Involvement of nitric oxide and prostacyclin in the antihypertensive effect of low-molecular weight procyanidin rich grape seed extract in male spontaneously hypertensive rats

Quiñones M[†], Guerrero L[†#], Fernández-Vallinas S[‡], Pons Z[†], Arola L[†§], Aleixandre A[‡], and Mugerza B[†§*].

[†] Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili, Tarragona, Spain.

[#] Department of Research, Nutrition and Innovation, ALPINA S.A, Bogotá, Colombia.

[‡] Department of Pharmacology, Universidad Complutense, Madrid, Spain.

[§] Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, Catalonia, Spain

Running title: The antihypertensive mechanism of a grape seed procyanidin extract

*Dra. Begoña Muguerza

Dpto. Bioquímica y Biotecnología, U. Rovira i Virgili, C/ Marcel·li Domingo s/n 43007 Tarragona, Spain. Centre Tecnològic de Nutrició i Salut (CTNS) (TECNIO), Avda. Universitat, 1 43204 Reus, Spain.

Telephone: 34-977 55 9566

Fax: 34-977 55 8232

E-mail: begona.muguerza@urv.cat

Abstract

The aim of this study was to evaluate the involvement of endothelial-relaxing factors as possible antihypertensive mechanism of low-molecular weight procyanidin rich grape seed extract (LM-GSPE).

Thirty 17-20-week-old male spontaneously hypertensive rats (SHR) were administered water or 375 mg/kg LM-GSPE by intragastric gavage. 1 mL of saline, 30 mg/kg Nw-nitro-L-arginine methyl ester (L-NAME) or 5 mg/kg indomethacin was administrated intraperitoneally. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded before and 6 hours after oral administration. The plasma concentration of 6-keto prostaglandin F1α (PGF1α) was evaluated. In addition, we evaluated the relaxation caused by LM-GSPE in different aorta preparations.

The antihypertensive effect of LM-GSPE was completely abolished by L-NAME, and the animals increased their values of SBP and DBP. Indomethacin partially inhibited the antihypertensive effect. In addition, plasma PGF1α was increased in the LM-GSPE-treated rats. Finally, LM-GSPE relaxed the intact aorta preparations but did not relax the endothelium-denuded aorta rings. L-NAME inhibited the relaxation caused by LM-GSPE in the SHR aorta rings, but indomethacin did not.

In conclusion, the antihypertensive effect of LM-GSPE in SHR is endothelium dependent, and it could be mediated by changes in endothelium-derived nitric oxide bioavailability. Nevertheless, prostacyclin could also contribute additionally to this effect.

Key words: Grape seed procyanidin extract; Nitric oxide; Polyphenols; Prostacyclin, Spontaneously hypertensive rats.

1. Introduction

Endothelial tissue regulates vascular tone and exerts finely tuned control over cardiovascular homeostasis, with nitric oxide (NO) being one of the best-characterised vasodilator endothelial factors. NO is synthesised in the endothelial cells by a Ca^{2+}-dependent constitutive isoform of the enzyme NO synthase (eNOS), which can be up-regulated by an elevation in arterial blood pressure or by the presence of insulin or hormones such as adiponectin, oestrogens or thyroid hormone (Vanhoutte, Shimokawa, Tang, & Feletou, 2009). In fact, the inhibition of eNOS synthesis will increase blood pressure, as has been previously demonstrated in animal experimental models (Quiñones, Muguerza, Miguel, & Aleixandre, 2011). The diet is also an important factor in the up-regulation of eNOS. It is known that flavonoid consumption potentiates NO endothelium-dependent relaxation (Duffy et al., 2001; Fisher, Hughes, Gerhard-Herman, & Hollenberg, 2003; Mukai & Sato, 2009; Schroeter et al., 2006; Stein, Keevil, Wiebe, Aeschlimann, & Folts, 1999; Yamamoto, Suzuki, & Hase, 2008).

Prostaglandin I2 (PGI_2), which is known as prostacyclin, is also an important vasodilator endothelial factor. PGI_2 is synthesised in the endothelial cells by cyclooxygenase 2, and this prostaglandin also plays an important role in limiting platelet-mediated thrombosis because it is also a potent inhibitor of platelet aggregation. Previous studies have suggested that a decrease in arterial blood pressure was caused by procyanidins and could be mediated at least in part by endothelial vascular relaxing factors (Quiñones et al., 2010; Quiñones, Sánchez, Muguerza, Miguel, & Aleixandre, 2011).

Our research group has previously demonstrated the antihypertensive effect of low-molecular weight procyanidin rich grape seed extract (LM-GSPE) in spontaneously hypertensive rats (SHR). This grape seed extract was found to have as the most abundant phenols low-molecular weight flavanols mainly monomers and dimers (Quiñones et al., 2013). This fact is considered important because although polyphenol bioavailability is relatively poor, flavanols with low molecular weight are among the most bioavailable flavanoid compounds (Tomas-Barberan et al., 2007). In addition, it is reported that the healthy properties attributed to food rich in flavanols, such as cocoa, seem to be related to the high amount of monomeric and dimeric compounds (Cooper et al., 2008). The aim of this study was to evaluate the involvement of endothelial-relaxing factors as possible antihypertensive mechanism of LM-GSPE. We used animals that had been alternatively treated with Nw-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO synthesis, or with indomethacin, an inhibitor of prostacyclin synthesis. Plasma PGI_2 concentrations were evaluated in LM-GSPE-administered rats. We also studied the involvement of endothelial factors on the vasorelaxing effect of LM-GSPE in the aorta rings of untreated SHRs.

2. Materials and Methods

2.1. Products

The procyanidin extract that was used in this study (LM-GSPE) was obtained from grape seeds by Les Dériveés Résiniques et Terpéniques, Dax, France. Table 1 shows the total polyphenol, phenolic compounds (flavan-3-ols and phenolic acids) and the antioxidant capacity of the extract used in this study (adapted from Quiñones et al., 2013).

N^w-nitro-L-arginine methyl ester (L-NAME) and indomethacin were purchased from Sigma (Barcelona, Spain).

2.2. Experimental Procedure in Rats

Thirty 17-20-week-old male SHRs with an average weight of 319.07 ± 2.38 g were used for the *in vivo* experiments. All these animals were obtained from Charles River Laboratories (Barcelona, Spain). The rats were maintained at a temperature of 22 °C in 12-hour light/dark cycles. They received tap water and a standard diet (A04 Panlab, Barcelona, Spain) *ad libitum* during the experiments and were divided into two groups that were administered distilled water or 375 mg/kg of LM-GSPE dissolved in distilled water by gastric intubation between 9 and 10 am. The total orally administered volume was always 1 mL, whether it was water or LM-GSPE water solution. Four hours after oral administration, 5 of the animals from each group were given 1 mL intraperitoneal saline, 30 mg/kg of L-NAME dissolved in 1 mL of saline or 5 mg/kg indomethacin dissolved in 1 mL of saline. Rat systolic blood pressure (SBP) and diastolic blood pressure (DBP) were taken by the tail cuff method before the initial oral administration and again at 6 hours afterwards. The rats were kept at 38°C for 10 minutes before the measurement to detect the pulse of the tail artery. The original method for measuring arterial blood pressure using the tail cuff provides only SBP values (Buñag, 1973), but the equipment used in this study, an LE 5001 (Letica, Spain), has a high sensitivity pulse transducer coupled with an accurate microprocessor program, which allowed us to distinguish between the SBP and the DBP. Five measurements were averaged to establish the values of the SBP and DBP. All measurements were taken by the same person in the same peaceful environment to minimise stress-induced variations in blood pressure. Moreover, to guarantee the reliability of the measurements, we established a training period of two weeks before the actual trial time, and the rats became accustomed to the procedure during this period.

Additionally, fifteen 22-week-old SHRs were sacrificed by decapitation after overnight fasting. Eight of them were treated with 375 mg/kg LM-GSPE 6 hours before being sacrificed, and the remaining animals (seven) were given water 6 hours before being sacrificed. The LM-GSPE and water were orally administered by gastric intubation between 9 and 10 a.m. Blood samples were collected from the sacrificed rats to quantify 6-keto prostaglandin F1α (PGF1α), a stable metabolite of PGI_2. The procedures that were used to evaluate all of these parameters are described below.

2.3. Prostacyclin determination

Blood samples from the sacrificed animals were collected in tubes containing the anticoagulant lithium heparin. These samples were centrifuged at 2000 g for 15 minutes at 4°C to obtain the plasma, which was divided into aliquots and stored at -80°C until the analysis. We measured the concentration of 6-keto prostaglandin F1α (PGF1α), a stable metabolite of PGI_2, by using a 6-keto prostaglandin F1α EIA (enzyme immunoassay) kit (Cayman Chemical Company, Tallinn, Estonia). The results are expressed as pg/mL of PGF1α.

2.4. Experiments in aorta rings

We used 17-22-week-old SHRs for these experiments. The animals were sacrificed by decapitation. The thorax was opened, and the aorta was rapidly excised from the aortic arch to the diaphragm. Excess fat and connective tissue were removed from the aorta, and the tissue was cut into rings (approximately 4 mm in length). The aortic rings were mounted between two steel hooks in isolated tissue chambers containing Krebs-Henseleit solution with the following composition (mM): NaCl, 118; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose, 10.0. The medium was maintained at 37°C and was continuously bubbled with a 95% O_2 and 5% CO_2 mixture with a pH of 7.4. An optimal resting tension of 2 g was applied to all the aortic segments. This tension was adjusted every 15 min during a 60-90 min equilibration period before adding the drugs. The isometric tension was recorded by using an isometric force displacement transducer connected to an acquisition system (Protos 5, Panlab, Spain). After the equilibration period, the rings were contracted with 80 mM KCl to assess their functionality, and when the contraction had reached a steady state (approximately 15 min after administration), the preparations were washed until their basal tension was recovered. The rings were then exposed to 10^{-6} M methoxamine, and LM-GSPE dose-response curves (10^{-6}-10^{-1} mg/mL) were performed in the methoxamine-precontracted rings. Relaxant responses to LM-GSPE are expressed as a percentage of the precontraction induced by methoxamine. The previously described procedure was applied to intact and endothelium-denuded tissue. It was also applied to another two groups of intact preparations; one with the addition of N^{W}-nitro-L-arginine methyl ester (L-NAME) (10^{-4} M), and the other with the addition of indomethacin (10^{-5} M). Both drugs were added to the organ bath 30 minutes before methoxamine administration. The denuded endothelium preparations were prepared by gently rubbing the tissue before it was cut into rings. The efficacy of the procedure that was used to remove the endothelial cells was judged by the loss of acetylcholine-induced relaxation when the aorta preparations were precontracted with methoxamine. According to a study by

Furchgott in 1980, the endothelial cells are necessary for this response (Furchgott & Zawadzki, 1980).

All the above-mentioned experiments were performed as authorised (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

2.5 Statistical analysis

The blood pressure results are expressed as the mean values ± the standard error of the mean (SEM), and they were analysed with a one-way analysis of variance (ANOVA) in GraphPad Prism software. The differences between the groups were assessed with the Bonferroni test. Plasma PGF1α data are also expressed as the mean values ± SEM, but they were analysed with Student´s t-test. Finally, the aorta ring results are expressed as the mean values ± SEM for at least 5 preparations and 5 animals. P values were estimated with a two-way ANOVA (Bonferroni Test).

Differences between the means were always considered to be significant when $p<0.05$.

3. Results

3.1. Effects of LM-GSPE on blood pressure in SHRs that were pretreated with N^{w}-nitro-L-arginine methyl ester (L-NAME) and indomethacin

The initial values of SBP and DBP in the SHRs were 201.99 ± 4.18 mm Hg and 181.22 ± 1.91 mm Hg, respectively. As expected, the rats that only received water or saline did not experience changes in their SBP and DBP, but the administration of 375 mg/kg LM-GSPE caused a significant decrease in the

SBP (-35.68 ± 5.39 mmHg; p<0.05) and DBP (-34.52 ± 5.45 mm Hg; p<0.05). These decreases were evident 6 hours post-LM-GSPE administration and are represented in Figure 1. Figure 1 also shows that 30 mg of L-NAME caused a clear increase in the SBP (+15.77 ± 4.09 mm Hg; p< 0.05) and DBP (+10.21 ± 1.62 mm Hg; p< 0.05) of the rats. The L-NAME effect was clearly evident two hours after the intraperitoneal administration of this arginine derivative. In addition, the LM-GSPE effect was not observed when the rats were treated four hours later with 30 mg/kg L-NAME. In fact, the antihypertensive effect of LM-GSPE was completely abolished by the intraperitoneal injection of this arginine derivative, and the animals that received LM-GSPE and L-NAME had increased SBP (+5.67 ± 5.94 mm Hg; p< 0.05) and DBP values (+11.13 ± 5.19 mm Hg; p< 0.05), as shown in Figures 1A and 1B.

Figures 2A and 2B demonstrate that the antihypertensive effect of LM-GSPE was also attenuated when the rats were treated with 5 mg/kg indomethacin four hours later, with SBP values of -12.13 ± 3.69 mm Hg (p< 0.05) and DBP values of -12.44 ± 5.15 mm Hg (p< 0.05). However, the intraperitoneal injection of this prostacyclin inhibitor did not modify arterial blood pressure in these animals in the group of rats that received water.

3.2. Effects of LM-GSPE on plasma prostacyclin concentration

As Figure 3 shows, the 6-keto prostaglandin F1α plasma levels in LM-GSPE-treated rats were significantly higher (27.68 ± 6.1 pg/mL) than in the water-administered rats (13.28 ± 2.5 pg/mL).

3.3. Effects of LM-GSPE in aorta rings

LM-GSPE induced the dose-dependent relaxation of the intact aorta ring preparations from untreated SHRs, but LM-GSPE did not relax the endothelium-denuded aorta ring preparations from these animals. In addition, the relaxation caused by LM-GSPE in the intact aorta ring preparations was completely abolished when the tissue had been exposed to 10^{-4} M L-NAME, mimicking the effect that was obtained in the endothelium-denuded aorta ring preparations. However, the presence of 10^{-5} M indomethacin in the bath solution did not modify the relaxations induced by LM-GSPE in the aorta tissue (see Figure 4).

4. Discussion

In previous study, our research group has demonstrated the multiple beneficial effects of LM-GSPE in the metabolic syndrome. LM-GSPE possesses potent hypolipidemic agents. LM-GSPE has shown to reduce plasma triglycerides levels, apo B and LDL cholesterol, as well as to increase the percentage of HDL cholesterol in rats (Del Bas et al., 2005). LM-GSPE has exhibited anti-inflammatory effect (Pallarès et al., 2013) and LM-GSPE also modulates proliferation and apoptosis of pancreatic beta-cells (Cedó et al., 2013). Recently, our research group also has demonstrated the antihypertensive effect of LM-GSPE in male SHRs (Quiñones et al., 2013). In this study, we have already corroborated that the single oral administration of 375 mg/kg LM-GSPE in SHRs, 6 h after-administration, caused a decrease in SBP that was similar to that of 50 mg/kg of Captopril, which is considered a very effective antihypertensive treatment in clinical practice (Quiñones et al., 2013).

The main objective in present study was performed to clarify the mechanisms implicated in the antihypertensive effect of LM-GSPE. This study evaluates the possible participation of endothelial-relaxing factors as possible antihypertensive mechanism of LM-GSPE.

The results provide clear evidence for the participation of NO in the vasodilator and antihypertensive effects of LM-GSPE. Our results are in accordance with the majority of available data, which support the idea that flavonoid-rich foods can improve NO release or NO availability in several animal models (Jiménez, Duarte, & Perez-Vizcaino, 2012; Quiñones et al., 2011).

L-NAME is an *in vivo* and *in vitro* inhibitor of eNOS (Moncada, Palmer, & Higgs, 1991; Rees, Schulz, Hodson, Palmer, & Moncada, 1990) and a clear increase in SBP and DBP was observed in the L-NAME-treated SHRs of this study. The inhibition of basal NO synthesis by L-NAME in these animals could justify these results, but in order to fulfil the aim of the present study, the most important finding was the observed inhibition of the LM-GSPE antihypertensive effect in the L-NAME-treated SHRs. These results provide clear evidence that antihypertensive effect of LM-GSPE is mediated by NO release in SHRs. A previous study carried out by our group (Quiñones, et al., 2011) has already suggested that the blood pressure-lowering effect of a low-molecular weight procyanidin-rich cocoa powder in SHRs was mediated through NO. In addition, an increment of aortic NO concentration in ouabain-induced hypertensive rats treated with a grape seed extract rich in procyanidins for 5 weeks with respect to animals not treated has been recently reported (Liu et al., 2012). Edirisinghe et al., (2008) also demonstrated that grape seed procyanidins endothelium-dependent relaxation of rabbit aorta rings was mediated by eNOS phosphorylation (Edirisinghe, Burton-Freeman, & Kappagoda, 2008). Moreover, other polyphenols such as (-)-epicatechin, epigallocatechin-3-gallate, delphinidin, resveratrol and procyanidins from grape skin have been reported to increase eNOS activation (Ramirez-Sanchez, Maya, Ceballos, & Villarreal, 2010; Auger, Chaabi, Anselm, Lobstein, & Schini−Kerth, 2010).

The aorta ring preparation results corroborate the importance of NO in the vascular effects of LM-GSPE and support our *in vivo* results with this extract. The effect of LM-GSPE was, in fact, endothelium dependent because LM-GSPE did not relax the endothelium-denuded aorta ring preparations from SHRs. Moreover, the effect of LM-GSPE on the aorta preparations was impaired when NOS was inhibited, and we were able to correlate the LM-GSPE activity in this tissue with the increased release of NO.

The endothelium also secretes other vasodilator agents than NO such as PGI_2. In fact, PGI_2 was the first described endothelium-derived relaxing substance. This prostaglandin is produced by the cyclooxygenase enzyme (COX) and PGI_2 synthase (Vane, Gryglewski, & Botting, 1987), and it is the major product of arachidonic acid metabolism in the vascular endothelium. PGI_2 is continuously released into the circulation by the lungs to counter platelet aggregation from the release of thromboxane A_2. The importance of PGI_2 is therefore not in doubt, but at the present time, NO is considered to be the more important vasodilator endothelial factor. We have also evaluated the effect of LM-GSPE in SHRs that were intraperitoneally injected with indomethacin, an inhibitor of COX enzyme and endothelial prostanoid biosynthesis. Indomethacin treatment did not modify SBP and DBP in the SHRs, but this compound partially abolished the LM-GSPE antihypertensive effect (Figure 2). Some studies have shown an increase in the release of PGI_2 in procyanidin-treated human aortic endothelial cells (Schramm et al., 2001) and increased the levels of prostacyclin in human plasma (Schramm et al., 2001) and in the plasma of rats that were fed a diet rich in procyanidins (Maffei, Carini, Aldini, Berti, Rossoni, & Morazzoni, 1999). In our study, we have demonstrated an increased plasma concentration of PGF1α in LM-GSPE-treated rats, and we know that PGF1α is a stable metabolite of PGI_2. Therefore, the rats that were treated with grape extract most likely also had increased plasma concentrations of PGI_2.

However, our results from the aorta ring preparations indicated that the vasodilator effect of LM-GSPE in this tissue was not mediated by PGI_2. In fact, LM-GSPE clearly relaxed the aorta tissue when the cyclooxygenase inhibitor indomethacin was present in the organ bath. Our *in vivo* and *in vitro* results are therefore not in accordance with results from our examination of the involvement of PGI_2 on the vascular effects of LM-GSPE, but we should not forget that the aorta is a conduit artery and resistance arteries determine the arterial blood pressure more than the large vessels. Therefore, we believe that PGI_2 may be involved in the LM-GSPE antihypertensive effect even if we could not demonstrate the involvement of this mediator in the extract effects within an actual artery. In any case, the vasodilator effect of PGI_2 could be a complementary that justifies the effect of this extract on the arterial blood pressure of SHRs.

5. Conclusion

Endothelial dysfunction could at least in part justify the increased arterial blood pressure of hypertensive subjects, and this study has demonstrated that the antihypertensive effect of LM-GSPE in SHRs is an endothelium-dependent effect that is mainly mediated by changes in endothelium-derived NO bioavailability. The vasodilator effect of PGI_2 may also be involved in the antihypertensive effect of LM-GSPE, but more studies are recommended to clarify the involvement of PGI_2 on the effects of this extract.

Acknowledgement

This study was supported by grants from the Ministerio de Educación y Ciencia of the Spanish Government (AGL 2008-00387/ALI) and the Universitat Rovira i Virgili-Banco Santander (2011 LINE-12).

~~Our research group has previously demonstrated that the greatest antihypertensive effect of LM-GSPE in male SHRs was obtained 6 h after administration of a dose of 375 mg/kg LM-GSPE (Quiñones et al., 2013). Therefore, in this study a dose of 375 mg/kg LM-GSPE was selected to be administered for 6 h. This study confirms the antihypertensive effect of LM-GSPE. Moreover, as previously was reported, the effect of 375 mg/kg LM-GSPE at 6h post-administration in male SHRs was similar to that of 50 mg/kg of Captopril, which is considered a very effective antihypertensive treatment in clinical practice (Quiñones et al., 2013)". In addition,~~

References

Auger, C., Chaabi, M., Anselm, E., Lobstein, A., & Schini-Kerth, V. B. (2010). The red wine extract-induced activation of endothelial nitric oxide synthase is mediated by a great variety of polyphenolic compounds. *Molecular nutrition & food research, 54*(S2), S171-S183.

Buñag, R. D. (1973). Validation in awake rats of a tail-cuff method for measuring systolic pressure. *Journal of applied physiology, 34*(2), 279-282.

Cedó, L., Castell-Auví, A., Pallarès, V., Blay, M., Ardévol, A., Arola, L., Pinent, M. (2013). Grape seed procyanidin extract modulates proliferation and apoptosis of pancreatic beta-cells. *Food Chemisty,* 138(1), 524-530.

Cooper, K.A., Donovan, J.L., Waterhouse, A.L., & Williamson, G. (2008). Cocoa and health: a decade of research. *British journal of nutrition, 99,* 1-11.

Del Bas, J.M., Fernández-Larrea, J., Blay, M., Ardèvol, A., Salvadó, M.J., Arola, L., Bladé, C. (2005). Grape seed procyanidins improve atherosclerotic risk index and induce liver CYP7A1 and SHP expression in healthy rats. *The FASEB Journal,* 19(3), 479-481.

Duffy, S. J., Keaney Jr, J. F., Holbrook, M., Gokce, N., Swerdloff, P. L., Frei, B., & Vita, J. A. (2001). Short-and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation, 104*(2), 151-156.

Edirisinghe, I., Burton-Freeman, B., & Kappagoda, T. (2008). Mechanism of the endothelium-dependent relaxation evoked by a grape seed extract. *Clinical science, 114*, 331-337.

Engeli, S., Schling, P., Gorzelniak, K., Boschmann, M., Janke, J., Ailhaud, G., Teboul, M., Massiéra, F., & Sharma, A. M. (2003). The adipose-tissue renin-angiotensin-aldosterone system: role in the metabolic syndrome? *The international journal of biochemistry & cell biology, 35*(6), 807-825.

Fisher, N. D. L., Hughes, M., Gerhard-Herman, M., & Hollenberg, N. K. (2003). Flavanol-rich cocoa induces nitric-oxide-dependent vasodilation in healthy humans. *Journal of hypertension, 21*(12), 2281-2286.

Furchgott, R. F., & Zawadzki, J. V. (1980). The obligatory role of endothelial cells in the relaxation of arterial smooth muscle by acetylcholine. *Nature, 288*, 373-376.

Jiménez, R., Duarte, J., & Perez-Vizcaino, F. (2012). Epicatechin: Endothelial Function and Blood Pressure. *Journal of Agricultural and Food Chemistry, 60*(36), 8823-8830.

Liu X, Qiu J, Zhao S, You B, Ji X, Wang Y, Cui X, Wang Q, Gao H. (2012). Grape seed proanthocyanidin extract alleviates ouabain-induced vascular remodeling through regulation of endothelial function. Mol Med Rep. 2012 Nov;6(5):949-54. doi: 10.3892/mmr.2012.1026. Epub 2012 Aug 8.

Maffei, R., Carini, M., Aldini, G., Berti, F., Rossoni, G. :. B., E., & Morazzoni, P. (1999). Diet enriched with procyanidins enhances antioxidant activity and reduces myocardial post-ischaemic damage in rats. *Life Sciences, 64*, 627-642.

Moncada, S., Palmer, R., & Higgs, E. (1991). Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev, 43*(2), 109-142.

Mukai, Y., & Sato, S. (2009). Polyphenol-containing azuki bean (Vigna angularis) extract attenuates blood pressure elevation and modulates nitric oxide synthase and caveolin-1 expressions in rats with hypertension. *Nutrition, Metabolism and Cardiovascular Diseases, 19*(7), 491-497.

Pallarès, V., Cedó, L., Castell-Auví, A., Pinent, M., Ardévol, A., Arola, L., Blay, M. (2013). Effects of grape seed procyanidin extract over low-grade chronic inflammation of obese Zucker fa/fa rats. *Food Research International,* 53(1), 319-324.

Quiñones, M., Guerrero, L., Suarez, M., Pons, Z., Aleixandre, A., Arola, L., & Muguerza, B. (2013). Low-molecular procyanidin rich grape seed extract exerts antihypertensive effect in males spontaneously hypertensive rats. *Food Research International, 51*(2), 587-595.

Quiñones, M., Muguerza, B., Miguel, M., & Aleixandre, A. (2011). Evidence that nitric oxide mediates the blood pressure lowering effect of a polyphenol-rich cocoa powder in spontaneously hypertensive rats. *Pharmacological Research, 64*(5), 478-481.

Quiñones, M., Sánchez, D., Muguerza, B., Miguel, M., & Aleixandre, A. (2011). Mechanisms for antihypertensive effect of CocoanOX, a polyphenol-rich cocoa powder, in spontaneously hypertensive rats. *Food Research International, 44*(5), 1203-1208.

Quiñones, M., Sánchez, D., Muguerza, B., Moulay, L., Laghi, S., Miguel, M., & Aleixandre, A. (2010). Long-term intake of CocoanOX attenuates the development of hypertension in spontaneously hypertensive rats. *Food Chemistry, 122*(4), 1013-1019.

Ramirez-Sanchez, I., Maya, L., Ceballos, G., & Villarreal, F. (2010). (−)-Epicatechin Activation of Endothelial Cell Endothelial Nitric Oxide Synthase, Nitric Oxide, and Related Signaling Pathways. *Hypertension, 55*(6), 1398-1405.

Rees, D. D., Schulz, R., Hodson, H. F., Palmer, R., & Moncada, S. (1990). Identification of some novel inhibitors of the vascular nitric oxide synthase in vivo and in vitro. In Anonymous *Nitric oxide from L-arginine: a bioregulatory system. Proceedings of a Symposium Held at the Royal Society, London, 14-15 September* (pp. 485-487). Amsterdam: Elsevier.

Schramm, D. D., Wang, J. F., Holt, R. R., Ensunsa, J. L., Gonsalves, J. L., Lazarus, S. A., Schmitz, H. H., German, J. B., & Keen, C. L. (2001). Chocolate procyanidins decrease the leukotriene-prostacyclin ratio in humans and human aortic endothelial cells. *The American Journal of Clinical Nutrition, 73*(1), 36-40.

Schroeter, H., Heiss, C., Balzer, J., Kleinbongard, P., Keen, C. L., Hollenberg, N. K., Sies, H., Kwik-Uribe, C., Schmitz, H. H., & Kelm, M. (2006). (–)-

Epicatechin mediates beneficial effects of flavanol-rich cocoa on vascular function in humans. *Proceedings of the National Academy of Sciences of the United States of America, 103*(4), 1024-1029.

Stein, J. H., Keevil, J. G., Wiebe, D. A., Aeschlimann, S., & Folts, J. D. (1999). Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. *Circulation, 100*(10), 1050-1055.

Tomas-Barberan, F.A., Cienfuegos-Jovellanos, E., Marin, A., Muguerza, B., Gil-Izquierdo, A., Cerdá, B., Zafrilla, P., Morillas, J., Mulero, J., Ibarra, A., Pasamar, M.A., Ramón, D., & Espín, J. C. (2007). A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans. *Journal of agricultural and food chemistry, 55,* 3926-3935.

Vane, J., Gryglewski, R., & Botting, R. (1987). The endothelial cell as a metabolic and endocrine organ. *Trends in pharmacological sciences, 8*(12), 491-496.

Vanhoutte, P. M., Shimokawa, H., Tang, E. H. C., & Feletou, M. (2009). Endothelial dysfunction and vascular disease. *Acta Physiologica, 196*(2), 193-222.

Yamamoto, M., Suzuki, A., & Hase, T. (2008). Short-term effects of glucosyl hesperidin and hesperetin on blood pressure and vascular endothelial function in spontaneously hypertensive rats. *Journal of nutritional science and vitaminology, 54*(1), 95-98.

Figure legends

Figure 1. Changes in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) caused in spontaneously hypertensive rats after different treatments: water + saline (□), 375 mg/kg LM-GSPE + saline (■), water + 30 mg/kg L-NAME (■) or 375 mg/kg LM-GSPE + 30 mg/kg L-NAME (⊟). Data are expressed as the mean ± SEM. The experimental groups always had 7 animals each. Different letters represent statistically significant differences ($p<0.05$). P was estimated by one-way ANOVA.

Figure 2. Changes in systolic blood pressure (SBP) (A) and diastolic blood pressure (DBP) (B) caused in spontaneously hypertensive rats after different treatments: water + saline (□), 375 mg/kg LM-GSPE + saline (■), water + 5 mg/kg indomethacin (■) or 375 mg/kg LM-GSPE + 5 mg/kg indomethacin (⊟). Data are expressed as the mean ± SEM. The experimental groups always had 7 animals. Different letters represent statistically significant differences ($p<0.05$). P was estimated by one-way ANOVA.

Figure 3. The plasma 6-keto prostaglandin F1α (PGF1α) concentration in spontaneously hypertensive rats treated with 375 mg/kg LM-GSPE-treated SHR (■) or water (□). Data are expressed as the mean ± SEM. The experimental group had 8 animals and the water group had 7 animals. The results were analysed with Student's t test, and the differences between the means were considered significant when $p<0.05$. The asterisk indicates statistically significant differences.

Figure 4. Grape Seed Procyanidin Extract (LM-GSPE) relaxation in different aorta ring preparations from spontaneously hypertensive rats: intact (■), endothelium denuded (□), intact incubated with L-NAME (10^{-4} M) (▲), intact incubated with indomethacin (10^{-5} M) (●). The aorta rings were precontracted with 10^{-6} mol/L methoxamine. Data are the mean values ± SEM for at least 5 preparations with 5 animals per group. P was estimated by two-way ANOVA (Bonferroni Test), and we considered the difference to be significant when $P < 0.05$. Similar letters represent no statistically significant differences.

Table 1. Total polyphenols (mg/g) and main phenolic compounds (flavan-3-ols and phenolic acids) (mg/g) of the grape seed low-molecular weight proacyanidin extract (LM-GSPE) used in this study.

	Amount
Total Polyphenols[1]	**516.8 ± 12.1**
Phenolic compounds[2]	
Gallic acid	17.7 ± 2.0
Protocatechuic acid	1.0 ± 0.1
Vanillic acid	0.1 ± 0.0
Procyanidin dimer B2[a]	68.5 ± 14.7
Procyanidin dimer B1+B3[a]	64.8 ± 15.2
Procyanidin dimer (others forms)[a]	10.8 ± 2.4
Catechin	90.7 ± 7.6
Epicatechin	55.0 ± 0.8
p-coumaric acid	0.1 ± 0.0
Dimer gallate B2[b]	27.3 ± 6.0
Dimer gallate B1+B3[b]	9.8 ± 0.9
Dimer gallate (others forms)[b]	2.5 ± 0.7
Epigallocatechin gallate[b]	0.4 ± 0.1
Procyanidin trimer[a]	28.4 ± 2.0
Procyanidin tetramer[a]	2.0 ± 0.2
Epicatechin gallate[b]	55.3 ± 1.5
Quercetin-3-*O*-galactoside	0.2 ± 0.0
Naringenin-7-glucoside	0.1 ± 0.0
Kaempferol-3-glucoside	0.1 ± 0.0
Quercetin	0.3 ± 0.0
Antioxidant capacity [3]	**16,936 ± 651**

The results are expressed on a wet basis as mean ± SD (n=3).
[1]Spectrophotometric method Folin-Ciocalteu. Results expressed as mg gallic acid equivalent/g of fresh LM-GSPE.
[2]HPLC-MS. Results expressed as mg of phenolic compound/g of fresh LM-GSPE
[3]Hydrophilic ORAC (H-ORAC) assay, expressed as µmol of Trolox equivalents (TE)/g of fresh LM-GSPE.
a Quantified using the calibration curve of procyanidin B2.
b Quantified using the calibration curve of epigallocatechin gallate.