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Antihypertensive effects of oleuropein-enriched olive leaf extract in spontaneously hypertensive rats

M. Romero\textsuperscript{a,b}, M. Toral\textsuperscript{a}, M. Gómez-Guzmán\textsuperscript{a}, R. Jiménez\textsuperscript{a,b}, P. Galindo\textsuperscript{a}, M. Sánchez\textsuperscript{a}, M. Olivares\textsuperscript{c}, J. Gálvez\textsuperscript{a,b,d} and J. Duarte*\textsuperscript{a,b}
Abstract

The effects of chronic consumption of oleuropein-enriched (15% w/w) olive leaf extract (OLE) in blood pressure, endothelial function, and vascular oxidative and inflammatory status in spontaneously hypertensive rats (SHR) were evaluated. Ten Wistar Kyoto rats (WKY) and twenty SHR were randomly assigned to three groups: a control WKY group, a control SHR group and a SHR group treated with OLE (30 mg/kg) for 5 weeks. Long-term administration of OLE reduced systolic blood pressure, heart rate, and cardiac and renal hypertrophy. OLE treatment reversed the impaired aortic endothelium-dependent relaxation to acetylcholine observed in SHR. OLE restored aortic eNOS phosphorylation at Ser-1177 and Thr-495 and increased eNOS activity. OLE abolished the increased aortic superoxide levels, and reduced the elevated NADPH oxidase activity, as a result of reduced NOX-1 and NOX-2 mRNA levels in SHR. OLE reduced the enhanced vascular TLR4 expression by inhibition of mitogen-activated protein kinase (MAPK) signaling with the subsequent reduction of proinflammatory cytokines. In conclusion, OLE exerts antihypertensive effects in genetic hypertension related to the improvement of vascular function as a result of reduced pro-oxidative and pro-inflammatory status.

Keywords: olive leaf extract, hypertension, endothelial dysfunction, NADPH oxidase, SHR.
Introduction

Hypertension is one of the most powerful risk factors for cardiovascular events, including myocardial infarction and stroke. Treatment with any commonly used antihypertensive regimen reduces the risk of total major cardiovascular events; the larger the reductions in blood pressure the larger reductions in risk. Nevertheless, most patients with hypertension will require two or more anti-hypertensive medications to achieve their blood pressure goals (<140/90 mmHg or <150/90 mmHg for in adults 60 years and older, and in patients with hypertension and diabetes, <140/90 mm Hg regardless of age), which, on the other hand, also means the increment of risks of adverse drug reaction and medication costs. One considerable alternative to bridge the efficacy and therapeutic costs is using potential herbal medicines.

The leaves of the olive tree (*Olea europaea* L.) have been used since ancient times to combat high blood pressure, atherosclerosis and diabetes and for other medicinal purposes. Our group described for the first time the hypotensive effects of a decoction of olive leaf in rats. The anti-hypertensive and cholesterol-lowering actions of olive leaves are well-documented in animal and human studies. In addition, the anti-hypertensive effect of the olive leaf extract (OLE) had demonstrated its superiority over the recommended life-style changes. Interestingly, the evaluation of safety parameters and occurrence of adverse events showed that OLE was safe and tolerable in patients with stage-1 hypertension. Olive leaf contains the active substances oleuropein (a polyphenolic iridoid glycoside), oleacein and oleanolic acid. EFLA® 943, a stable olive leaf extract which is standardized to oleuropein, has been preclinically studied for its safety and anti-hypertensive effects. However, the mechanism of action by which OLE exerts its antihypertensive effects in not clear. The antihypertensive effects of active constituents in OLE, such as oleuropein and oleacein,
might be associated with inhibition of angiotensin converting enzyme.\textsuperscript{14} Oleuropein has been recognized as one of the responsible components of a decoction of olive leaf having acute endothelium-independent vasodilatory effects in isolated rat aorta.\textsuperscript{4} We also showed that oleuropein decreases sinus node function and guinea-pig atria contractile response, which cannot be attributed to an inhibitory effects on calcium entry through L-type channels.\textsuperscript{15} In contrast, in a latest study using isolated rabbit’s heart and rat’s cardiomyocytes, it was reported that OLE suppressed the L-type calcium channel directly and reversibly.\textsuperscript{16} Very recently, chronic oleuropein treatment in rats with simultaneous type 2 diabetes and renal hypertension showed antihypertensive effects which seems to be partly mediated by improving the release of nitric oxide (NO), and antioxidant and sympathoplegic activities.\textsuperscript{17} Other minor components, such as triterpenic compounds (oleanolic and maslinic acids), induce acute vasorelaxation of the aorta from spontaneously hypertensive rats (SHR) that involves calcium-independent release of endothelial nitric oxide (NO).\textsuperscript{18,19} Chronic pomace olive oil supplemented in oleanolic acid improve endothelial function in conductance\textsuperscript{20} and resistance arteries\textsuperscript{21} from SHR by increasing endothelial NO synthase (eNOS) protein expression.

Based on the positive results observed in the preclinical and human studies, the current study was designed to primarily confirm the anti-hypertensive effect of the OLE in SHR, an established model of genetic hypertension. Secondary objectives of the study were to investigate mechanisms involved on its antihypertensive effects focusing on the improvement of vascular function and the possible antioxidant and anti-inflammatory properties in vascular wall.
Results

Effects of OLE on blood pressure, and morphological variables

At the end of the 5 weeks of administration, OLE induced a progressive reduction in both systolic blood pressure (SBP) (-21.6 ± 5.5 mmHg) (Fig. 1A), and heart rate (HR) (-47.0 ± 11.6 bpm) (Fig. 1B). The effect on SBP was only seen after 3 weeks and that of HR at 4 weeks.

Body weight (BW) increased in both Wistar Kyoto rats (WKY) and SHR control groups after 5 weeks (7.2 ± 0.5 % and 6.2 ± 0.6 %, respectively). SHR treated with OLE showed similar final BW than control SHR (Table 1). Absolute heart weight (HW) and left ventricle weight (LVW), as well as HW, LVW, and kidney (KW) relative to tibia length (TL) were higher in SHR control group as compared with WKY control group. Treatment with OLE weakly, but significantly, reduced HW/TL and LVW/TL, and abolished the increase in KW/TL (Table 1).

OLE treatment improves endothelial function in SHR

Aortae from control SHR showed significant reduced endothelium-dependent vasodilator responses to acetylcholine in arteries stimulated by phenylephrine as compared with aortae from control WKY (Emax = 31 ± 5 % vs 57 ± 6 %, respectively). OLE consumption produced a significant increase in the relaxation induced by acetylcholine in SHR rats (Emax = 50 ± 7 %, P<0.05 vs SHR control) (Fig. 2A). The relaxant response induced by acetylcholine was fully inhibited by N^G^-nitro-L-arginine methyl ester (L-NAME) in all experimental groups (Fig. 2B), showing that in this vessel
acetylcholine-induced relaxation in both WKY and SHR was entirely dependent on endothelium-derived NO.

To analyze whether the impaired response to endothelial-derived NO is due to a defect in the signaling of NO in vascular smooth muscle, we analyzed the effects of nitroprusside, which directly activates soluble guanylyl cyclase in vascular smooth muscle, mimicking the effects of endogenous NO. The endothelium-independent vasodilator responses to nitroprusside were not different among groups (Fig. 2C).

Endothelial NO synthase gene (Fig. 3A) and protein (Fig. 3B) expression in the aorta were similar in control SHR as compared with WKY rats. However, lower eNOS at Ser1177 phosphorylation (Fig. 3B) and higher eNOS phosphorylation at Thr495 (Fig. 3C) were found in aorta from SHR animals as compared to control WKY. In addition, the phosphorylation of Akt at Ser473 was also reduced in SHR (Fig. 3D). OLE treatment restored the levels of eNOS phosphorylation.

**OLE reduces vascular reactive oxygen species (ROS) levels in SHR by reducing NADPH oxidase activity**

Red fluorescence could be observed in adventitial, medial and endothelial cells from sections of aorta incubated with dihydroethidium (DHE) (Fig. 4A). Nuclear red fluorescence was quantified and normalized to the blue fluorescence of the nuclear stain 4,6-diamidino-2-phenylindole dichlorohydrate (DAPI), allowing comparisons between different sections. Rings from SHR showed marked increased staining in adventitial, medial and endothelial cells as compared with WKY rats which was significantly reduced by OLE treatments (Fig. 4A, 4B).
NADPH oxidase activity was increased in aortic rings from SHR as compared with WKY rats (Fig. 4C). OLE prevented this increase in NADPH oxidase activity in SHR. Significant increase in mRNA levels of NADPH oxidase subunits, NOX-1 (Fig. 5D) and NOX-2 (Fig. 4E) was observed in aortic tissue from SHR as compared with WKY rats, without change in NOX-4 (Fig. 5F). Again, OLE treatments reduced gene expression of both NOX-1 and NOX-2 in SHR.

**OLE reduces vascular inflammatory response in SHR**

The expression of phospho-ERK1/2 (Fig. 5A) and phospho-p38 (Fig. 5B) proteins was increased in aorta from SHR control group as compared with WKY control rats. Chronic OLE treatment reduced mitogen-activated protein kinases (MAPKs) phosphorylation in SHR.

The mRNA levels of toll-like receptor 4 (TLR4) mRNA level (Fig. 5C), and its downstream phospho-IκBα (Fig. 5D) in aortic homogenates were higher in SHR as compared with WKY. In addition, the expression of the proinflammatory cytokines tumor necrosis factor α (TNFα) (Fig. 5E), interleukin 1β (IL-1β) (Fig. 5F) and interleukin 6 (IL-6) (Fig. 5G), as well as of inducible NOS (iNOS) (Fig. 5H) were also increased in SHR. OLE consumption also reduced the higher TLR4 mRNA level and IκBα phosphorylation and restored mRNA levels of proinflammatory cytokines and iNOS to values similar to WKY.

**Discussion**
The present study has demonstrated that chronic oral administration of OLE improved endothelial dysfunction, vascular inflammation, vascular oxidative stress, cardiac and renal hypertrophy and reduced high BP in genetic hypertension.

Endothelial dysfunction is a hallmark underlying vascular disease. In the aorta, NO is the major factor accounting for endothelium-dependent relaxation\textsuperscript{22} as denoted by the almost full inhibitory action of L-NAME in this study. Thus, the diminished acetylcholine-induced relaxation indicates an impaired agonist-induced NO bioactivity. OLE treatment abolished the altered responses to acetylcholine observed in aortae from SHR, indicating a protective role on agonist-induced NO bioactivity. These results are in agreement with previous data showing that chronic oleuropein treatment, the main component of OLE, reversed the hypertension/diabetes-induced impairment of NO release in rat aorta.\textsuperscript{17} Additionally, hydroxytyrosol, the main metabolite of oleuropein,\textsuperscript{23} also reversed the reduced intracellular NO levels stimulated by acetylcholine in endothelial cells incubated \textit{in vitro} with high glucose and free fatty acids.\textsuperscript{24} eNOS is a constitutive enzyme, controlled at transcriptional and post-transcriptional level. The post-transcriptional eNOS regulation is dependent on the phosphorylation state, mainly on a serine residue (Ser-1177) on the reductase domain and on a threonine residue (Thr-495) within the calcium/calmodulin residue.\textsuperscript{25} In fact, in our experiment, changes in eNOS activity correlate with simultaneous changes in eNOS phosphorylation at the activator site Ser-1177 and the inhibitor site Thr-495, since in control SHR, with lower eNOS activity, the level of Ser-1177 and Thr-495 phosphorylation was reduced and increased, respectively. In addition, lower eNOS phosphorylation at Ser-1177 also correlates with lower activity of its activator Akt in SHR. In vitro, oleanolic acid, a minor component of OLE, evoked endothelium-derived NO release by increasing phosphorylation of Akt kinase at Ser-473 and eNOS at Ser-1177.\textsuperscript{21} eNOS Thr-495
residue is constitutively phosphorylated in all endothelial cells and is a negative regulatory site. The link between phosphorylation and NO production can be explained by interference with the binding of calmodulin to calmodulin-binding domain. The constitutively active kinase which phosphorylates eNOS Thr-495 is most probably protein kinase C\textsuperscript{25}, and under inflammatory conditions Rho-kinase.\textsuperscript{26} When endothelial cells are stimulated with agonists like acetylcholine, substantially more calmodulin binds to eNOS if Thr-495 is dephosphorylated.\textsuperscript{27} The impaired relaxant response to acetylcholine found in SHR might be related, at least in part, to higher eNOS Thr-495 phosphorylation, which was reversed by OLE treatment via eNOS Thr-495 dephosphorylation. Also, protein kinase C inhibition by hydroxytyrosol\textsuperscript{28} might be involved on the reduced eNOS Thr-495 phosphorylation induced by OLE. The present study demonstrates that chronic OLE consumption stimulates eNOS activity, primarily through non-genomic changes in eNOS expression, but through post-transcriptional eNOS phosphorylation at Ser-1177, via Akt, and eNOS dephosphorylation at Thr-495.

In the present study, the relaxant response to the activator of soluble guanylyl cyclase nitroprusside was similar in aorta from all experimental groups, indicating that alterations in cyclic GMP do not seem to contribute to endothelial dysfunction in SHR. Thus, the functional changes observed in endothelium-dependent relaxation should be attributed to an alteration in NO synthesis and/or its bioavailability. A key mechanism of endothelial dysfunction in hypertension involves the vascular production of ROS, particularly superoxide (O_2^-), which reacts rapidly with NO and inactivates it.\textsuperscript{29-31} In aorta from SHR group incubated with DHE there was a marked increase in red fluorescence indicating increased vascular O_2^- levels. We described that OLE treatment abolished this increase in SHR, showing antioxidant properties \textit{in vivo}. These data are in agreement with the reduced serum levels of malondialdehyde, a marker of systemic
oxidative stress, found in rat fed high carbohydrate-high fat diet treated with an olive leaf extract enriched in oleuropein,\textsuperscript{32} and in hypertensive/diabetic rats treated with oleuropein.\textsuperscript{17} In addition, reduced intracellular ROS levels were found in endothelial cells exposed to hyperglycemia and free fatty acids and treated with hydroxytyrosine.\textsuperscript{24} These data might also be consistent with the potent $O_2^-$ scavenging effect of hydroxytirosol and oleuropein described previously in \textit{in vitro} conditions.\textsuperscript{33}

Increased $O_2^-$ production via NADPH oxidase is thought to contribute to hypertension and endothelial dysfunction in SHR and in essential hypertension.\textsuperscript{34-36} We found increased NADPH oxidase activity in aorta from SHR as compared to WKY, which was suppressed by OLE treatment. The NADPH oxidase is a multi-enzymatic complex formed by gp91\textsuperscript{phox} or its vascular homologous NOX-1, NOX-2 and NOX-4, rac, p22\textsuperscript{phox}, p47\textsuperscript{phox}, and p67\textsuperscript{phox}. We found a marked increase in the expression of the catalytic NADPH oxidase subunits NOX-1 and NOX-2 in aortas from SHR, which was also abolished by OLE consumption, and this could be involved on the NADPH oxidase inhibition and in the reduction of vascular ROS level induced by OLE. Protein kinase C inhibition by hydroxytyrosol\textsuperscript{28} might be also involved on the reduced NADPH oxidase activity.

The enhancement of ROS production, in particular $O_2^-$, affects endothelial function not only by reducing NO bioavailability, but also by promoting inflammation.\textsuperscript{37} MAPK signaling pathways are associated with the vascular inflammation that is modulated by ROS.\textsuperscript{38} However, in vascular tissue, angiotensin II, thrombin or fibroblast growth factor-2 (bFGF)-induced MAPKs phosphorylation of cJun-N-terminal kinase (JNK) and p38, but not ERK1/2, is modulated by NOX enzymes. Responses in smooth muscle cells are mediated by NOX-1 and in endothelial cell by NOX-2.\textsuperscript{39} Our data are in agreement with the fact that vascular ROS increase
links with amplified pro-inflammatory vascular status through MAPKs activation, since the higher aortic ROS content, from NOX-1 and NOX-2, found in SHR correlates with higher p38 phosphorylation and higher mRNA level of the proinflammatory cytokines TNFα, IL-1β, and IL-6 as compared to WKY control group. In addition, TNFα and IL-1β are able to induce iNOS in the blood vessel, which was also increased in aorta from SHR. Moreover, OLE administration, which reduced ROS level, also reduced p38 activation as well as the higher mRNA levels of these cytokines and iNOS enzyme found in aorta from SHR. Of note, ERK1/2 signaling is also upregulated in SHR. ERK1/2 activation is associated with vascular contraction and growth, important determinants of vascular dysfunction and arterial remodeling in hypertension.\textsuperscript{40} In fact, normalization of ERK1/2 activity improved endothelial-dependent relaxation in SHR.\textsuperscript{40}

In the present study we show that phosphorylation of ERK1/2 in aorta is increased in SHR compared with WKY, which was normalized after OLE treatment. This reduced ERK1/2 phosphorylation might be involved on the improvement of acetylcholine-induced relaxation showed by OLE in SHR.

It has been reported that olive oil polyphenols, including oleuropein and hydroxytyrosol, inhibited NF-κB activation induced by bacterial lipopolysaccharide (LPS) in endothelial cells.\textsuperscript{41} LPS stimulates the expression of TLR4 in the vasculature, which resulted in increased NADPH oxidase-dependent O$_2^-$ production and inflammation.\textsuperscript{42,43} TLR4 activation contributes to increased BP and low-grade vascular inflammation displayed by SHR. In fact, in the present study, aortic mRNA levels of TLR4 where higher in SHR than in WKY, and were reduced by OLE treatment. Enhanced TLR4 expression and mRNA stabilization in human aortic smooth muscle cells is mediated by NADPH oxidase-related ROS production and MAPK signalling pathway \textit{in vitro}.\textsuperscript{44} In agreement with these \textit{in vitro} results we found that the protective
effects of OLE in vascular inflammation might be related to interference in TLR4 expression, as a result of reduced vascular NADPH oxidase-mediated ROS production and p38 MAPK activation, and TLR4 signaling, showed by lower IκBα phosphorylation and pro-inflammatory cytokines production. Our data strongly argues that antioxidants act downstream on any membrane receptor, at a step common to all proinflammatory agents, reducing ROS-mediated MAPK activation and subsequent inflammation.

Sustained high blood pressure is one of the most powerful determinants of the development of cardiac and renal hypertrophy.45 In our study, SHR showed increased left ventricular and renal weight indices as compared to normotensive WKY rats. Chronic OLE treatment also significantly reduced this organ target damage. Thus, the beneficial effect of OLE on cardiovascular structure seems to be related to its blood pressure lowering effect. However, other effects beyond the antihypertensive properties, such as the decrease in heart rate, the antioxidant effects and the protection from NO break-down, might also play a role in the prevention of morphological changes observed in SHR rats treated with OLE.

Experimental

This study was carried out in accordance with the regulations and requirements of the European Union concerning the protection of animals used for scientific purposes, and the experimental protocol was approved by the Ethic Committee of Laboratory Animals of the University of Granada (Spain; permit number 459-bis-CEEA-2012).

Animals and experimental groups
Twelve-week old, male SHR and WKY were obtained from Harlan Laboratories (Barcelona, Spain). All rats were fed ad libitum throughout the whole experiment period and on standard rat chow. An adaptation period of two weeks for blood pressure measurements was allowed before the initiation of the experimental protocols. Ten WKY and twenty SHR were randomly assigned to three groups (n =10): a control WKY group and a control SHR groups both received water as vehicle, and a SHR group treated with OLE (30 mg/kg/day by oral gavage) for 5 weeks. OLE consisting of an optimized mixture of compounds derived from olive leaf, standarized for its oleuropein content (15% w/w), medial triterpenic acids content (10% w/w), and hydroxytyrosol content (1% w/w), supplied by Biosearch S.A. (Granada, Spain). OLE is manufactured in accordance with food grade and food safety standards embraced by the Global Food Safety Initiative (FSSC 22000). The OLE treatment was stopped 24 hours before the end of the experiment in order to study the long-term effects of OLE without the involvement of the effects of acute administration. Food and water intake was recorded daily for all groups. During the experimental periods rats had free access to tap water and chow. Body weight was measured every week.

**Blood pressure measurements**

SBP and heart rate HR was measured weekly at room temperature using tail-cuff plethysmography as described previously.\(^{46}\)

**Cardiac and renal weight indices**

When the experimental period was completed, 18 h fasting animals were anaesthetized with 2.5 mL/kg equitensin (i.p.) and blood was collected from the abdominal aorta.
Finally, the rats were killed by exsanguination. The kidneys and ventricles were then removed and weighed. The heart was divided into right ventricle and left ventricle plus septum.

**Vascular reactivity studies**

Segments of thoracic aortic rings were mounted in an organ chambers filled with Krebs solution (composition in mM: NaCl 118, KCl 4.75, NaHCO₃ 25, MgSO₄ 1.2, CaCl₂ 2, KH₂PO₄ 1.2 and glucose 11) at 37 ºC and gassed with 95% O₂ and 5% CO₂ and maintained at a resting tension of 2 g. Isometric tension was recorded using an isometric force-displacement transducer (Letigraph 2000) connected to an acquisition system, as previously described.⁴⁶

The concentration-relaxation response curves to acetylcholine (10⁻⁹ M-10⁻⁴ M) were performed in rings pre-contracted to the same tension with phenylephrine (0.3 x 10⁻⁶ and 10⁻⁶ M, in WKY and SHR, respectively). The concentration-relaxation response curves to nitroprusside (10⁻¹⁰ -10⁻⁶ M) were performed in the dark in rings without endothelium pre-contracted to the same tension with phenylephrine.

**Measurement of ex vivo vascular ROS levels**

We used DHE, an oxidative fluorescent dye, to localize ROS in aortic segments *in situ*, as previously described.⁴⁷ Briefly, the aorta segments were included in optimum cutting temperature compound medium (Tissue-Tek; Sakura Finetchnical, Tokyo, Japan), quickly frozen, and cut into 10 µm thick sections in a cryostat (Microm International Model HM500 OM). Sections were incubated at room temperature for 30 min with 10⁻⁵
M DHE in the dark, counterstained with the nuclear stain DAPI (3 x 10^-7 M) and in the following 24 h examined on a fluorescence microscope (Leica DM IRB, Wetzlar, Germany). Sections were photographed and ethidium and DAPI fluorescence were quantified using ImageJ (version 1.32j, NIH, http://rsb.info.nih/ij/). ROS production was estimated from the ratio of ethidium/DAPI fluorescence.

**NADPH oxidase activity**

The lucigenin-enhanced chemiluminescence assay was used to determine NADPH oxidase activity in intact aortic rings, as previously described. Aortic rings from all experimental groups were incubated for 30 minutes at 37 °C in HEPES-containing physiological salt solution (pH 7.4) of the following composition (in mM): NaCl 119, HEPES 20, KCl 4.6, MgSO_4_1, Na_2HPO_4_0.15, KH_2PO_4_0.4, NaHCO_3_1, CaCl_2_1.2 and glucose 5.5. Aortic production of O_2^- was stimulated by addition of NADPH (100 µM). Rings were then placed in tubes containing physiological salt solution, with or without NADPH, and lucigenin was injected automatically at a final concentration of 5 µM. NADPH oxidase activity were determined by measuring luminescence over 200 s in a scintillation counter (Lumat LB 9507, Berthold, Germany) in 5-s intervals and was calculated by subtracting the basal values from those in the presence of NADPH. Vessels were then dried, and dry weight was determined. NADPH oxidase activity is expressed as relative luminescence units (RLU)/min/mg dry aortic tissue.
Western blotting analysis

We examined the state of eNOS, protein kinase B (Akt), ERK1/2, and IκBα phosphorylation in aortae homogenates. Aortic homogenates were run on a sodium dodecyl sulphate (SDS)-polyacrilamide electrophoresis (25 µg of protein per lane), then proteins were transferred to polyvinylidene difluoride membranes (PVDF). Phosphorylated eNOS (Ser\textsuperscript{1177}, and Thr\textsuperscript{495}), Akt (Ser\textsuperscript{473}), ERK1/2 (Thr\textsuperscript{183} and Tyr\textsuperscript{185}), IκBα (Ser\textsuperscript{32/36}), eNOS, Akt, and ERK1/2, were detected after the membranes were incubated with the respective primary antibodies: rabbit monoclonal anti-p-eNOS-ser-1177 (Cell Signalling Technology, MA, USA), rabbit monoclonal anti-p-eNOS-thr-495 (Millipore, Darmstadt, Germany), mouse monoclonal anti-eNOS (Transduction Laboratories, San Diego, California, USA), rabbit anti-p-Akt-ser-473, rabbit anti-Akt, rabbit anti-ERK1/2 (Cell Signalling Technology, MA, USA), mouse anti-p-ERK1/2-Thr183 and Tyr185 (Sigma-Aldrich, Barcelona, Spain), and rabbit polyclonal anti-p-IκBα-Ser-32/36 (Santa Cruz Biotechnology, Santa Cruz, USA). All were used at 1/1000 dilution and incubated overnight at 4°C. The membranes were then incubated with secondary peroxidase conjugated goat anti-rabbit or goat anti-mouse antibodies (1:3000, Santa Cruz Biotechnology, Santa Cruz, USA), respectively. Antibody binding was detected by an ECL system (Amersham Pharmacia Biotech, Amersham, UK) and densitometric analysis was performed using Scion Image-Release Beta 4.02 software (http://www.scioncorp.com).\textsuperscript{49} Phospho-eNOS/eNOS, p-Akt/Akt, pERK1/2/ERK1/2, and p-IκBα/α-actin abundance ratio was calculated and data is expressed as a percentage of the values in control aorta from the same gel. Samples were re-probed for expression of smooth muscle α-actin.
Gene expression analysis in aorta

The analysis of gene expression in the aorta was performed by RT-PCR, as previously described. For this purpose total RNA was extracted from aorta by homogenization and converted to cDNA by standard methods. Polymerase chain reaction was performed with a Techne Techgene thermocycler (Techne, Cambridge, UK). A quantitative real-time RT-PCR technique was used to analyze mRNA expression of eNOS, iNOX, NOX-1, NOX-4, TNFα, IL-1β, IL6, and TLR4. The sequences of the sense and antisense primers used for amplification are described in Table 2. The efficiency of the PCR reaction was determined using a dilution series of standard vascular samples. To normalize mRNA expression, the expression of the housekeeping gene β-actin was used. The mRNA relative quantification was calculated using the ∆∆Ct method.

Drugs

All drugs used were obtained from Sigma (Alcobendas, Madrid, Spain). All drugs and chemicals were dissolved in distilled deionized water.

Statistical analysis

Results are expressed as means ± SEM of measurements. The evolution of tail SBP with time was compared using the nested design, with treatment and days as fixed factors and the rat as random factor. When the overall difference was significant comparisons were made using Bonferroni’s method with an appropriate error. Analysis of the nested
design was also carried out with groups and concentrations to compare the concentration-response curves to acetylcholine. The remaining variables were compared using a two way factor design, where group and treatment were fixed effect factors with unequal sample sizes in the different groups. When interaction was significant Bonferroni’s method was used for pairwise comparisons. \( P < 0.05 \) was considered statistically significant.

**Conclusion**

Chronic OLE treatment reduces high BP in genetic hypertensive rats. This effect was related to endothelial dysfunction improvement, as a result of reduced vascular pro-inflammatory and pro-oxidative status (Fig. 6).

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aDepartamento de Farmacología. Facultad de Farmacia. Universidad de Granada.

bInstituto de Investigación Biosanitaria de Granada (ibs.GRANADA). Granada. Spain

cLaboratorio de Descubrimiento y Preclínica. Departamento de Investigación
BIOSEARCH S.A. Granada. Spain.

Spain.

*Correspondence to J. Duarte. Department of Pharmacology, School of Pharmacy,
University of Granada, 18071 Granada, Spain. Tel: (34)-958241791, Fax: (34)-958248264, Email: jmduarte@ugr.es


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Table 1. Body and organ weights and cardiac and renal indices.

<table>
<thead>
<tr>
<th></th>
<th>WKY (n=10)</th>
<th>SHR (n=9)</th>
<th>SHR-OLE (n=9)</th>
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<tbody>
<tr>
<td>BW (g)</td>
<td>397 ± 6</td>
<td>374 ± 7*</td>
<td>368 ± 5**</td>
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<td>HW (mg)</td>
<td>1169 ± 29</td>
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<td>LVW/TL (mg/cm)</td>
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<td>KW/TL (mg/cm)</td>
<td>2.38 ± 0.05</td>
<td>2.52 ± 0.03##</td>
<td>2.38 ± 0.06*</td>
</tr>
</tbody>
</table>

BW, body weight; HW, heart weight; LVW, left ventricle weight; KW, kidney weight. Values are expressed as mean ± SEM of n rats. # P<0.05 y ##P<0.01 vs WKY and *P<0.05 vs SHR-control.

Table 2. Oligonucleotides for real-time RT-PCR.

<table>
<thead>
<tr>
<th>mRNA targets</th>
<th>Descriptions</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>eNOS</em></td>
<td>Endothelial nitric oxide synthase</td>
<td>ATGGATGAGCCAATCAAGG</td>
<td>TGTCGTGTAATCGGTCTTGC</td>
</tr>
<tr>
<td><em>INOS</em></td>
<td>Inducible nitric oxide synthase</td>
<td>TCTGTGCCCTTGCTCATGAC</td>
<td>CATGGTAGACGTTCTTGG</td>
</tr>
<tr>
<td><em>NOX-1</em></td>
<td>NOX-1 subunit of NADPH oxidase</td>
<td>TCTTGTGGTTGACAATCTTG</td>
<td>TATGGAGTGGAATCTTGG</td>
</tr>
<tr>
<td><em>NOX-4</em></td>
<td>NOX-1 subunit of NADPH oxidase</td>
<td>ACAGGCTCCCTTGCTAACTTGC</td>
<td>TTCGGAGATCATCTTGG</td>
</tr>
<tr>
<td><em>TNF-α</em></td>
<td>tumor necrosis factor-alpha</td>
<td>ACGATGCTCAAGAACACAG</td>
<td>CGATGGAGACGTTTGG</td>
</tr>
<tr>
<td><em>IL-6</em></td>
<td>interleukin-6</td>
<td>GATGGATGCTTCAAATCTGG</td>
<td>AGGAGAGCATGAGTAGG</td>
</tr>
<tr>
<td><em>IL-1β</em></td>
<td>interleukin-1β</td>
<td>GTACATCTGTCGTGCTTGG</td>
<td>GCAGTGCTGCGAGTAGG</td>
</tr>
<tr>
<td><em>TLR4</em></td>
<td>Toll-like receptor-4</td>
<td>GCCCTCAGGGAATTAAGCTCC</td>
<td>AGATCAACCGATGGACGTGTA</td>
</tr>
<tr>
<td><em>Actb</em></td>
<td>Beta actin</td>
<td>AATCGATGTCGACATCAAAG</td>
<td>ATGCCACAGGATCCCATAACC</td>
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</table>
**Figure Legends**

**Fig. 1.** Effects of long-term OLE administration on (A) systolic blood pressure (SBP) and (B) heart rate (HR) as measured by tail-cuff plethysmography in SHR rats. Values are expressed as mean ± SEM (n = 10 rats). # and ## indicate P<0.05 and P<0.01, respectively, compared with the WKY control group, * and ** P<0.05 and P<0.01, respectively, compared with SHR control group.

**Fig. 2.** Effects of OLE on endothelial function. Vascular relaxant responses induced by acetylcholine (ACh) (A, B) and by sodium nitroprusside (SNP) (C) in aortae pre-contracted by 10^-6 M phenylephrine in the absence (A, C) and presence (B) of L-NAME (10^-4 M) in rings from all experimental groups. Values are expressed as mean ± SEM (n = 8-9 rings from different rats). # and ## indicate P<0.05 and P<0.01, respectively, compared with the respective WKY control group. * and ** indicate p < 0.05 and p < 0.01, respectively, compared with SHR control rats.

**Fig. 3.** Effects of OLE on the expression of eNOS at the level of mRNA by RT-PCR (A) and protein phosphorylation of eNOS at Ser1177 (B) and at Thr495 (C), and AKT at Ser473 (D) by Western blot in SHR. Values are expressed as mean ± SEM (n = 8-9 rings from different rats for RT-PCR and 4-6 for Western blot). # and ## indicate P<0.05 and P<0.01, respectively, compared with the respective WKY control group. * indicates P < 0.05 compared with SHR control rats.
**Fig. 4.** Effects of OLE on the $\text{O}_2^-$ levels and NADPH oxidase pathway. (A) Pictures show arteries incubated in the presence of DHE which produces a red fluorescence when oxidized to ethidium by $\text{O}_2^-$, blue fluorescence of the nuclear stain DAPI (B) Averaged values, mean ± SEM (n = 5-7 rings from different rats) of the red ethidium fluorescence normalized to the blue DAPI fluorescence. NADPH oxidase activity measured by lucigenin-enhanced chemiluminescence (C), and mRNA levels of NADPH oxidase subunits NOX-1 (D), NOX-2 (E), and NOX-4 (F) in aorta from all experimental groups. Values are expressed as mean ± SEM (n= 8-9 rings from different rats). # and ## indicate P<0.05 and P<0.01, respectively, compared with the WKY control group. * and ** indicates P< 0.05 and P<0.01, respectively, compared with the SHR control group.

**Fig. 5.** Effects of OLE on vascular inflammation in SHR. Effects on phosphorylation of ERK1/2 (A) and p38 (B) MAPKs and IkBa (D) measured by western blots, and aortic mRNA levels of TLR4 (C) and pro-inflammatory cytokines TNFα (E), IL-1β (F), IL-6 (G), and iNOS (H) by RT-PCR. Data presented as a ratio of arbitrary units of mRNA ($2^{-\Delta\DeltaCt}$). Values are expressed as mean ± SEM (n= 8-9 rings from different rats for RT-PCR and 4-6 for Western blot). # and ## indicate P<0.05 and P<0.01, respectively, compared with the respective WKY control group. * indicates P < 0.05 compared with SHR control rats.

**Fig. 6.** Scheme showing the pathways involved in the protective effect of OLE.
Fig. 1

190x142mm (300 x 300 DPI)
Fig. 2

190x142mm (300 x 300 DPI)
Fig. 3
Fig. 4
Fig. 5

190x142mm (300 x 300 DPI)
Fig. 6

190x142mm (300 x 300 DPI)