

Accepted Manuscript

(-)-Epicatechin reduces blood pressure increase in high fructose-fed rats: effects on the determinants of nitric oxide bioavailability

Maria C. Litterio, Marcela A. Vazquez-Prieto, Ana M. Adamo, Rosana Elesgaray, Patricia I. Oteiza, Monica Galleano, Cesar G. Fraga

PII: S0955-2863(15)00065-0
DOI: doi: [10.1016/j.jnutbio.2015.02.004](https://doi.org/10.1016/j.jnutbio.2015.02.004)
Reference: JNB 7326

To appear in: *The Journal of Nutritional Biochemistry*

Received date: 26 September 2014
Revised date: 30 December 2014
Accepted date: 6 February 2015

Please cite this article as: Litterio Maria C., Vazquez-Prieto Marcela A., Adamo Ana M., Elesgaray Rosana, Oteiza Patricia I., Galleano Monica, Fraga Cesar G., (-)-Epicatechin reduces blood pressure increase in high fructose-fed rats: effects on the determinants of nitric oxide bioavailability, *The Journal of Nutritional Biochemistry* (2015), doi: [10.1016/j.jnutbio.2015.02.004](https://doi.org/10.1016/j.jnutbio.2015.02.004)

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



(-)-Epicatechin reduces blood pressure increase in high fructose-fed rats: effects on the determinants of nitric oxide bioavailability

Maria C. Litterio^a, Marcela A. Vazquez-Prieto^b, Ana M. Adamo^c, Rosana Elesgaray^d, Patricia I. Oteiza^{e,f}, Monica Galleano^{a,*}, Cesar G. Fraga^{a,e}.

^a Physical Chemistry-Institute for Molecular Biochemistry and Molecular Medicine (IBIMOL) School of Pharmacy and Biochemistry, University of Buenos Aires-National Council of Scientific and Technological Research (CONICET), Argentina.

^b Department of Pathology, School of Medicine, National University of Cuyo and Institute of Medicine and Experimental Biology–CONICET, Mendoza, Argentina

^c Department of Biological Chemistry (IQUIFIB), School of Pharmacy and Biochemistry, University of Buenos Aires-National Council of Scientific and Technological Research (CONICET), Argentina.

^d Physiology-Institute of Drug Chemistry and Metabolism (IQUIMEFA), School of Pharmacy and Biochemistry, University of Buenos Aires-National Council of Scientific and Technological Research (CONICET), Argentina.

^e Departments of Nutrition and of ^f Environmental Toxicology, University of California at Davis, Davis, CA 95616, USA.

*Corresponding author:

Dr Monica Galleano

Phone.: +54 11 4964 2845 Ext 107

Fax: +54 11 4964 8245 Ext 102

E-mail address: mgallean@ffyb.uba.ar

Abstract

This work investigated the blood pressure (BP) lowering effect of the flavanol (-)-epicatechin in a model of metabolic syndrome. Rats were fed a regular chow diet without (Control) or with 10% (w/v) fructose in the drinking water (high fructose, HF) for 8 w. A subgroup of the HF-fed rats was supplemented with (-)-epicatechin 20 mg/kg body weight (HF-EC). Dietary (-)-epicatechin reverted the increase in BP caused by the fructose treatment. In aorta, superoxide anion production and the expression of the NADPH oxidase (NOX) subunits p47^{phox} and p22^{phox} were enhanced in the HF-fed rats. The increase was prevented by (-)-epicatechin. Similar profile was observed for NOX4 expression. The activity of aorta nitric oxide synthase (NOS) was increased in the HF group and was even higher in the HF-EC rats. These effects were paralleled by increased endothelial NOS phosphorylation at the activation site Ser1177. Among the more relevant MAPKs pathways in vascular tissue, JNK was shown to be activated in the aorta of the HF-fed rats and (-)-epicatechin supplementation mitigated this activation.

Thus, the results suggest that dietary (-)-epicatechin supplementation prevented hypertension in HF-fed rats decreasing superoxide anion production and elevating NOS activity favoring an increase in NO bioavailability.

Keywords: flavonoids; fructose; hypertension; metabolic syndrome; oxidants; superoxide anion.

Abbreviations: BP, blood pressure; BSA, bovine serum albumin; EC, (-)-epicatechin; ERK, mitogenic extracellular signal-regulated protein kinase; HDL, high density lipoprotein; HF, high fructose; LDL, low density lipoprotein; JNK, c-Jun-N-terminal kinase; L-NAME, *N*^ω-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; MS, metabolic syndrome; NOS, nitric oxide synthase; NOX, NADPH oxidase; PBS, phosphate buffered saline.

1. Introduction

Increased blood pressure (BP) is found with high frequency in metabolic syndrome (MS) patients [1] and it is associated to endothelial dysfunction [2]. Endothelial dysfunction, mechanistically defined as an impairment of endothelium-dependent vasorelaxation, is caused by a decrease in nitric oxide (NO) bioavailability in the vascular wall [3].

A decline in NO bioavailability may be caused by an ineffective NO synthesis and/or accelerated NO degradation. Under physiological conditions NO is mainly produced in the vasculature by the endothelial nitric oxide synthase (eNOS) isoform [4-5]. NO is degraded by superoxide anion in a near diffusion-limited rate reaction [6-8]. eNOS activity is regulated by substrate availability, phosphorylation, protein-protein interactions and subcellular localization [9]. The NOX family is a major source of superoxide anion in the vascular wall through the NOX1 and NOX2 isoforms [10-13]. Flavonoids are polyphenols present at large amounts in the human diet [14]. An increased intake of flavonoid-rich foods, particularly of the flavanol subfamily, has been associated with the prevention and/or amelioration of MS-associated risk factors (reviewed in [15,16]). Regarding antihypertensive effects, in overweight/obese subjects, a polyphenol-rich dark chocolate significantly reduced BP [17] and the intake of high-flavanol cocoa reduced BP in association with improved flow-mediated dilatation [18]. Consumption of flavanol-rich dark chocolate by hypertensive patients with impaired glucose tolerance, ameliorated insulin sensitivity and decreased BP [19]. It has been shown that the beneficial effects of cocoa on human vascular function may be mediated by the flavanol (-)-epicatechin [20]. (-)-Epicatechin is one of the most abundant flavonoids in human diets, being present in high concentrations in grapes, cocoa, tea, and many other fruits and vegetables. Mechanistic studies by using pure (-)-epicatechin were carried out in experimental models. Dietary (-)-epicatechin was effective in reducing BP in a rat model of decreased NO production-induced hypertension [21,22] and in spontaneously hypertensive rats [23]. The administration of (-)-epicatechin did not improve the increase in BP caused by the chronic inhibition of NO synthesis with L-NAME in rats [24]. Nevertheless, the same (-)-epicatechin treatment was effective in reducing BP in deoxycorticosterone acetate-salt-induced hypertension rats [25].

In this study we observed that (-)-epicatechin supplementation prevented the increase in BP associated with high fructose consumption. This effect can be in part ascribed to a restoration of NO bioavailability through the attenuation of superoxide

anion production (concomitant with decreased levels of NOX subunits expression) and augmentation of NOS activity. In addition, (-)-epicatechin attenuated c-Jun-N-terminal kinase (JNK) activation produced by high fructose consumption.

2. Materials and Methods

2.1. Materials

Primary antibodies for p47^{phox} (sc-7660), p22^{phox} (sc-11712), eNOS (sc-654), caveolin-1 (sc-70516), p-JNK (Thr183/Tyr185) (sc-6254), JNK (sc-572), p-ERK (Tyr204) (sc-7383), ERK 1 (sc-93), p38 (sc-7149) and β -actin (sc-47778) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Primary antibodies for p-eNOS (Ser1177) (#9570) and p-p38 (Thr180/Tyr182) (#9211) were from Cell Signalling Technology (Boston, MA, USA). Cy3-conjugated rabbit anti-goat was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). (-)-Epicatechin, β -nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt hydrate (NADPH); N,N'-dimethyl-9,9'-biacridinium dinitrate (lucigenin), and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO, USA). Fructose was obtained from Droguería Saporiti (Buenos Aires, Argentina). Commercial rat chow was from Gepsa-Feeds (Buenos Aires, Argentina). [¹⁴C] L-arginine was from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA).

2.2. Animals and animal care

All procedures were in agreement with standards for the care of laboratory animals as outlined in the NIH Guide for the Care and Use of Laboratory Animals. All procedures were performed according to institutional guidelines for animal experimentation and were approved by the Technical and Science Secretary at the National University of Cuyo School of Medicine. Rats were housed under conditions of controlled temperature (21-25°C) and humidity with a 12 h light/dark cycle. Thirty-days-old male Sprague-Dawley rats, weighing 100-130 g, were randomly divided into 3 groups (10 rats per group) that were fed a standard rat chow diet and water ad libitum (control group, Control), the chow diet and water supplemented with 10% (w/v) fructose (high fructose group, HF), or the chow diet supplemented with 20 mg (-)-epicatechin (EC)/kg body weight [26], and the fructose-supplemented water (HF-EC). Food and water intake were recorded twice per week. Systolic BP was

measured by tail plethysmography in conscious, prewarmed, slightly restrained rats and recorded on a Grass Model 7 polygraph (Grass Instruments Co., Quincy, MA, USA) at the beginning (0 w), middle (4 w), and end (8 w) of the study. After 8 w on treatment, and after an overnight fast, rats were weighted, anesthetized with ketamine (50 mg/kg body weight) and acepromazine (1 mg/kg body weight). Blood was collected from the abdominal aorta into heparinized tubes, and plasma was obtained after centrifugation at 1,000 x g for 15 min at 4°C. Aortas were dissected and placed into Krebs solution (20 mM HEPES buffer, pH 7.4, containing 119 mM NaCl, 4.7 mM KCl, 1 mM MgSO₄, 0.4 mM NaH₂PO₄, 0.15 mM Na₂HPO₄, 5 mM NaHCO₃, 1.25 mM CaCl₂, 5.5 mM glucose) and stored at -80°C for subsequent biochemical procedures or processed for immunohistochemistry.

2.3. Metabolic measurements

Glucose, triglycerides, total and HDL-cholesterol levels were measured in plasma using commercially available kits (GTLab, Rosario, Argentina) following the manufacturer's protocol.

2.4. Aorta morphometry

Part of the thoracic aorta was fixed in 10% (w/v) formaldehyde and embedded in paraffin. Tissues sections (5 µm) were stained with hematoxylin-eosin. Micrographs were collected with a Cool Snap digital camera using an Olympus BX50 microscope. Aorta wall thickness and lumen area were calculated using Image J (National Institute of Health, Bethesda, Maryland, USA) according to Xiong et al [27].

2.5. Determination of vascular NOS activity

Vascular NOS activity was measured using [¹⁴C]L-arginine as substrate in aortic slices [28]. Specific NOS activity was assessed in the presence of 10⁻⁴ M L-NAME. NO production (measured as pmol of [¹⁴C]L-citrulline) in each tube was normalized to the weight of the tissue slices incubated with the substrate during equal periods of time and expressed as pmol/g.min.

2.6. Determination of vascular NADPH-dependent superoxide anion production

NADPH-dependent superoxide anion production in aorta homogenates was measured using the lucigenin-enhanced chemiluminescence assay [29]. Sample aliquots containing 200 μg of aorta protein, were placed on vials containing warm (37°C) Krebs solution, and subsequently added with NADPH (40 μM final) and lucigenin (25 μM final) in a volume of 1 ml. Each sample was measured in the absence and presence of superoxide dismutase (SOD) (100 U/ml). Light emission was measured for 10 min using a LKB Wallac 1209 Rackbeta Liquid Scintillation Counter (Turku, Finland) in the chemiluminescence mode, and the area under the curve (AUC) was calculated. Results are expressed as the difference between AUC in the absence and in the presence of SOD, representing the total amount of superoxide anion production.

2.7. Western blots

Aorta homogenates were added with a 2X solution of SDS-sample buffer (62.5 mM Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 25% (v/v) glycerol, 5% (v/v) β -mercaptoethanol, and 0.01% (w/v) bromophenol blue), and then heated at 95°C for 2 min. Aliquots containing 30-50 μg of protein were separated by reducing 10% (w/v) polyacrylamide gel electrophoresis, and electroblotted to polyvinylidene difluoride membranes. Colored molecular weight standards (GE Healthcare, Piscataway, NJ, USA) were run in simultaneous. Membranes were blocked for 2 h in 5% (w/v) nonfat milk and incubated overnight in the presence of the corresponding antibodies (1:1,000 dilution) in 5% (w/v) BSA in PBS containing 0.1% (v/v) Tween 20. After incubation for 90 min at room temperature in the presence of the corresponding HRP-conjugated secondary antibody (1:10,000 dilution), complexes were visualized by chemiluminescence detection. Films were scanned and densitometric analysis was performed using Image J (National Institute of Health, Bethesda, Maryland, USA).

2.8. Immunohistochemistry

Aortas were post-fixed in a solution of 4 % (w/v) paraformaldehyde in PBS for 20 min at room temperature, followed by washing in PBS, and cryoprotection in 30 % (w/v) sucrose in PBS for 24 h. Tissues were frozen at -80°C and sectioned (18 μm) using

a Leica CM 1850 cryotome (Leica Microsystems Inc., Buffalo Grove, IL, USA). For immunohistochemistry, cryotome sections were rinsed twice with PBS, followed by a third rinse with PBS containing 0.3 % (v/v) Tween 20 (PBST), and then blocked for 2 h at 37°C with a solution containing 5% (w/v) BSA in PBST. Incubation with the primary antibody for p47^{phox} (1:50) was done overnight at 4°C in a humidified chamber. Sections were incubated with a Cy3-conjugated rabbit anti-goat antibody (1:1000) for 2 h, cell nuclei were stained with Hoechst 33342 [30]. Microscopic observations were done by epifluorescence using an Olympus BX50 microscope, and photography was carried out with a Cool Snap digital camera. Integrated optical density was measured as relative to area and was determined for each sample in 5 randomly selected fields. Evaluation of the data was carried out using the Image-Pro Plus version 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using StatView 5.0 (SAS Institute, Cary, NC, USA). Fisher's least significance difference test was used to examine differences between group means. A $p < 0.05$ was considered statistically significant. Data are shown as mean \pm SEM.

3. Results

3.1. (-)-Epicatechin improves BP and metabolic parameters in HF-fed rats

No differences in total body weight and cardiac weight index were found between the three experimental groups (Table 1). The ratio total cholesterol /HDL-cholesterol was significantly higher in the HF-group respect to Control and HF-EC groups. Consumption of fructose in the drinking water resulted in increased systolic BP in the HF group recorded after 4 and 8 w in treatment. This increase was not observed in the HF-EC group. The ratio between wall thickness and lumen diameter in aorta, determined to evaluate changes in aorta architecture, resulted similar among the groups indicating the absence of remodeling processes.

3.2. (-)-Epicatechin lowers superoxide anion production in HF-fed rats.

Superoxide anion production was evaluated measuring NADPH-driven SOD-inhibitable lucigenin chemiluminescence. Chemiluminescence was significantly higher in the HF group compared to the Control group (Control = 1.0 ± 0.1 AUC/mg prot, HF = 1.8 ± 0.1 AUC/mg prot, $p < 0.05$) and this higher level was not observed in the HF-EC group (Fig. 1A).

The expression of NOX subunits p47^{phox}, p22^{phox} and NOX4 (Fig. 2) was higher in the aortas from HF group respect to Control and HF-EC groups. The expression/distribution of p47^{phox} was evaluated by immunohistochemistry across the aortic wall (Fig. 1 B-C). The fluorescent signal was observed in the intima and adventitia layers of the aorta and was markedly higher in HF rats compared to controls, whereas this signal was decreased by (-)-epicatechin treatment.

3.3 (-)-Epicatechin increases arterial NOS activity in HF-fed rats

Figure 3 shows NO related parameters in aorta from the three experimental groups. NOS activity was slightly but significantly higher in the HF group compared to controls; in the HF-EC group NOS activity was significantly higher than in both, Control and HF groups (Fig. 3A). To assess the possible mechanisms involved in aorta NO production changes, we studied eNOS protein expression and posttranslational modifications. Although similar eNOS protein abundance was observed in the three experimental groups (Fig. 3B), HF and HF-EC groups exhibited significantly enhanced eNOS phosphorylation ratio at the activation site Ser1177 in comparison with the Control group (Fig. 3C). With the purpose of evaluating the most relevant protein-protein interaction involved in eNOS activity regulation (caveolin-1/eNOS), caveolin-1 expression was measured by Western blot in aorta homogenates (Fig. 3D). Results showed an augmentation in caveolin-1 expression in the HF respect to the Control group that was not observed in the HF-EC group.

3.4. (-)-Epicatechin decreases the activation of the c-Jun-N-terminal kinase pathway in HF-fed rats

MAPK activation was evaluated in aorta homogenates through the determination of the ratio phosphorylated/total levels by Western blot (Fig. 4). Fructose administration caused an increase in c-Jun-N-terminal kinase (JNK) phosphorylation (Thr 183/Tyr 185), not affecting ERK (Tyr 204), and p38 (Thr 180/Tyr 182) phosphorylation (Fig.

4). (-)-Epicatechin dietary supplementation prevented the fructose-induced increase in JNK phosphorylation.

4. Discussion

This work shows that the dietary administration of (-)-epicatechin prevents the increase in BP induced by long-term high fructose consumption in rats. (-)-Epicatechin regulates NO bioavailability in part through the modulation of superoxide anion production (and NOX subunits expression), and NOS activity.

Under the used experimental conditions, and in agreement with previous reports [31], BP augmentation in HF group was not associated with cardiac hypertrophy or aorta remodeling. According to the NO bioavailability hypothesis, the quantitative relationship between superoxide anion and NO is crucial in defining NO steady state levels, and as a consequence, its bioavailability. A higher production of superoxide anion is associated to hypertension [32] and compromises NO bioavailability.

Explaining the antihypertensive effects of (-)-epicatechin through direct antioxidant mechanisms, such as free radical scavenging, is unlikely [33-35], due to the low concentration of (-)-epicatechin and its metabolites present in plasma and potentially reaching endothelial cells [22,36,37]. Therefore, the regulation of translational and posttranslational processes by (-)-epicatechin, in this case related with NOS and NOX expression/activity, could provide a better explanation [38-41].

NOX1 and NOX2 activities are regulated by the abundance of both stabilizer (e.g. p22^{phox}) and activator subunits (e.g. p47^{phox}) [42]. Under our experimental conditions, the increase in aortic superoxide anion production observed in the HF group was associated with higher expression of both p22^{phox} and p47^{phox}. In line with this, the decrease in superoxide anion production in aorta detected in the HF-EC respect to the HF group should be ascribed to a lower assembly/activity of NOX1 and/or NOX2 as a consequence of the downregulation of p22^{phox} and/or p47^{phox}. In agreement, similar changes in NOX subunits expression were associated with dietary (-)-epicatechin supplementation in other hypertension models [22,25,43].

Concerning NO production, the increase in aortic NOS activity in fructose treated rats could be ascribed to a compensatory mechanism in response to the elevation of BP [44]. Interestingly, this compensation was not paralleled with an increased eNOS expression (expected for a long-term adaptative response), but with increased eNOS phosphorylation. Molecular mechanisms of eNOS activity regulation includes phosphorylation at activation sites (Ser1177, Ser633, and Ser614) and inhibitory

sites (Thr 495) [45]. In vitro studies showed that 1 μ M (-)-epicatechin treatment of human coronary artery endothelial cells in culture increased phosphorylation at Ser1177 and Ser633 and decreased phosphorylation at Thr495 [46]. We currently observed a higher phospho-eNOS (Ser1177)/eNOS ratio in the aorta of the group receiving simultaneously fructose and (-)-epicatechin (HF-EC) respect to the Control group, in agreement with previous studies [24,25]. However, as in the group treated with fructose alone (HF group) there was also an increased in Ser1177-phosphorylation, it is not possible to unequivocally attribute the higher NOS activity found in the HF-EC group to an increased phosphorylation.

In addition, eNOS activity in endothelial cells is tightly regulated at a posttranslational level by a protein-protein interaction with caveolin-1 [47]. In this regard, the increased expression of caveolin-1 observed in the HF group would be associated with a negative modulation of eNOS in endothelial cells. Accordingly, similar caveolin-1 levels in the Control and HF-EC groups suggest a lack of negative modulation.

A role for caveolin-1 in NOX assembly has also been proposed in vascular smooth muscle cells as well as in endothelial cells [48]. Thus, increased abundance of caveolin-1 is associated to lower eNOS activity and higher assembly of NOX1 and NOX2.

Mechanisms of NOX4 modulation in endothelial and smooth muscle cells are controversial [32]. NOX4 expression has been found increased in vessels of diabetic mice [49] and some authors consider that NOX4 contributes to vascular injury [50]. In line with this, the increased NOX4 expression observed in this work appears associated to not desirable effects. The proposed primarily product of NOX4 enzymatic activity, hydrogen peroxide, participates in signal transduction regulation, in particular of the MAPK pathways.

Among the more relevant MAPKs pathways in vascular tissue, JNK was shown to be activated in the aorta of the rats fed with high fructose diet. Accordingly, JNK activation by fructose consumption was previously showed in liver [26,51], skeletal muscle [52], and adipose tissue [26,52]. In this study, EC supplementation mitigated HF-mediated JNK activation in aorta, as in liver and adipose tissue of rats under the same experimental model [26].

At vascular level, superoxide anion [53], hydrogen peroxide [54] and even peroxynitrite [55] have been considered as responsible for JNK activation. Moreover, NOX inhibitors antagonized the stimulatory effect of angiotensin II on JNK [53,56], supporting a role for NOX produced oxygen active species in this process.

In this regard, NOX and JNK activation upon high fructose consumption suggest a perturbation of cellular redox status which in the long term could lead to oxidative damage and irreversible changes in vascular health.

The amount of (-)-epicatechin provided to the rats (equivalent to 200 mg/d for a 70 kg human being) is 8-16 times higher than that estimated for the diet of most non-vegetarian populations [57,58]. These levels of consumption could be realistically reached by: i) increasing fruits and vegetables consumption (most world populations consume less than half that the recommended 5 portions/d); ii) increasing specifically the consumption of (-)-epicatechin- and/or flavanol-rich fruits and vegetables; and/or iii) supplementing the diet or by pharmacological administration of pure (-)-epicatechin or flavanols.

In summary, dietary (-)-epicatechin supplementation prevents hypertension developed in high fructose-fed rats concomitantly with modifications in superoxide anion and NO metabolism that results in an increase in NO bioavailability. Considering the abundance of (-)-epicatechin in some edible plants and products derived from them, it is feasible to develop diets enriched in those foods to avoid or prevent the increase in BP that occurs with age and is associated to metabolic syndrome. Moreover, the application of pure (-)-epicatechin as a pharmacological agent to treat hypertension and other vascular diseases could be considered.

Acknowledgements

This study was supported by grants from the Universidad de Buenos Aires (UBACyT 20020130100760BA, 20020120100177, 20020120100132); Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) PIP-0612; and Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT BID PICT 2012-0765, 2011-1957).

References

- [1] Ervin RB. Prevalence of metabolic syndrome among adults 20 years of age and over, by sex, age, race and ethnicity, and body mass index: United States, 2003-2006. Natl Health Stat Report 2009;1-7.
- [2] Lteif AA, Han K, Mather KJ. Obesity, insulin resistance, and the metabolic syndrome: determinants of endothelial dysfunction in whites and blacks. Circulation 2005;112:32-8.

- [3] Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 2000;87:840-4.
- [4] Govers R, Rabelink TJ. Cellular regulation of endothelial nitric oxide synthase. *Am J Physiol Renal Physiol* 2001;280:F193-206.
- [5] Forstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I et al. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning, and functions. *Hypertension* 1994;23:1121-31.
- [6] Griendling KK, FitzGerald GA. Oxidative stress and cardiovascular injury: Part I: basic mechanisms and in vivo monitoring of ROS. *Circulation* 2003;108:1912-6.
- [7] Forstermann U, Munzel T. Endothelial nitric oxide synthase in vascular disease: from marvel to menace. *Circulation* 2006;113:1708-14.
- [8] Goldstein S, Czapski G. The reaction of NO. with O₂.- and HO₂.: a pulse radiolysis study. *Free Radic Biol Med* 1995;19:505-10.
- [9] Mount PF, Power DA. Nitric oxide in the kidney: functions and regulation of synthesis. *Acta Physiol (Oxf)* 2006;187:433-46.
- [10] Paravicini TM, Touyz RM. NADPH oxidases, reactive oxygen species, and hypertension: clinical implications and therapeutic possibilities. *Diabetes Care* 2008;31 Suppl 2:S170-80.
- [11] Pendyala S, Usatyuk PV, Gorshkova IA, Garcia JG, Natarajan V. Regulation of NADPH oxidase in vascular endothelium: the role of phospholipases, protein kinases, and cytoskeletal proteins. *Antioxid Redox Signal* 2009;11:841-60.
- [12] Drummond GR, Selemidis S, Griendling KK, Sobey CG. Combating oxidative stress in vascular disease: NADPH oxidases as therapeutic targets. *Nat Rev Drug Discov* 2011;10:453-71.
- [13] Csanyi G, Taylor WR, Pagano PJ. NOX and inflammation in the vascular adventitia. *Free Radic Biol Med* 2009;47:1254-66.
- [14] Crozier A, Jaganath IB, Clifford MN. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat Prod Rep* 2009;26:1001-43.
- [15] Galleano M, Calabro V, Prince PD, Litterio MC, Piotrkowski B, Vazquez-Prieto MA et al. Flavonoids and metabolic syndrome. *Ann N Y Acad Sci* 2012;1259:87-94.
- [16] Alam MA, Subhan N, Rahman MM, Uddin SJ, Reza HM, Sarker SD. Effect of citrus flavonoids, naringin and naringenin, on metabolic syndrome and their mechanisms of action. *Adv Nutr* 2014;5:404-17.
- [17] Almoosawi S, Fyfe L, Ho C, Al-Dujaili E. The effect of polyphenol-rich dark chocolate on fasting capillary whole blood glucose, total cholesterol, blood pressure

and glucocorticoids in healthy overweight and obese subjects. *Br J Nutr* 2010;103:842-50.

[18] Davison K, Coates AM, Buckley JD, Howe PR. Effect of cocoa flavanols and exercise on cardiometabolic risk factors in overweight and obese subjects. *Int J Obes (Lond)* 2008;32:1289-96.

[19] Grassi D, Desideri G, Necozione S, Lippi C, Casale R, Properzi G et al. Blood pressure is reduced and insulin sensitivity increased in glucose-intolerant, hypertensive subjects after 15 days of consuming high-polyphenol dark chocolate. *J Nutr* 2008;138:1671-6.

[20] Schroeter H, Heiss C, Balzer J, Kleinbongard P, Keen CL, Hollenberg NK et al. (-)-Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proc Natl Acad Sci U S A* 2006;103:1024-9.

[21] Fraga CG, Litterio MC, Prince PD, Calabro V, Piotrkowski B, Galleano M. Cocoa flavanols: effects on vascular nitric oxide and blood pressure. *J Clin Biochem Nutr* 2011;48:63-7.

[22] Litterio MC, Jagers G, Sagdicoglu Celep G, Adamo AM, Costa MA, Oteiza PI et al. Blood pressure-lowering effect of dietary (-)-epicatechin administration in L-NAME-treated rats is associated with restored nitric oxide levels. *Free Radic Biol Med* 2012;53:1894-902.

[23] Galleano M, Bernatova I, Puzserova A, Balis P, Sestakova N, Pechanova O et al. (-)-Epicatechin reduces blood pressure and improves vasorelaxation in spontaneously hypertensive rats by NO-mediated mechanism. *IUBMB Life* 2013;65:710-5.

[24] Gomez-Guzman M, Jimenez R, Sanchez M, Romero M, O'Valle F, Lopez-Sepulveda R et al. Chronic (-)-epicatechin improves vascular oxidative and inflammatory status but not hypertension in chronic nitric oxide-deficient rats. *Br J Nutr* 2011;106:1337-48.

[25] Gomez-Guzman M, Jimenez R, Sanchez M, Zarzuelo MJ, Galindo P, Quintela AM et al. Epicatechin lowers blood pressure, restores endothelial function, and decreases oxidative stress and endothelin-1 and NADPH oxidase activity in DOCA-salt hypertension. *Free Radic Biol Med* 2012;52:70-9.

[26] Bettaieb A, Vazquez Prieto MA, Rodriguez Lanzi C, Miatello RM, Haj FG, Fraga CG et al. (-)-Epicatechin mitigates high-fructose-associated insulin resistance by modulating redox signaling and endoplasmic reticulum stress. *Free Radic Biol Med* 2014;72:247-56.

- [27] Xiong X, You C, Feng QC, Yin T, Chen ZB, Ball P et al. Pulse width modulation electro-acupuncture on cardiovascular remodeling and plasma nitric oxide in spontaneously hypertensive rats. *Evid Based Complement Alternat Med* 2011;2011:812160.
- [28] Costa MA, Loria A, Elesgaray R, Balaszczuk AM, Arranz C. Role of nitric oxide pathway in hypotensive and renal effects of furosemide during extracellular volume expansion. *J Hypertens* 2004;22:1561-9.
- [29] Li JM, Wheatcroft S, Fan LM, Kearney MT, Shah AM. Opposing roles of p47phox in basal versus angiotensin II-stimulated alterations in vascular O₂-production, vascular tone, and mitogen-activated protein kinase activation. *Circulation* 2004;109:1307-13.
- [30] Oberhammer F, Fritsch G, Schmied M, Pavelka M, Printz D, Purchio T et al. Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. *J Cell Sci* 1993;104 (Pt 2):317-26.
- [31] Zakula Z, Koricanac G, Tepavcevic S, Stojiljkovic M, Milosavljevic T, Isenovic ER. Impairment of cardiac insulin signaling in fructose-fed ovariectomized female Wistar rats. *Eur J Nutr* 2011;50:543-51.
- [32] Lassegue B, Griendling KK. NADPH oxidases: functions and pathologies in the vasculature. *Arterioscler Thromb Vasc Biol* 2010;30:653-61.
- [33] Fraga CG. Plant polyphenols: how to translate their in vitro antioxidant actions to in vivo conditions. *IUBMB Life* 2007;59:308-15.
- [34] Galleano M, Verstraeten SV, Oteiza PI, Fraga CG. Antioxidant actions of flavonoids: thermodynamic and kinetic analysis. *Arch Biochem Biophys* 2010;501:23-30.
- [35] Fraga CG, Galleano M, Verstraeten SV, Oteiza PI. Basic biochemical mechanisms behind the health benefits of polyphenols. *Mol Aspects Med* 2010;31:435-45.
- [36] Actis-Goretta L, Leveques A, Giuffrida F, Romanov-Michailidis F, Viton F, Barron D et al. Elucidation of (-)-epicatechin metabolites after ingestion of chocolate by healthy humans. *Free Radic Biol Med* 2012;53:787-95.
- [37] Ottaviani JI, Momma TY, Kuhnle GK, Keen CL, Schroeter H. Structurally related (-)-epicatechin metabolites in humans: assessment using de novo chemically synthesized authentic standards. *Free Radic Biol Med* 2012;52:1403-12.
- [38] Verstraeten SV, Mackenzie GG, Oteiza PI, Fraga CG. (-)-Epicatechin and related procyanidins modulate intracellular calcium and prevent oxidation in Jurkat T cells. *Free Radic Res* 2008;42:864-72.

- [39] Mackenzie GG, Delfino JM, Keen CL, Fraga CG, Oteiza PI. Dimeric procyanidins are inhibitors of NF-kappaB-DNA binding. *Biochem Pharmacol* 2009;78:1252-62.
- [40] Fraga CG, Oteiza PI. Dietary flavonoids: Role of (-)-epicatechin and related procyanidins in cell signaling. *Free Radic Biol Med* 2011;51:813-23.
- [41] Vazquez-Prieto MA, Bettaieb A, Haj FG, Fraga CG, Oteiza PI. (-)-Epicatechin prevents TNFalpha-induced activation of signaling cascades involved in inflammation and insulin sensitivity in 3T3-L1 adipocytes. *Arch Biochem Biophys* 2012;527:113-8.
- [42] Bedard K, Krause KH. The NOX family of ROS-generating NADPH oxidases: physiology and pathophysiology. *Physiol Rev* 2007;87:245-313.
- [43] Piotrkowski B, Calabró V, Galleano M, Fraga CG. (-)-Epicatechin prevents alterations in the metabolism of superoxide anion and nitric oxide in the hearts of L-NAME-treated rats. *Food Funct.* 2014 Oct 31. [Epub ahead of print]
- [44] Costa MA, Elesgaray R, Caniffi C, Fellet A, Mac Laughlin M, Arranz C. Role of nitric oxide as a key mediator on cardiovascular actions of atrial natriuretic peptide in spontaneously hypertensive rats. *Am J Physiol Heart Circ Physiol* 2010;298:H778-86.
- [45] Chen CA, Druhan LJ, Varadharaj S, Chen YR, Zweier JL. Phosphorylation of endothelial nitric-oxide synthase regulates superoxide generation from the enzyme. *J Biol Chem* 2008;283:27038-47.
- [46] Ramirez-Sanchez I, Maya L, Ceballos G, Villarreal F. (-)-Epicatechin induces calcium and translocation independent eNOS activation in arterial endothelial cells. *Am J Physiol Cell Physiol* 2011;300:C880-7.
- [47] Garcia-Cardena G, Martasek P, Masters BS, Skidd PM, Couet J, Li S et al. Dissecting the interaction between nitric oxide synthase (NOS) and caveolin. Functional significance of the nos caveolin binding domain in vivo. *J Biol Chem* 1997;272:25437-40.
- [48] Lobysheva I, Rath G, Sekkali B, Bouzin C, Feron O, Gallez B et al. Moderate caveolin-1 downregulation prevents NADPH oxidase-dependent endothelial nitric oxide synthase uncoupling by angiotensin II in endothelial cells. *Arterioscler Thromb Vasc Biol* 2011;31:2098-105.
- [49] Ding H, Hashem M, Triggle C. Increased oxidative stress in the streptozotocin-induced diabetic apoE-deficient mouse: changes in expression of NADPH oxidase subunits and eNOS. *Eur J Pharmacol* 2007;561:121-8.
- [50] Ginnan R, Jourd'heuil FL, Guikema B, Simons M, Singer HA, Jourd'heuil D. NADPH oxidase 4 is required for interleukin-1beta-mediated activation of protein

kinase Cdelta and downstream activation of c-jun N-terminal kinase signaling in smooth muscle. *Free Radic Biol Med* 2013;54:125-34.

[51] Kelley GL, Allan G, Azhar S. High dietary fructose induces a hepatic stress response resulting in cholesterol and lipid dysregulation. *Endocrinology* 2004;145:548-55.

[52] Giani JF, Mayer MA, Munoz MC, Silberman EA, Hocht C, Taira CA et al. Chronic infusion of angiotensin-(1-7) improves insulin resistance and hypertension induced by a high-fructose diet in rats. *Am J Physiol Endocrinol Metab* 2009;296:E262-71.

[53] Touyz RM, Yao G, Viel E, Amiri F, Schiffrin EL. Angiotensin II and endothelin-1 regulate MAP kinases through different redox-dependent mechanisms in human vascular smooth muscle cells. *J Hypertens* 2004;22:1141-9.

[54] Yoshizumi M, Abe J, Haendeler J, Huang Q, Berk BC. Src and Cas mediate JNK activation but not ERK1/2 and p38 kinases by reactive oxygen species. *J Biol Chem* 2000;275:11706-12.

[55] Go YM, Patel RP, Maland MC, Park H, Beckman JS, Darley-Usmar VM et al. Evidence for peroxynitrite as a signaling molecule in flow-dependent activation of c-Jun NH(2)-terminal kinase. *Am J Physiol* 1999;277:H1647-53.

[56] Viedt C, Soto U, Krieger-Brauer HI, Fei J, Elsing C, Kubler W et al. Differential activation of mitogen-activated protein kinases in smooth muscle cells by angiotensin II: involvement of p22phox and reactive oxygen species. *Arterioscler Thromb Vasc Biol* 2000;20:940-8.

[57]. Vogiatzoglou A, Mulligan AA, Luben RN, Lentjes MA, Heiss C, Kelm M et al. Assessment of the dietary intake of total flavan-3-ols, monomeric flavan-3-ols, proanthocyanidins and theaflavins in the European Union. *Br J Nutr* 2014;111:1463-73.

[58]. Knaze V, Zamora-Ros R, Luján-Barroso L, Romieu I, Scalbert A, Slimani N et al. Intake estimation of total and individual flavan-3-ols, proanthocyanidins and theaflavins, their food sources and determinants in the European Prospective Investigation into Cancer and Nutrition (EPIC) study. *Br J Nutr* 2012;108:1095-108.

Legends to Figures

Fig. 1.

Effect of dietary (-)-epicatechin on aorta NADPH-dependent superoxide anion production and p47^{phox} subunit expression in HF-fed rats. (A) Superoxide anion production measured by lucigenin chemiluminescence in aortic homogenates from Control, HF and HF-EC groups after 8 w of treatment. Results are expressed as the difference between area under the curve (AUC) in the presence and the absence of SOD. (B-C) Quantification and representative images of p47^{phox} immunohistochemistry from Control, HF and HF-EC groups. Left column shows p47^{phox} protein (red). Central column shows elastin autofluorescence (green). Right column shows the p47^{phox} protein (red) merged with elastin autofluorescence (green). In all the images blue fluorescence indicates nuclear staining with Hoechst. Values are shown as means \pm ESM (n = 4 per group). * p < 0.05 with respect to Control and HF-EC groups.

Fig. 2.

Effect of dietary (-)-epicatechin on aortic expression of NOX subunits in HF-fed rats. Western blot of (A) p47^{phox}, (B) p22^{phox}, and (C) NOX4 in aortic homogenates from Control, HF and HF-EC groups after 8 w of treatment. β -tubulin levels were measured as loading control. Values are shown as means \pm ESM (n = 6 per group). * p < 0.05 with respect to Control and HF-EC groups.

Fig. 3.

Effect of dietary (-)-epicatechin on NO production related parameters in aorta from HF-fed rats. (A) NOS activity; Western blot of (B) eNOS, (C) p-eNOS (Ser1177) and (D) caveolin-1 in aortic homogenates from Control, HF and HF-EC groups after 8 w of treatment. Phosphorylated/total ratio was calculated for p-eNOS and eNOS and caveolin-1 were normalized to β -tubulin content. Values are shown as means \pm ESM (n = 5 per group). * p < 0,05 with respect to Control and HF-EC groups. & p < 0.05 with respect to Control and HF groups. # p < 0.05 with respect to Control.

Fig. 4.

Effect of dietary (-)-epicatechin on aorta MAPKs signaling pathways in HF-fed rats. Western blot of (A) p-ERK (Tyr204), (B) p-JNK (Thr183/Tyr185) and (C) p-p38 (Thr180/Tyr182) in aortic homogenates from Control, HF and HF-EC groups after 8 w of treatment. Results are expressed as the ratio of phosphorylated/total protein level. Values are shown as means \pm ESM (n = 5 per group). * p < 0.05 with respect to Control and HF-EC groups.

Table 1

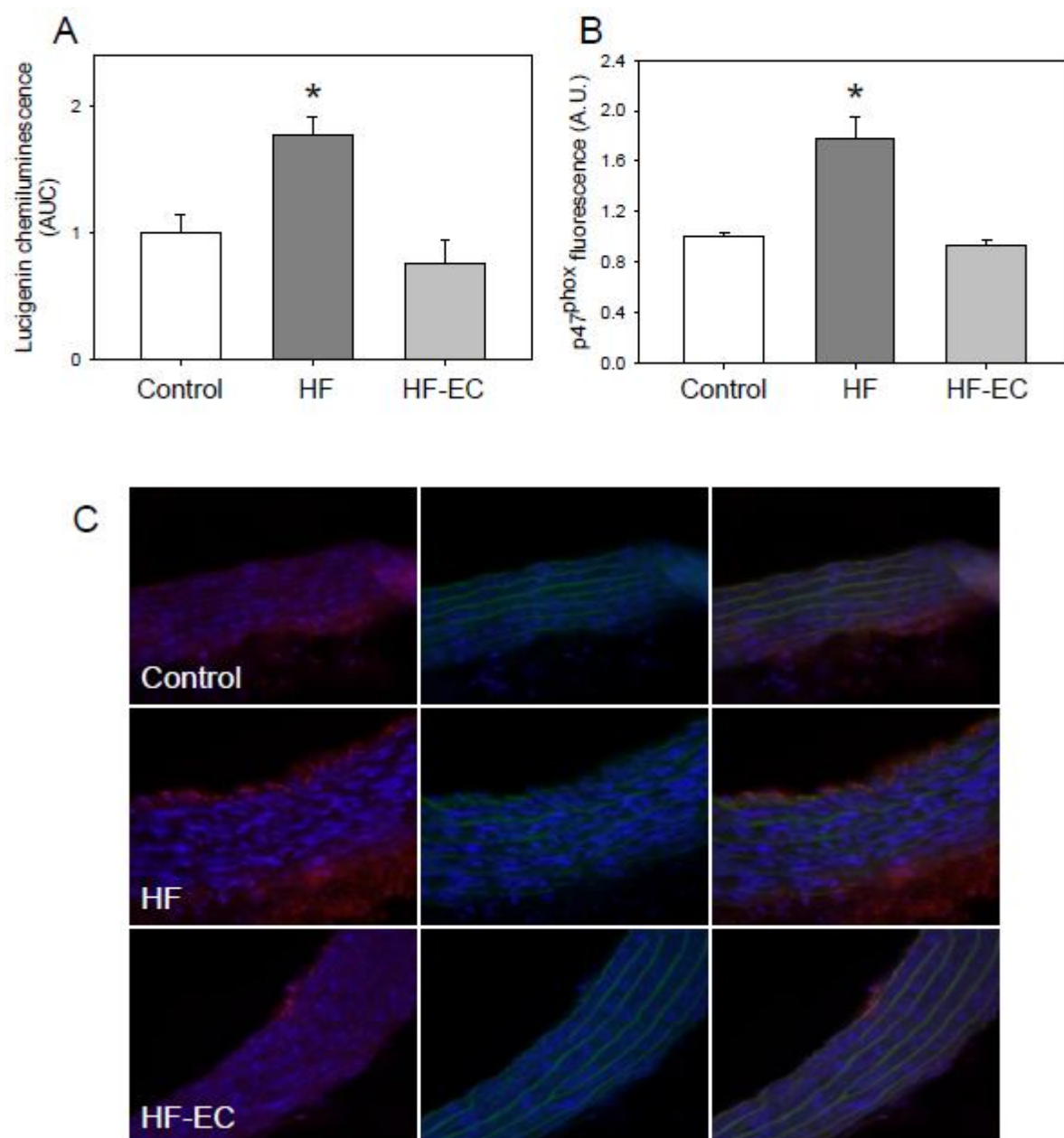
Body weight, heart weight, cardiac weight index, plasma total cholesterol/HDL cholesterol ratio, and aorta wall thickness/lumen diameter ratio after 8 w on treatments and systolic blood pressure at 0, 4 and 8 w on treatments.

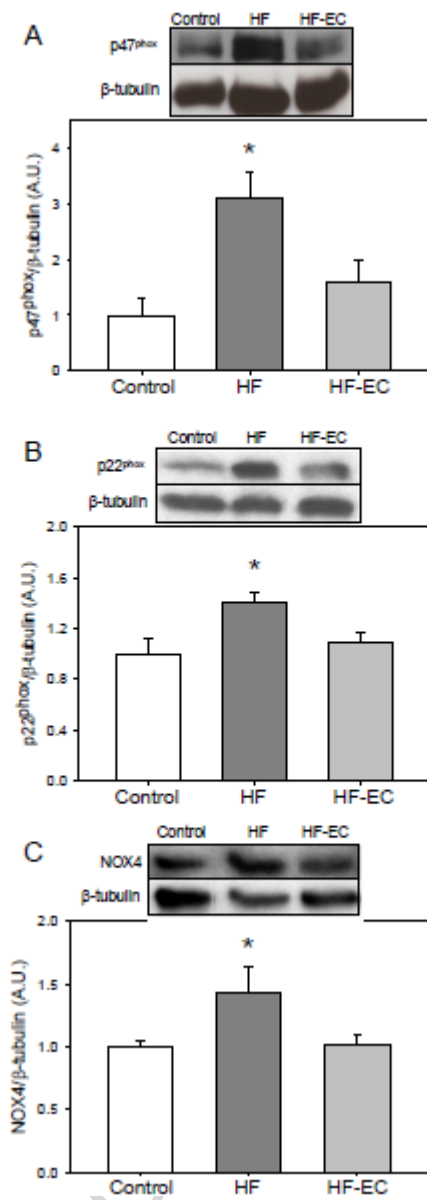
Parameters	Control	HF	HF-EC
BW (g)	336 ± 13	310 ± 13	322 ± 14
HW (g)	1.14 ± 0.06	1.02 ± 0.05	1.13 ± 0.05
HW/BW	3.4 ± 0.1	3.29 ± 0.09	3.51 ± 0.05
Plasma TC/HDL-C	2.4 ± 0.1	3.5 ± 0.4*	1.72 ± 0.08
WT/LD (µm/mm)	65 ± 1	59 ± 3	66 ± 2
SBP (mmHg)			
0 w	109 ± 2	112 ± 2	112 ± 2
4 w	121 ± 2	132 ± 2*	124 ± 1
8 w	130 ± 4	142 ± 3*	130 ± 2

BW: body weight; HW: heart weight; TC/HDL-C: ratio total cholesterol/HDL cholesterol; WT/LD: ratio wall thickness/lumen diameter; SBP: blood pressure; Values are shown as means ± ESM (n = 10 per group).

* p < 0.05 with respect to Control and HF-EC groups.

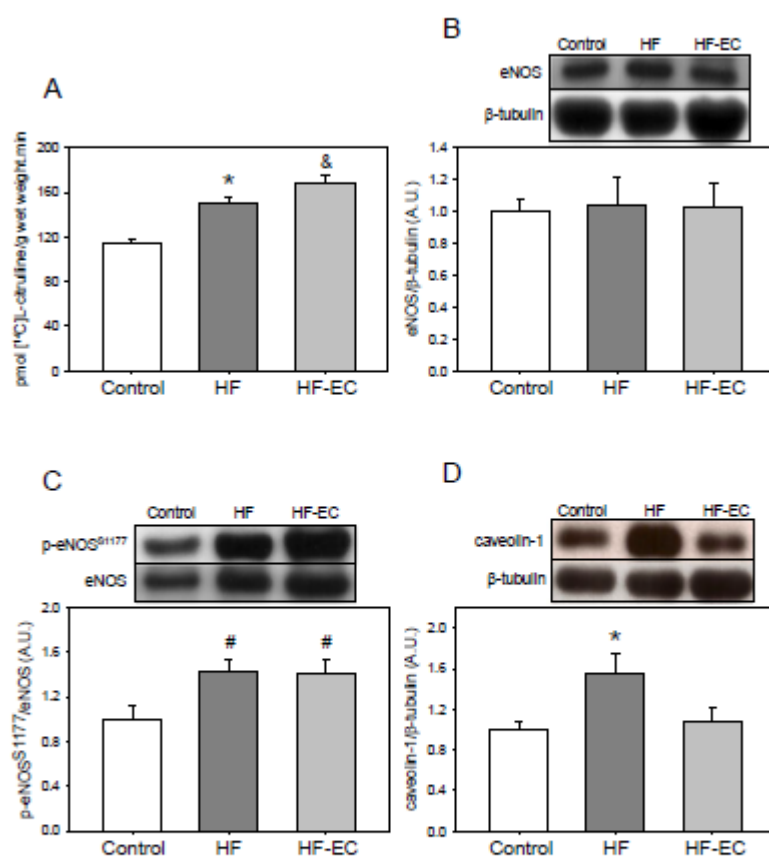
Fig. 1.



Figure(s)
Fig. 2.

Figure(e)

Fig. 3.



Figure(s)
Fig. 4.