^-. Right pictures show blue fluorescence of the nuclear stain 4′-6-diamidino-2-phenylindole (DAPI) Q14 (400× magnification). (b) Averaged values, mean with their standard errors (*n* 5–7 rings from different rats) of the red ethidium fluorescence normalised to the blue DAPI fluorescence in aortae from control (C), N^G-nitro-L-arginine methyl ester (L-NAME), L-NAME + 2 mg/kg Epi (Epi 2) and L-NAME + 10 mg/kg Epi (Epi 10) groups. Values are means, with their standard errors represented by vertical bars. **Mean values were significantly different between L-NAME and C group ($P<0·01$). Mean values were significantly different between L-NAME-Epi and L-NAME group: †$P<0·05$; ††$P<0·01$.

393 treated with L-NAME because: (1) the concentrations of (−)-epicatechin in plasma after 2 or 10 mg/kg (−)-epicatechin[26] are below its active range of concentrations as vasodilators and (2) its relaxant response would be small under conditions of inhibited eNOS. Therefore, the lack of antihypertensive effect of (−)-epicatechin in this model of hypertension may be related, at least in part, to the absence of acute vasodilator effect. 400

401 Renal and cardiac injury

402 Renal injury has been consistently reported after chronic inhibition of NO synthesis[20]. In our study, the L-NAME group presented moderate/severe kidney injury, especially in the vasculature, and with low tubular casts and mild tubular atrophy. The main and most intense vascular lesion in L-NAME rats was hyaline arteriopathy and thickening of vascular wall (proliferative arteriopathy) with moderate decrease of lumen, which was observed almost always in medium-sized vessels. These histological findings were associated with proteinuria, indicating functional impairment of the glomerular wall barrier. (−)-Epicatechin, at the higher dose used, partially prevented renal parenchyma and vascular lesions and proteinuria, indicating that (−)-epicatechin protects, at least partially, from L-NAME-induced renal injury, despite the lack of antihypertensive effect. A modest left ventricular hypertrophy has also been found in this model of hypertension[27]. In our study, the heart and left ventricular weight indices 418 were significantly increased in L-NAME-treated rats and these effects were significantly prevented by 10 mg/kg (−)-epicatechin. These protective effects would be at least partly due to a reduction in ROS and proinflammatory cytokines induced by (−)-epicatechin, which are potent stimulus for cardiac growth and renal injury[28,29]. 424

425 Endothelial dysfunction, NO and cyclo-oxygenase-derived vasoconstrictors 426

427 Administration of L-NAME is associated with endothelial dysfunction[20]. As expected, L-NAME-treated rats showed reduced endothelium-dependent vasodilator responses induced by acetylcholine in both small arteries and in aorta, with similar endothelium-independent relaxant response to the NO donor nitroprusside. (−)-Epicatechin 10 mg/kg only weakly prevented this effect in both vascular beds, without affecting NO sensitivity, since it did not modify the vasodilation induced by nitroprusside. 435

436 L-NAME inhibits the constitutive Ca^{2+}-dependent NOS isoforms (eNOS and nNOS) and the Ca^{2+}-independent inducible NOS isoform. However, these inhibitory effects on NOS activity were associated with increased eNOS expression in aortic tissues, which may be viewed as a compensatory mechanism to maintain the production of bioactive NO in the face of increased oxidant stress[30]. (−)-Epicatechin reduced eNOS gene and protein overexpression, possibly as a result of the reduced vascular superoxide levels in the aortae. Interestingly, 444

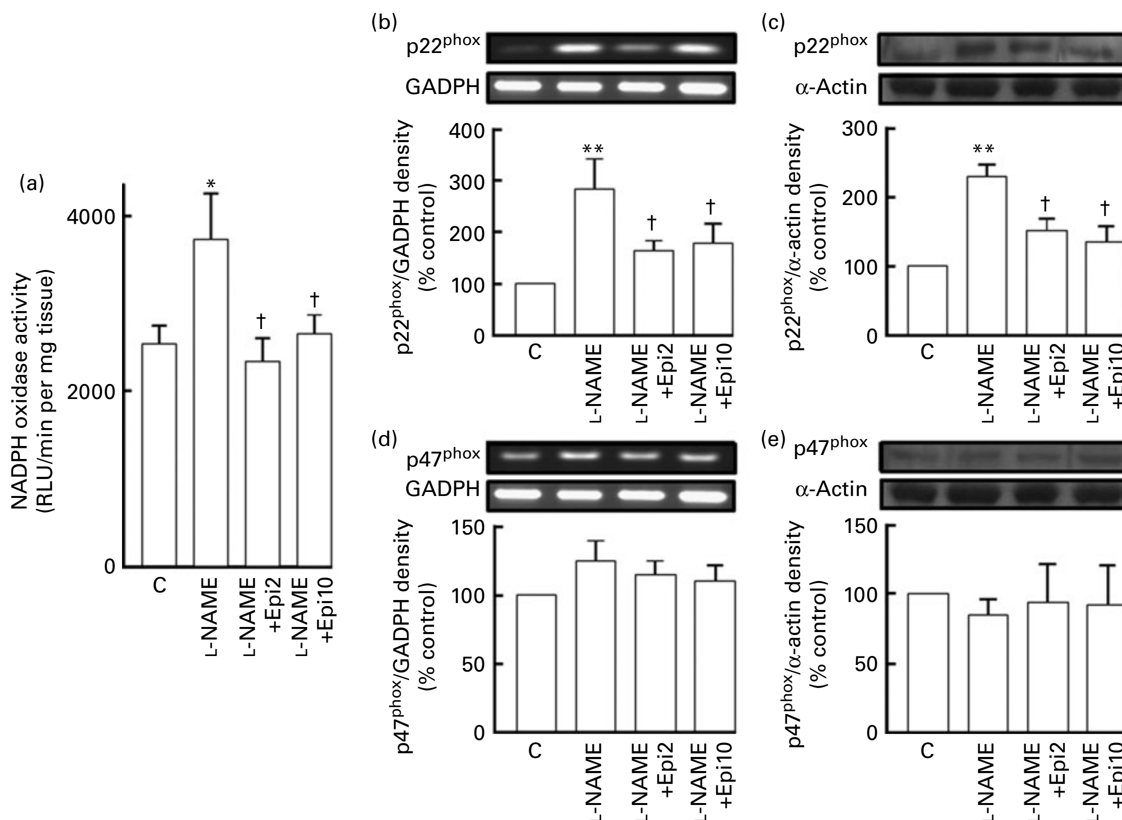


Fig. 8. Effects of epicatechin (Epi) in NADPH oxidase pathway. (a) NADPH oxidase activity measured by lucigenin-enhanced chemiluninescence, and expression of NADPH oxidase subunits p22^{phox} and p47^{phox} at the level of (b and d) mRNA by RT-PCR and (c and e) protein by Western blot in aortae from control (C), N^G-nitro-L-arginine methyl ester (L-NAME), L-NAME + 2 mg/kg Epi (Epi 2) and L-NAME + 10 mg/kg Epi (Epi 10) groups. Panels show representative bands and histograms represent densitometric values normalised to the corresponding RT-PCR products of (b and d) glyceraldehyde 3-phosphate dehydrogenase (GADPH) or normalized to the corresponding (c and e) α-actin. Values are means, with their standard errors represented by vertical bars (n 3–5). Mean values were significantly different between L-NAME and C group: * $P<0·05$, ** $P<0·01$. † Mean values were significantly different between L-NAME-Epi and L-NAME group ($P<0·05$). RLU, remote line unit.

Q14 Q3

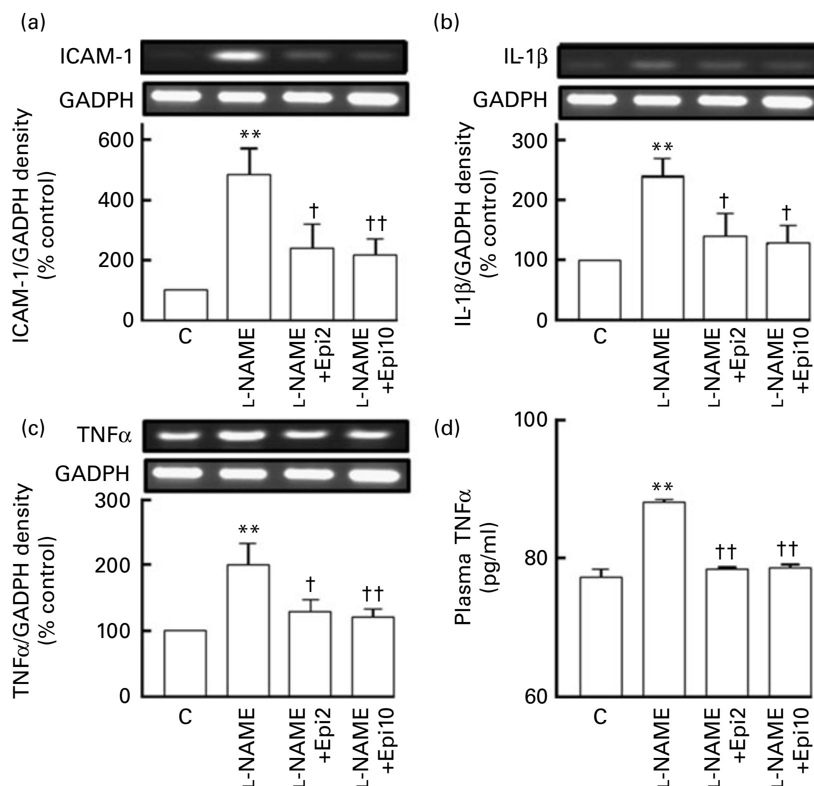
445 we also found an increased eNOS phosphorylation of Ser1177, associated with an increased vascular Akt phosphorylation in both groups of rats treated with (−)-epicatechin. These *in vivo* results are consistent with previous *in vitro* observations showing (−)-epicatechin-induced eNOS activation via PI3K/Akt-mediated phosphorylation in human endothelial cells[17].

452 In addition, increased endothelium-dependent vasoconstriction induced by acetylcholine in the presence of L-NAME in the organ chamber was also observed in aorta from L-NAME-treated rats. These contractions have been previously attributed to increased endothelial release of COX-derived vasoconstrictor prostanoids (such as PG endoperoxides or thromboxane A_2)[31,32]. Q3 Likewise, COX-2 overexpression was found in aorta from L-NAME-treated rats. (−)-Epicatechin 10 mg/kg reduced acetylcholine-induced vasoconstriction and prevented the increase in COX-2, suggesting that (−)-epicatechin inhibits the release of COX-derived metabolites by down-regulating COX-2. Thus, although chronic (−)-epicatechin prevented the secondary changes in endothelial function (due to increased release of endothelial vasoconstrictors), it was unable to restore the primary endothelial defect (i.e. deficient NO production). 467

Systemic and vascular reactive oxygen species and inflammatory markers 468 469

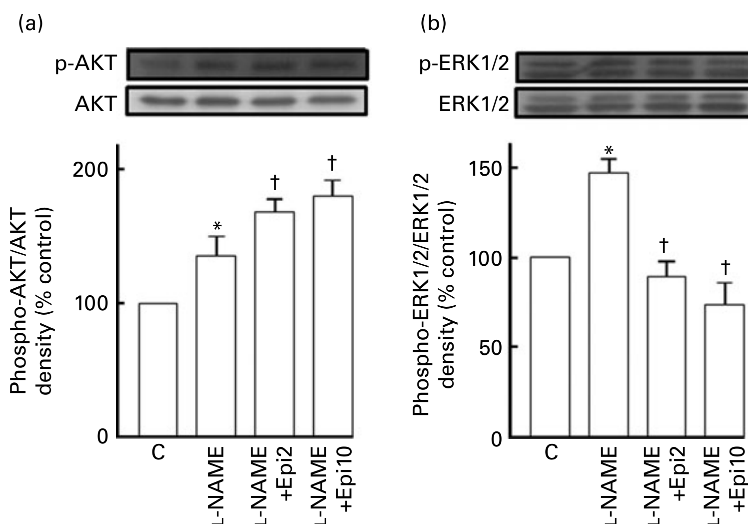
470 ROS have been suggested to contribute to the genesis of atherosclerosis, diabetes, IHD, heart failure and hypertension. The L-NAME model of hypertension has been also associated with increased systemic oxidative stress[32]. In fact, plasma MDA values which reflect a general index of the oxidative status and lipid peroxidation and the 24 h urinary levels of isoprostane $F_{2\alpha}$, a PG-like compound produced by the reaction of arachidonic acid and superoxide[33], were increased in the present study. (−)-Epicatechin *in vitro* is known to possess antioxidant properties[34] and reduces the MDA content in erythrocytes from hypertensive patients[35]. In agreement with these data, in our experiment, long-term (−)-epicatechin treatment reduced plasma MDA levels and the urinary levels of isoprostanes. 483

484 In agreement with other models of hypertension[36], hypertension induced by chronic blockade of NO production was associated with an overproduction of ROS also within the arterial wall[37]. In our *in situ* detection of O_2^- production study, we found that rings from the L-NAME group showed marked staining as compared to control rats, and it was distributed in all layers of the aortic wall. This overproduction 490



Q14 **Fig. 9.** Effects of epicatechin (Epi) in proinflammatory genes. Panels show representative bands and histograms represent densitometric values normalised to the corresponding RT-PCR products of (a) glyceraldehyde 3-phosphate dehydrogenase (GADPH) for intercellular adhesion molecule-1 (ICAM-1), (b) IL-1 Iβ and (c) TNFα (*n* 3–5) in aortae from control (C), N^G-nitro-L-arginine methyl ester (L-NAME), L-NAME + 2 mg/kg Epi (Epi 2) and L-NAME + 10 mg/kg Epi (Epi 10) groups. (d) Plasma TNFα levels in all experimental groups. Values are means, with their standard errors represented by vertical bars. **Mean values were significantly different between L-NAME and C group ($P < 0.01$). Mean values were significantly different between L-NAME-Epi and L-NAME group: †$P < 0.05$, ††$P < 0.01$.

491 may be due, at least in part, to an increase in NADPH 492 oxidase activity, as suggested by both NADPH-stimulated luci-493 genin-enhanced chemiluminescence and an increase in 494 p22^phox expression (present results and Gonzalez *et al.*[37]). (−)-Epicatechin, at both doses, strongly reduced ethidium flu- 495 orescence, NADPH oxidase activity and p22^phox up-regulation 496 in aortic rings from L-NAME-treated rats. Similar results in 497 the vascular wall were also described using a higher dose 498



Q11 Q14 **Fig. 10.** Effects of epicatechin (Epi) in Akt and extracellular-signal-regulated kinase (ERK) pathways. (a and b) Representative bands and histograms represent densitometric values of phospho-Akt and phospho-ERK1/2 (p-ERK1/2) relative to total Akt and ERK1/2 protein levels (*n* 3–5) in aortae from control (C), N^G-nitro-L-arginine methyl ester (L-NAME), L-NAME + 2 mg/kg Epi (Epi 2) and L-NAME + 10 mg/kg Epi (Epi 10) groups. Values are means, with their standard errors represented by vertical bars. * Mean values were significantly different between L-NAME and C group ($P < 0.05$). † Mean values were significantly different between L-NAME-Epi and L-NAME group ($P < 0.05$).

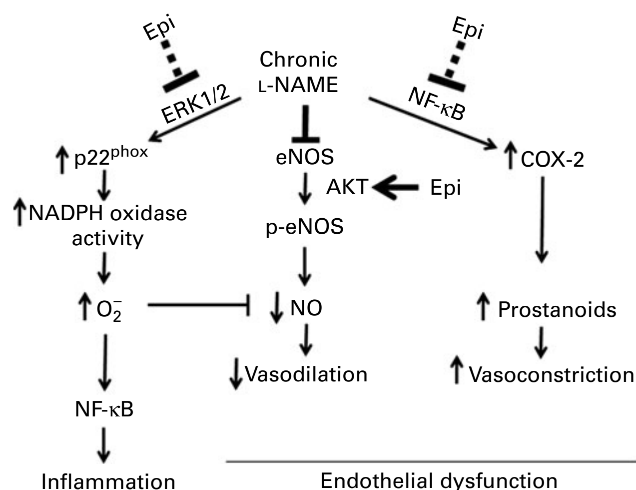


Fig. 11. Schematic diagram representing the mechanism of action of epicatechin (Epi) on the inflammatory and vascular dysfunction pathways in nitric oxide (NO) deficient hypertensive rats. L-NAME, N^G-nitro-L-arginine methyl ester; eNOS, endothelial NO synthase; p-eNOS, phospho-eNOS; COX-2, cyclo-oxygenase 2; O_2^-, superoxide anion; ERK1/2, extracellular-signal-regulated kinase 1/2.

(30 mg/kg) of its stereoisomer catechin in prediabetic Otsuka Long Evans Tokushima Fatty rats[16]. In our study, chronic NO inhibition also induced increased ERK1/2 phosphorylation, an effect which was prevented by (−)-epicatechin and may be involved in its inhibitory effects on NADPH oxidase activity.

Monocyte recruitment is one of the early steps in hypertension-induced arteriosclerosis and perivascular fibrosis[38]. Chronic NO suppression induced an increase in *ex vivo* monocyte endoluminal adhesion and in *in vivo* perivascular macrophage accumulation, in concert with increases in oxidative stress and inflammatory cytokines in the arterial wall[19]. IL-1β, ICAM-1 and TNFα mRNA levels were increased in the vascular wall of the L-NAME-treated rats, suggesting that proinflammatory signals come from the arterial wall. The expression of adhesion molecules and proinflammatory cytokines are mainly the products of inducible genes that are usually controlled, at least in part, by the redox-sensitive NF-κB pathway[39]. The increase in oxidative stress in the vascular wall of L-NAME-treated rats probably activates the NF-κB system, which, in turn, induces the expression of proinflammatory cytokines[40]. Our results further support this hypothesis, since (−)-epicatechin, which reduced aortic superoxide levels also inhibited the vascular expression of these proinflammatory and proatherogenic markers.

In the present model in which NO bioactivity is impaired, no change in blood pressure and only marginal effects on endothelial function were found with (−)-epicatechin. This contrasts with human intervention studies with tea[1] and with the effects of epicatechin in other animal models of hypertension (unpublished results) indicating that NO plays an essential role in the effects of (−)-epicatechin on blood pressure and endothelial function. Our results are consistent with favourable effects of tea and other flavanol-containing food on cardiovascular risk factors[5]. However, caution should be taken with the potential use of green tea extracts as supplements, rich in (−)-epicatechin, because of reports of potential liver damage at high doses.

In conclusion, the present study demonstrates that chronic (−)-epicatechin treatment, at doses equivalent to those that can be achieved in the human diet, prevented cardiac hypertrophy, renal parenchyma and vascular lesions and proteinuria, and blunted the prostanoid-mediated enhanced endothelium-dependent vasoconstrictor responses in the model of chronic inhibition of NO synthesis with L-NAME. Furthermore, (−)-epicatechin also reduced the vascular oxidative stress and proinflammatory status, early events involved in atherosclerosis development.

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