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Cathleen London, M.D., P.C.

Board certified Family Medicine Physician and graduate of Yale University School of Medicine and Brown University.

The safety of patented MegaNatural®-BP Grape Seed Extract is documented in a GRAS petition reviewed by the Food and Drug Administration. MegaNatural®-BP Grape Seed Extract has been the subject of published studies conducted at the Department of Internal Medicine of the University of California at Davis.

- **Effect of Grape Seed Extract on Blood Pressure in Subjects with the Metabolic Syndrome**

Sivaprakasapillai B^a, Edirisinghe I^c, Randolph J^b, Steinberg F^b, Kappagoda T^a.
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- **Mechanism of the Endothelium-Dependent Relaxation Evoked by a Grape Seed Extract**

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QUESTIONS TO ASK YOUR DOCTOR:

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5. How soon after starting a daily dose of MegaNatural®-BP are we likely to see benefits?

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Mechanism of the endothelium-dependent relaxation evoked by a grape seed extract

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A B S T R A C T

GSEs (grape seed extracts) which contain polyphenolic compounds cause an endothelium-dependent relaxation of blood vessels. The aim of the present study was to examine the mechanisms involved in this response. A well-characterized GSE was applied to rabbit aortic rings suspended in organ baths containing Krebs–Henseleit buffer maintained at 37 °C. In aortic rings pre-contacted with noradrenaline (norepinephrine), the extract produced a dose-dependent relaxation. The maximum relaxations elicited by the extract ($71.9 \pm 1.0\%$) were similar to those elicited by acetylcholine ($64.2 \pm 1.5\%$) ($n = 12$ for each). As expected, the relaxations were abolished by removal of the endothelium and by prior incubation with L-NAME (N^G -nitro-L-arginine methyl ester), confirming the essential role of eNOS (endothelial NO synthase) in the response. The responses to the GSE were also abolished by incubation with wortmannin and LY294002, which are inhibitors of PI3K (phosphoinositide 3-kinase). These compounds had no effect on the responses to acetylcholine. Using immunoblotting, we also demonstrated that the GSE induced the phosphorylation of both Akt and eNOS in HUVECs (human umbilical vein endothelial cells). Finally, the extract was modified by methylation of the hydroxy groups in the polyphenolic groups and was applied to the aortic rings. The modified extract failed to cause a relaxation. Taken together, these findings suggest that the endothelium-dependent relaxation induced by the GSE was mediated by activation of the PI3K/Akt signalling pathway through a redox-sensitive mechanism, resulting in phosphorylation of eNOS.

INTRODUCTION

There is evidence that a diet rich in vegetables and fruit has a beneficial effect on blood pressure. This effect has been attributed to phenolic compounds present in the plants. These compounds have also been shown to influence endothelial function in a variety of experimental situations [1–3]. In humans, extracts of fruits and vegetables have been shown to enhance flow-mediated vasodilation in the brachial artery [4].

Of all of the phenolic products, those derived from grapes appear to have received the most attention, possibly because of their involvement with the French paradox [5,6]. Grapes and grape products derived from the skin, seeds, pulp and stem are good sources of polyphenolic compounds; however, it has been found that >70% of polyphenolic compound are concentrated in the seeds [7]. GSEs (grape seed extracts) cause an EDR (endothelium-dependent relaxation) of aortic rings *in vitro* (for example, [2,8,9]). Similarly, these extracts

Key words: bioactive phenolic, endothelium-dependent relaxation, endothelial nitric oxide synthase (eNOS), grape seed extract, phosphoinositide 3-kinase (PI3K), reactive oxygen species (ROS).

Abbreviations: EDR, endothelium-dependent relaxation; GSE, grape seed extract; HUVEC, human umbilical vein endothelial cell; KH buffer, Krebs–Henseleit buffer; L-NAME, N^G -nitro-L-arginine methyl ester; NOS, NO synthase; eNOS, endothelial NOS; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; SNP, sodium nitroprusside.

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have also been shown to activate eNOS [endothelial NOS (NO synthase)] [2,10] and up-regulate eNOS in cultured endothelial cells [11].

The mechanism mediating this response to GSEs has not been established with certainty. Grape juice [12] and extracts of red wine [13] have been shown to cause EDR, which was abolished by blocking the PI3K (phosphoinositide 3-kinase)/Akt pathway. In the present study, we have examined the effect of a well-characterized GSE, which has been shown previously to cause EDR in guinea pig aortic rings [14] and reduce blood pressure in humans [15], on the PI3K/Akt signalling pathway and phosphorylation of eNOS. The studies were undertaken on both rabbit aortic rings and HUVECs (human umbilical vein endothelial cells).

MATERIALS AND METHODS

Study design and procedures

This study was approved by Animal Use and Care Administrative Advisory Committee, University of California, Davis, CA, U.S.A. Male New Zealand rabbits, weighing 3–3.5 kg, were sedated by intramuscular injection of acepromazine. After 5 min, a lethal dose of sodium pentobarbitone (50 mg/kg of body weight; Abbott Laboratory) was administered through the lateral ear vein. A thoracotomy was performed and the descending thoracic aorta was excised carefully. The aorta was flushed twice with fresh ice-cold KH (Krebs–Henseleit) buffer (118 mmol/l NaCl, 5.4 mmol/l KCl, 1.2 mmol/l, MgCl₂ 2.5 mmol/l CaCl₂, 22 mmol/l NaHCO₃, 1.2 mmol/l NaH₂PO₄ and 10.1 mmol/l glucose; using Sigma analytical grade reagents) and placed in a dissecting tray filled with the same buffer. All surrounding connective tissues and fat were removed carefully.

The GSE used in the present study is a water extract prepared by Polyphenolics Inc (Meganatural-BP[®]; patent pending). The extract is made up of polymers of catechin and has an average degree of polymerization of 2.3. The extract was dissolved in KH buffer, and the concentrations of the solution were based on a nominal M_r of 1000. The phenol content of the GSE solution (1 mg/ml) was measured using the Folin–Ciocalteu assay and was found to be 39.2 ± 0.65 mmol/l gallic acid units ($n=5$). The characterization of the extract is given in Supplementary material available at <http://www.clinsci.org/cs/114/cs1140331add.htm>.

Measurement of EDR

EDR was assessed as described previously [16]. Briefly, the aorta was segmented into rings (5 mm in length) which were mounted between two tungsten wire triangles. One triangle was attached to a strain-gauge transducer and the other to the bottom of an organ bath (20 ml) containing KH buffer maintained at 37°C and

oxygenated with a mixture of 95% O₂/5% CO₂. A pre-load of 8 g was applied to the rings, and the tissues were allowed to equilibrate for 60 min. The transducer was connected to a pen recorder (Gould-2400S recorder), and the changes in tensions were monitored using a Windaq computer program (2003 version; Dataq Instruments).

After equilibration for 60 min at a pre-load of 8 g, the aortic rings were pre-contracted with 10 μmol/l noradrenaline (norepinephrine; Sigma). Acetylcholine (Sigma) was added in an incremental manner to achieve bath concentrations from 0.1–10 μmol/l to obtain dose–response curves for EDR. The relaxations were expressed as a percentage of the contraction induced by noradrenaline.

GSE-induced EDR

After demonstrating EDR evoked by acetylcholine, the rings were treated with increasing concentrations of the GSE following pre-contraction with noradrenaline. In additional experiments, the effect of removing the endothelium on relaxation evoked by acetylcholine and the GSE were examined to establish the endothelium-dependent nature of the relaxation. In these experiments, after demonstrating the absence of relaxation, the rings were treated with SNP (sodium nitroprusside; Sigma) to establish the ability of the aortic smooth muscle to relax. As a further control, the effect of incubation with L-NAME (*N*^G-nitro-L-arginine methyl ester; bath concentration, 1 mmol/l; Sigma), a competitive inhibitor of NOS, was examined to demonstrate the involvement of NOS in the relaxation of the rings.

Effect of blocking the PI3K/Akt pathway on EDR induced by the GSE

Previous studies have shown that the EDR evoked by polyphenolic compounds derived from grapes was abolished by inhibitors of the PI3K/Akt pathway [13,17]. In the present study, the effect of the GSE was examined after incubating the aortic rings with wortmannin (30 nmol/l; Sigma) and LY294002 (30 μmol/l; Sigma) in KH buffer. Both wortmannin and LY294002 are potent and specific PI3K inhibitors. In testing the effect of each inhibitor, three aortic rings were tested simultaneously according to the sequence shown in Table 1. This protocol was based on a previous finding that prior exposure to the GSE and other phenolic compounds (e.g. cocoa) attenuated the effect of subsequent exposure [2,10]. Thus it is not possible to expose a ring to the same extract twice, before and after exposure to the inhibitor, and obtain meaningful data.

Step 1 was done to establish responsiveness of the rings to a standard concentration of acetylcholine, step 2 provided a baseline dose–response curve to acetylcholine, and steps 3 and 4 established the effect of the inhibitors.

Ring 1 was used to examine the effect of the extract after incubation with the inhibitor, and ring 2 was used to demonstrate the response to the extract without prior

Table 1 Protocol for testing the effect of the PI3K inhibitors on EDR induced by the GSE

Step	Ring 1	Ring 2	Ring 3
1	Acetylcholine (10 $\mu\text{mol/l}$)	Acetylcholine (10 $\mu\text{mol/l}$)	Acetylcholine (10 $\mu\text{mol/l}$)
2	Dose-response curve with acetylcholine	Dose-response curve with acetylcholine	Dose-response curve with acetylcholine
3	Incubate with PI3K inhibitor for 30 min	No incubation, KH buffer alone	Incubate with PI3K inhibitor for 30 min
4	Dose-response curve with the GSE	Dose-response curve with the GSE	Dose-response curve with acetylcholine

exposure to the inhibitor. It also showed that prior exposure to acetylcholine did not influence the response to the extract (i.e. the maximal responses were similar). Ring 3 was used to demonstrate that the response to acetylcholine was unaltered with time (time control) and that exposure to the blocker did not affect the ability of eNOS to be activated by acetylcholine. This protocol avoided the application of the extract twice in succession to a ring.

Effect of wortmannin and LY294002 on phosphorylation of eNOS and Akt

HUVECs were grown in EGM-2 medium (Cambrex) with 10% (v/v) fetal bovine serum. Cells were grown to confluence (approx. 90%) and starved for 6 h in serum-free medium before the cells were treated with the GSE (10 $\mu\text{mol/l}$). Some wells were treated with LY294002 (30 $\mu\text{mol/l}$) or wortmannin (30 nmol/l) for 30 min before exposure to the GSE. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂ for 10 min. The reaction was stopped by adding ice-cold PBS, washed twice with PBS and cell lysates were prepared in RIPA buffer [20 mmol/l Tris/HCl (pH 7.5), 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Nonidet P40, 1% sodium deoxycholate, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β -glycerophosphate, 1 mmol/l sodium orthovanadate and 1 $\mu\text{g/ml}$ leupeptin]. Total proteins (30 μg) were separated by SDS/PAGE [7.5% (w/v) polyacrylamide gels] and were transferred electrophoretically on to nitrocellulose membranes (Amersham Biosciences). Membranes were blocked with blocking buffer containing 5% (w/v) non-fat milk in TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1 h. Phosphorylated Akt (at Ser⁴⁷³), phosphorylated eNOS (at Ser¹¹⁷⁷), Akt and eNOS were detected after the membranes were incubated with the respective primary antibodies {rabbit anti-[phospho-eNOS (Ser¹¹⁷⁷)], anti-eNOS, anti-[phospho-Akt (Ser⁴⁷³)] and anti-Akt; 1:1000 dilution; Cell Signaling Technology} overnight at 4°C. Membranes were washed three times (10 min each) and incubated with the secondary antibody [HRP (horseradish peroxidase)-labelled anti-(rabbit IgG); 1:20 000 dilution; Cell Signaling Technology] at room temperature (25°C) for 60 min. Membranes were washed three times again (10 min each) and the specific protein bands were visualized using ECL[®] (Amersham

Biosciences). All four proteins were detected on the same blot, and the membranes were washed with stripping buffer (Pierce Biotechnology) for 30 min in 37°C before being incubated with the next primary antibody.

Effect of methylated GSE on EDR

An additional series of experiments were undertaken to study the effect of methylated GSE on rings of rabbit aorta. In each experiment, two rings were prepared as described above. Ring 1 was exposed to acetylcholine (10 $\mu\text{mol/l}$) and the GSE (100 $\mu\text{mol/l}$), and ring 2 was treated sequentially with acetylcholine (10 $\mu\text{mol/l}$), methylated GSE and the GSE (both 100 $\mu\text{mol/l}$). The methylation procedure is outlined in the Supplementary material available at <http://www.clinsci.org/cs/114/cs1140331add.htm>.

Statistical analysis

Group data are expressed as means \pm S.E.M. Comparisons between groups were compared using a paired Student's *t* test or ANOVA depending on the number of groups being examined. Dose-response curves were compared using repeated measures ANOVA. Data were analysed using Sigma Stat (version 3, 2003) statistical software. Statistical significance among treatments was determined as $P < 0.05$.

RESULTS

Effect of the GSE on EDR

The GSE produced a dose-dependent relaxation of the aortic rings. The maximum relaxations observed were similar to those produced by acetylcholine (Figure 1). Removal of the endothelium abolished the responses evoked by acetylcholine and the GSE, confirming the obligatory role of the endothelium. Incubation with L-NAME, a competitive eNOS inhibitor, also abolished the relaxation responses to acetylcholine and the GSE. However, thereafter the rings remained responsive to SNP, which is a non-endothelium-dependent relaxant of smooth muscle (Figure 2). The maximum relaxations observed in the rings under the different conditions are summarized in Table 2. These results confirmed that the GSE causes EDR in rings of rabbit aorta.

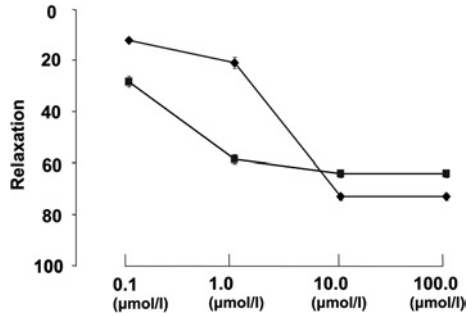


Figure 1 Dose-dependent relaxation of aortic rings induced by acetylcholine and the GSE

Dose-response curves relating relaxation (as a percentage of contraction to 10 μmol/l noradrenaline) and concentration of the agonists in the organ bath. Dose-dependent relaxations were evoked by acetylcholine (◆) and the GSE (■). Values are means ± S.E.M. ($n = 12$).

Effect of inhibitors of the PI3K/Akt pathway

Incubation of aortic rings which had been previously shown to be responsive to acetylcholine with wortmannin or LY294002 significantly attenuated the relaxation induced by the GSE. The responses evoked by acetylcholine were unaffected. The sequence of treatments described in the Materials and methods section were used in these experiments. An example of an experiment with each blocker is shown in Figure 3, showing that the GSE-induced dose-dependent EDR was significantly attenuated in rings exposed previously to a PI3K inhibitor. The responses induced by the highest concentration of the GSE in these experiments are shown in Figure 4. It was also confirmed that acetylcholine-induced EDR was unaffected by PI3K inhibitors. Therefore it is apparent that prior exposure to a PI3K inhibitor attenuated EDR evoked by the GSE, suggesting that EDR induced by

Table 2 Summary of the maximum relaxations observed in the aortic rings

Values are means ± S.E.M. of maximum percentage relaxation evoked by different treatments ($n = 6$). Values with different superscripts are significantly different, as determined by ANOVA ($P < 0.05$).

Agent	Maximum relaxation (%)
Acetylcholine (10 μmol/l)	64.2 ± 1.5 ^a
GSE (100 μmol/l)	71.9 ± 1.0 ^b
L-NAME (1 mmol/l) + GSE (100 μmol/l)	6.3 ± 1.0 ^c
SNP (10 μmol/l)	81.4 ± 1.6 ^d

the GSE is mediated by the activation of the PI3K/Akt pathway.

GSE induces the phosphorylation of Akt and eNOS in HUVECs

EDR is caused by NO produced by the phosphorylation of eNOS. Therefore we investigated whether the GSE induced the phosphorylation of Akt (on Ser⁴⁷³) and eNOS (on Ser¹¹⁷⁷) *in vitro* in HUVECs. The GSE-induced phosphorylation of Akt and eNOS was shown by immunoblotting. Prior exposure to the PI3K inhibitor LY294002 abolished the phosphorylation of Akt and eNOS in HUVECs (Figure 5). These results suggested that the GSE phosphorylates eNOS through a PI3K/Akt pathway.

Effect of methylation of the GSE on EDR

It was found that methylated GSE failed to produce an EDR in the aortic rings. Subsequent exposure to the GSE (100 μmol/l) produced a significant relaxation, which was similar to that evoked by acetylcholine (10 μmol/l) (Figure 6).

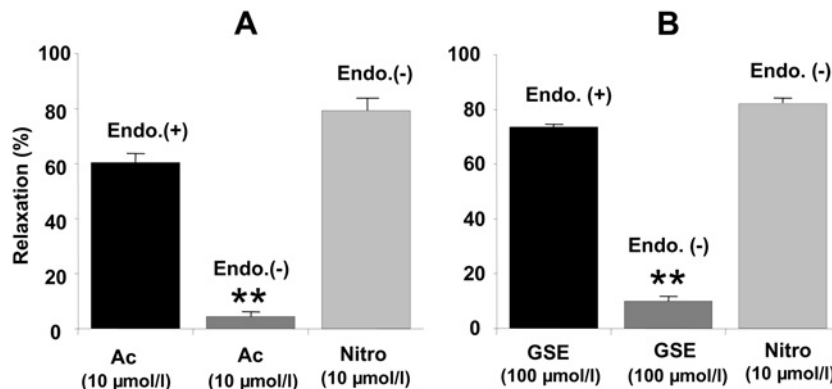


Figure 2 Effect of removal of endothelium on the maximum relaxation

(A) Responses induced by acetylcholine (Ac). (B) Responses induced by the GSE. Removal of endothelium abolished the responses elicited by acetylcholine and the GSE. The rings remained responsive to SNP (Nitro), which is a non-endothelium-dependent relaxant of smooth muscle. Values are means ± S.E.M. ($n = 4$). ** $P < 0.01$ compared with the treatment with the endothelium present and SNP with the endothelium removed. Endo.(+), endothelium present; Endo.(-), endothelium removed.

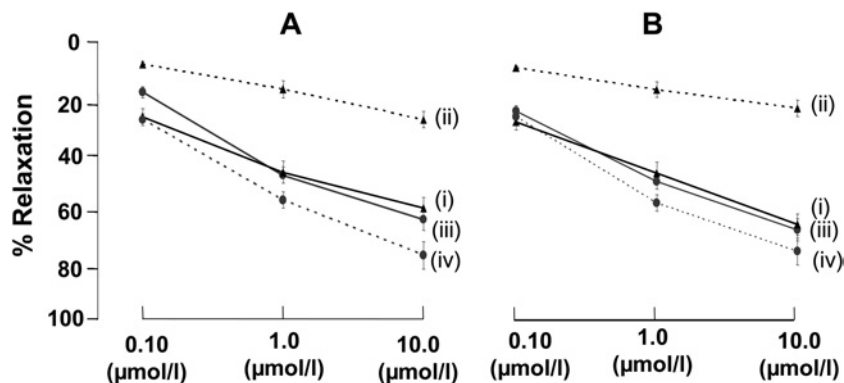


Figure 3 Dose–response curves evoked by the GSE after incubation with (A) wortmannin and (B) LY294002

Treatment with the agonists and inhibitors are described in the Materials and methods section and Table 1. (A) Initial response to acetylcholine [curve (i)], response to the GSE after incubation with wortmannin (30 nmol/l) for 30 min [curve (ii)], initial response to acetylcholine [curve (iii)], and response to GSE without prior incubation with wortmannin [curve (iv)]. Dose–response curves (i) and (ii) were generated from ring 1, and curves (iii) and (iv) were generated from ring 2. (B) Initial response to acetylcholine [curve (i)], response to the GSE after incubation with LY294002 (30 μmol/l) for 30 min [curve (ii)], initial response to acetylcholine [curve (iii)], and response to the GSE without incubation with LY294002 [curve (iv)]. Dose–response curves (i) and (ii) were generated from ring 1, and curves (iii) and (iv) were generated from ring 2. Curve (ii) is different from other three in both (A) and (B). Values are means \pm S.E.M. ($n = 4$) in both A and B. Results from ring 3 are not shown.

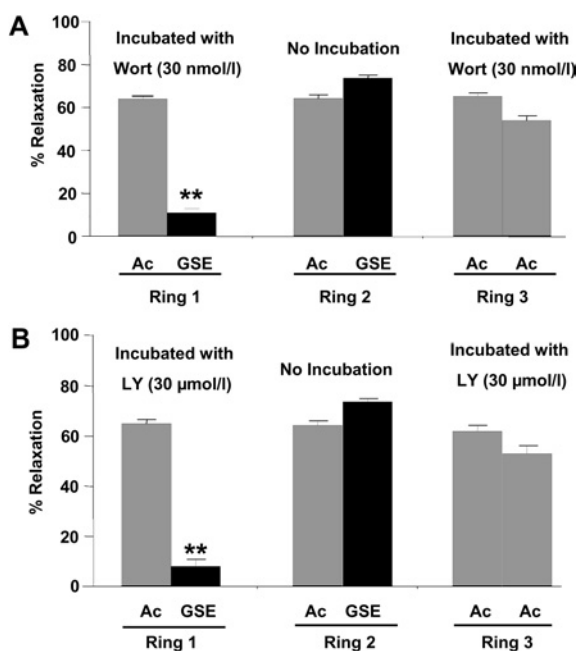


Figure 4 Effect of PI3K inhibitors on the maximum relaxation produced by GSE

(A) All three rings responded to acetylcholine initially. Ring 1, which was incubated with wortmannin (30 nmol/l for 30 min) and tested with the GSE, had a significantly attenuated relaxation (** $P < 0.01$ compared with GSE alone). Ring 2, which was not incubated with wortmannin, had a similar relaxation with the GSE. Ring 3, which was also incubated with wortmannin, had no significant change in the responses to acetylcholine. (B) Similar findings were observed with LY294002 (30 μmol/l) for the effect induced by the GSE. The relaxation evoked by the GSE was significantly decreased (** $P < 0.01$ compared with GSE alone). All values are means \pm S.E.M. ($n = 4$).

DISCUSSION

The present study has shown that the GSE used produced EDR in the rabbit aorta, which was significantly attenuated by prior incubation with the PI3K inhibitors wortmannin and LY294002. In these respects, the response is similar to that evoked by other derivatives of grapes which have been investigated extensively [12,13,18]. The novel aspects of the present study are the following: (i) the concurrent phosphorylation of both Akt and eNOS; (ii) modifying the antioxidant activity of the extract by methylation removed the ability to cause EDR; and (iii) we have used a compound that is very high in phenols (>90%), unlike GSEs used in other studies. Overall, the GSE used in the present study has been analysed in much greater detail than ones used by other investigators (see Supplementary material available at <http://www.clinsci.org/cs/114/cs1140331add.htm>).

Potential mechanism of action

Akt is a serine/threonine protein kinase that is recruited to the (endothelial) membrane by binding to PI3K-produced phosphoinositides. At the membrane, Akt is phosphorylated and activates eNOS (by phosphorylation at Ser¹¹⁷⁷ in humans), leading to the production of NO [17]. It has also been shown that the production of NO in response to fluid shear stress is controlled by Akt-dependent phosphorylation of eNOS [19]. However, recent studies performed in cell culture have established that polyphenolic compounds in red wine also affect the level of phosphorylation of Akt in a PI3K-dependent manner, which in turn phosphorylates

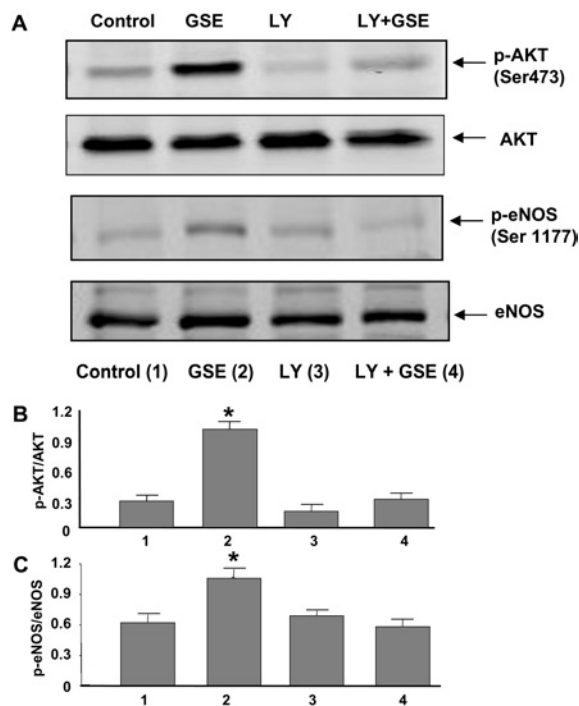


Figure 5 Effect of the GSE on eNOS and Akt phosphorylation

(A) HUVECs were treated with vehicle (control; lane 1), GSE (lane 2), LY294002 alone (lane 3) and GSE plus LY294002 (LY+GSE). In the controls, low levels of phosphorylated Akt and eNOS were observed, which were increased after incubation with GSE (lane 2). LY294002 alone (lane 3) had no effect on phosphorylation compared with the controls, but inhibited GSE-induced phosphorylation of Akt and eNOS when incubated with GSE (lane 4). (B and C) Quantification of the immunoblots of phosphorylated Akt (p-Akt) (B) and phosphorylated eNOS (p-eNOS) (C) using densitometry ($n = 4$). The results are ratios of the phosphorylated and non-phosphorylated form of each enzyme. * $P < 0.05$ compared with the control.

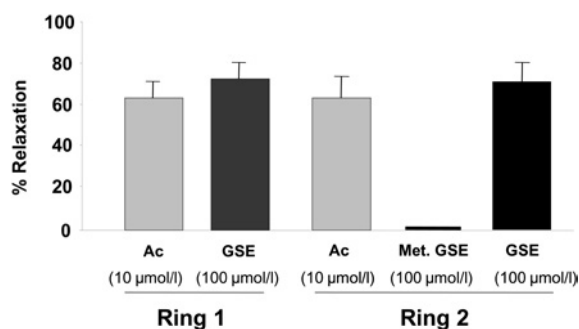


Figure 6 Effect of methylation of the GSE on EDR

The responses induced by acetylcholine (Ac), the GSE and methylated GSE (Met. GSE) are shown. Ring 1 had acetylcholine and the GSE applied in sequence, and Ring 2 had acetylcholine, methylated GSE and the GSE applied in sequence. No relaxation was evoked by methylated GSE ($n = 4$).

eNOS, resulting in an increased formation of NO [13]. PI3K, which is a redox-sensitive protein kinase, appears to be activated by the redox sensitivity of polyphenols,

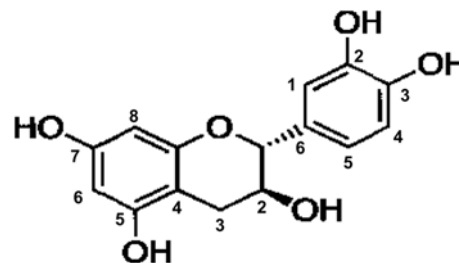


Figure 7 Basic structure of flavanoids

Proanthocyanidins are polymeric phenolic compounds characterized by a flavanoid with the basic three-ring structure.

leading to the production of NO. It has also been shown that, in endothelial cells, phosphorylation induced by polyphenols occurs on Ser¹¹⁷⁷ of eNOS and dephosphorylation at Thr⁴⁹⁵ within a few minutes of exposure. These changes in the phosphorylation level of eNOS were maintained for at least 30 min.

The GSE used in the present study is a relatively 'pure' one, with phenolic compounds forming 93% of its constituents. These compounds are mostly proanthocyanidins which occur as mixtures of oligomers and polymers of catechin and epicatechin (see Supplementary material available at <http://www.clinsci.org/cs/114/cs1140331add.htm>). Some of the larger polymeric compounds have the capacity to complex with proteins to form tannins. Plant tannins are divided into hydrolysable and condensed forms. The former contains gallic acid and a dimeric condensation product (hexahydroxydiphenic acid) that is esterified to a polyol such as glucose. The condensed tannins are high-molecular-mass oligomers and polymers of the monomeric unit flavanol-3-ol and their gallic acid esters. The monomeric units themselves are formed through oxidative condensation by carbon-carbon bonds, normally between carbon-4 of the heterocycle carbon ring and carbon-8 of the adjacent units (Figure 7). The GSE used in the present study consisted mainly of dimers and trimers (see Supplementary material available at <http://www.clinsci.org/cs/114/cs1140331add.htm>) and was devoid of gallic acid residues.

Polyphenolic compounds are generally considered to be antioxidants [20,21]; however, under certain circumstances, they have pro-oxidant properties attributable to the hydroxy groups in the phenolic rings. For instance, treatment of cell cultures with polyphenolic compounds significantly increased the production of ROS (reactive oxygen species) such as H₂O₂ [22,23]. It has been proposed that H₂O₂ is generated by auto-oxidation of hydroxy groups present in phenolic compounds (see Figure 1 in [23]), which subsequently activate PI3K [13,23]. Ndaye et al. [13] have shown that removal of hydroxyl radicals derived from H₂O₂

by enhancing endogenous SOD (superoxide dismutase) abolished the EDR produced by GSEs. In the present study, we have demonstrated that the removal of the hydroxy groups from the GSE by prior methylation also abolished EDR, thus supporting the important role of the hydroxy groups in producing EDR.

Conclusions

In the present study, we provide evidence to suggest that EDR evoked by the GSE is mediated by the activation of the PI3K/Akt signalling pathway, resulting in the phosphorylation of eNOS. Previous studies have suggested that GSEs activate PI3K and downstream signalling via Akt and activate eNOS through a redox-sensitive mechanism [13]. Furthermore, we found that removal of the antioxidant activity from the GSE by methylation of the hydroxy groups abolished the EDR induced by GSEs. These results support the suggestion that ROS produced by GSEs can activate eNOS to produce NO and cause vasodilation.

ACKNOWLEDGMENTS

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REFERENCES

- Aldini, G., Carini, M., Piccoli, A., Rossoni, G. and Facino, R. M. (2003) Procyanidins from grape seeds protect endothelial cells from peroxynitrite damage and enhance endothelium-dependent relaxation in human artery: new evidences for cardio-protection. *Life Sci.* **73**, 2883–2898
- Cishek, M. B., Galloway, M. T., Karim, M., German, J. B. and Kappagoda, C. T. (1997) Effect of red wine on endothelium-dependent relaxation in rabbits. *Clin. Sci.* **93**, 507–511
- Stoclet, J. C., Chataigneau, T., Ndiaye, M. et al. (2004) Vascular protection by dietary polyphenols. *Eur. J. Pharmacol.* **500**, 299–313
- Plotnick, G. D., Corretti, M. C., Vogel, R. A., Hesslink, Jr, R. and Wise, J. A. (2003) Effect of supplemental phytonutrients on impairment of the flow-mediated brachial artery vasoactivity after a single high-fat meal. *J. Am. Coll. Cardiol.* **41**, 1744–1749
- de Lorgeril, M., Salen, P., Paillard, F., Laporte, F., Boucher, F. and de Leiris, J. (2002) Mediterranean diet and the French paradox: two distinct biogeographic concepts for one consolidated scientific theory on the role of nutrition in coronary heart disease. *Cardiovasc. Res.* **54**, 503–515
- Chawla, R. (2004) Regular drinking might explain the French paradox. *Br. Med. J.* **329**, 1308
- Pastrana-Bonilla, E., Akoh, C. C., Sellappan, S. and Krewer, G. (2003) Phenolic content and antioxidant capacity of muscadine grapes. *J. Agric. Food Chem.* **51**, 5497–5503
- Fitzpatrick, D. F., Fleming, R. C., Bing, B., Maggi, D. A. and O'Malley, R. M. (2000) Isolation and characterization of endothelium-dependent vasorelaxing compounds from grape seeds. *J. Agric. Food Chem.* **48**, 6384–6390
- Fitzpatrick, D. F., Bing, B., Maggi, D. A., Fleming, R. C. and O'Malley, R. M. (2002) Vasodilating procyanidins derived from grape seeds. *Ann. N.Y. Acad. Sci.* **957**, 78–89
- Karim, M., McCormick, K. and Kappagoda, C. T. (2000) Effects of cocoa extracts on endothelium-dependent relaxation. *J. Nutr.* **130**, 2105S–2108S
- Wallerath, T., Deckert, G., Ternes, T. et al. (2002) Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation* **106**, 1652–1658
- Anselm, E., Chataigneau, M., Ndiaye, M., Chataigneau, T. and Schini-Kerth, V. B. (2007) Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src- and Akt-dependent activation of eNOS. *Cardiovasc. Res.* **73**, 404–413
- Ndiaye, M., Chataigneau, M., Lobysheva, I., Chataigneau, T. and Schini-Kerth, V. B. (2004) Red wine polyphenol-induced, endothelium-dependent NO-mediated relaxation is due to the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of endothelial NO-synthase in the isolated porcine coronary artery. *FASEB J.* **19**, 455–457
- Edirisinghe, I., Siva, B., Randolph, J., Steinberg, F., Skepper, J. and Kappagoda, T. (2006) A diet rich in saturated fat impairs endothelium dependent relaxation (EDR) in guinea pig (GP). *FASEB J.* **20**, A287
- Siva, B., Edirisinghe, I., Randolph, J., Steinberg, F. and Kappagoda, T. (2006) Effect of a polyphenolics extracts of grape seeds (GSE) on blood pressure (BP) in patients with the metabolic syndrome (MetS). *FASEB J.* **20**, A305
- Edirisinghe, I., McCormick-Hallam, K. and Kappagoda, C. T. (2006) Effect of fatty acids on endothelium-dependent relaxation in the rabbit aorta. *Clin. Sci.* **111**, 145–151
- Kawasaki, K., Smith, R. S., Hsieh, C. M. and Chao, J. (2003) Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis. *Mol. Cell. Biol.* **23**, 5726–5737
- Ndiaye, M., Chataigneau, T., Andriantsitohaina, R., Stoclet, J. C. and Schini-Kerth, V. B. (2003) Red wine polyphenols cause endothelium-dependent EDHF-mediated relaxations in porcine coronary arteries via a redox-sensitive mechanism. *Biochem. Biophys. Res. Commun.* **310**, 371–377
- Jin, Z. G., Ueba, H., Tanimoto, T., Lungu, A. O., Frame, M. D. and Berk, B. C. (2003) Ligand-independent activation of vascular endothelial growth factor receptor 2 by fluid shear stress regulates activation of endothelial nitric oxide synthase. *Circ. Res.* **93**, 354–363
- Ariga, T. (2004) The antioxidative function, preventive action on disease and utilization of proanthocyanidins. *Biofactors* **21**, 197–201
- Koga, T., Moro, K., Nakamori, K. et al. (1999) Increase of antioxidative potential of rat plasma by oral administration of proanthocyanidin-rich extract from grape seeds. *J. Agric. Food Chem.* **47**, 1892–1897
- Yang, G. Y., Liao, J., Li, C. et al. (2000) Effect of black and green tea polyphenols on c-jun phosphorylation and H₂O₂ production in transformed and non-transformed human bronchial cell lines: possible mechanisms of cell growth inhibition and apoptosis induction *Carcinogenesis* **21**, 2035–2039
- Akagawa, M., Shigemitsu, T. and Suyama, K. (2003) Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. *Biosci. Biotechnol. Biochem.* **67**, 2632–2640

Effect of grape seed extract on blood pressure in subjects with the metabolic syndrome

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Abstract

This study was undertaken to determine whether grape seed extracts (GSE) that contain powerful vasodilator phenolic compounds lower blood pressure in subjects with the metabolic syndrome. The subjects were randomized into 3 groups—(a) placebo, (b) 150 mg GSE per day, and (c) 300 mg GSE per day—and treated for 4 weeks. Serum lipids and blood glucose were measured at the beginning of the study and at the end. Blood pressure was recorded using an ambulatory monitoring device at the start of the treatment period and at the end. Both the systolic and diastolic blood pressures were lowered after treatment with GSE as compared with placebo. There were no significant changes in serum lipids or blood glucose values. These findings suggest that GSE could be used as a nutraceutical in a lifestyle modification program for patients with the metabolic syndrome.

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1. Introduction

There is evidence that a diet rich in vegetables and fruit has a beneficial effect on blood pressure. This effect has been attributed to the presence of phenolic compounds in the plant products. These compounds have also been shown to have vasodilator effects [1]. Of all the phenolic compounds, those derived from grape seeds appear to have received the most attention, possibly because of their involvement with the French paradox [2]. These extracts have also been shown to activate endothelial nitric oxide synthase (eNOS) [1,3], up-regulate eNOS in cultured endothelial cells [4], and cause an endothelium-dependent relaxation (EDR) of blood vessels [3].

We have shown recently that an extract of grape seed (Meganatural BP; Polyphenolics, Fresno, CA) that is rich in polyphenolic compounds causes an EDR that is mediated by

activation of the Akt/PI3 kinase signaling pathway resulting in phosphorylation of eNOS [3]. Abolition of the EDR by removal of antioxidant activity from the extract by methylation of the OH groups supported the contention that activation of eNOS is mediated through a redox-sensitive mechanism [5].

These data suggested that this extract had the potential to lower blood pressure in human subjects. Recent studies in a spontaneously hypertensive rat had demonstrated that polyphenols present in red wine lower blood pressure and enhance nitric oxide bioactivity without up-regulating eNOS [6]. The definition of the *metabolic syndrome* was that adopted by the National Cholesterol Education Program Adult Treatment Panel III [7]. It was diagnosed when 3 of the following factors were present: abdominal obesity, elevated serum triglycerides, low serum high-density lipoprotein (HDL) concentration, hypertension, and elevated blood glucose [8]. All these factors are associated with impaired endothelial function resulting from the combined effects of a decrease in systemic antioxidant activity and an increase in reactive oxygen/nitrogen species [9,10]. It has been shown that polyphenolics derived from

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grapes increase the antioxidant levels and improve the endothelial function [6,11]. Therefore, the investigation reported here was undertaken to examine the effect of this extract on blood pressure in subjects with the metabolic syndrome as proof of this concept. The study was a placebo-controlled double-blind trial.

2. Materials and methods

The study was approved by the Institutional Review Board of the University of California, Davis. The study was conducted on a sample of 27 adults (age, 25–80 years) with the metabolic syndrome [8]. The exclusion criteria were as follows: smokers (abstinence for <1 year); clinical evidence of coronary artery, pulmonary, gastrointestinal, or renal disease; and consumption of prescription medications and vitamin preparations.

After initial screening, the subjects were randomized into 3 groups to receive a placebo, 300 mg/d of grape seed extract (GSE), or 150 mg of GSE (Meganatural BP). The phenol content of this extract is 94%. A detailed analysis of the extract is archived at a previous publication [3]. Before starting treatment, they were fitted with an ambulatory blood pressure measurement device that recorded the daytime blood pressure at intervals of 1 hour over a 12-hour period (Model SE-25S; Sein Electronics, Koyang, South Korea). This system has been evaluated using a protocol approved by the British Hypertension Society (www.tiba.medical.com).

The subjects were advised to maintain their usual level of activity and diet. The latter was monitored by examining a 4-day food diary that was completed at the start and at the end of the study. After 4 weeks, a final 12-hour daytime ambulatory blood pressure was recorded. Fasting blood samples were collected for the following measurements at the start of the study and at the end: hemoglobin, white cell count with differential, serum lipids, chemistry panel including a serum glucose, insulin, and oxidized low-density lipoprotein (Ox-LDL). Additional samples were obtained from 5 subjects in the placebo group and in the group that received 300 mg/d for measurement of plasma catechin concentrations. These samples were obtained immediately before and 90 minutes after ingestion of a capsule.

Table 1
Baseline parameters in the 27 subjects

Parameters	Placebo group	150-mg/d group	300-mg/d group	Significance
Age (y)	46 ± 3	45 ± 3	47 ± 4	NS
Male/female	3/6	4/5	4/5	NS
Waist circumference (in)	43 ± 1.8	44 ± 1.7	42 ± 1.8	NS
BMI (kg/m ²)	36 ± 2.4	36 ± 1.4	37 ± 2.1	NS
Glucose (mg/dL)	98 ± 5.9	101 ± 11	105 ± 10	NS
Insulin (μU/L)	25 ± 4	26 ± 5	30 ± 4	NS
Ox-LDL (U/L)	58 ± 4	60 ± 6	62 ± 7	NS

NS indicates not significant; BMI, body mass index.

Table 2
Initial and final blood pressures (in millimeters of mercury)

	Placebo		GSE (150 mg/d)		GSE (300 mg/d)	
	Systolic	Diastolic	Systolic	Diastolic	Systolic	Diastolic
Start	123 ± 4	74 ± 4	134 ± 5	83 ± 3	127 ± 4	78 ± 3
4 wk	121 ± 4	70 ± 4	123 ± 4	77 ± 2	116 ± 3	71 ± 3
n	9		9		9	
P ^a	.2	.1	.003	.01	.007	.007
Power	NS	NS	>0.8	0.8	^b	>0.8

^a Comparisons between initial and final blood pressures.

^b Signed rank test.

The Ox-LDL concentration in plasma was measured using an mAb-4E6–based enzyme-linked immunosorbent assay (Merckodia, Uppsala, Sweden). Plasma insulin concentrations were measured using human-specific radioimmunoassay (Millipore, Billerica, MA). The plasma catechin concentrations were measured using a high-performance liquid chromatography technique [12] (“Acknowledgment”).

2.1. Statistical analysis

The primary end points were the mean daytime systolic and diastolic blood pressures. Secondary end points were the changes in serum lipids and Ox-LDL. Baseline values in the 3 groups were compared using a 1-way analysis of variance (ANOVA). The changes in blood pressure in the subjects in the 3 groups were also compared using a 1-way ANOVA. A 2-way ANOVA was used to compare the blood pressures at the start of the study and at the end to demonstrate a time effect.

3. Results

Twenty-seven subjects who met the criteria for the metabolic syndrome were randomized. The baseline clinical data are given in Table 1. There were no significant differences in baseline parameters in the subjects assigned to the 3 groups (ANOVA, $P > .05$).

At the end of 4 weeks, both systolic and diastolic blood pressures decreased in the groups that received GSE, whereas the group that received the placebo showed no effect (2-way ANOVA, $P < .05$). The changes in blood pressure in the 3 groups were also analyzed, and it was found that the effect of the placebo was significantly different from the effects of the 2 doses of GSE (ANOVA, $P < .05$). The effects of 150- and 300-mg/d doses were similar. The findings on blood pressure are summarized in Table 2 and Fig. 1. There were no significant changes in heart rate in the 3 groups.

There were no changes in the serum total, LDL, and HDL cholesterol values in 3 groups (Table 3). We observed decreased levels of Ox-LDL in the groups that consumed 150 and 300 mg/d of GSE, but these differences were not significant compared with the placebo group. However, the change in Ox-LDL appeared to be inversely related to the

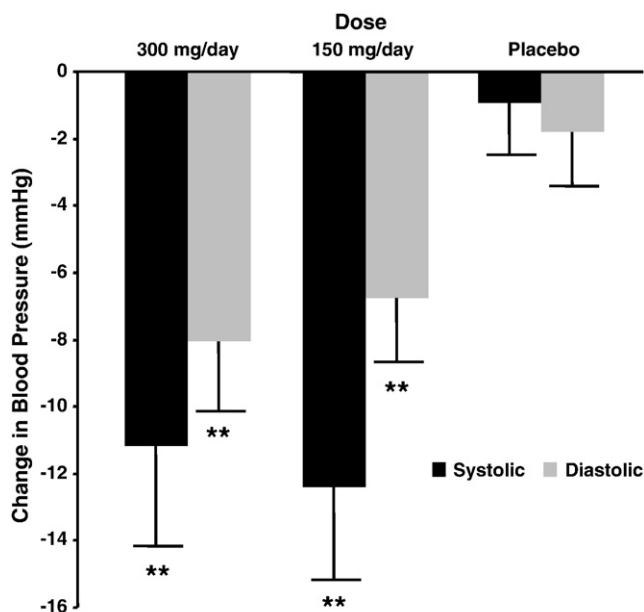


Fig. 1. Changes in blood pressure after administration of GSE for 4 weeks. Changes in systolic and diastolic blood pressure after 1 month of treatment. **Significantly different from corresponding placebo value ($P < .01$). Bars represent mean changes in the blood pressure \pm SEM.

baseline (initial) concentration before starting treatment. This relationship was statistically significant in the group given 300 mg of GSE per day ($P < .05$) (Fig. 2).

The serum sodium, potassium, bicarbonate, creatinine, and blood urea nitrogen were also measured and were unchanged during the study. There were also no changes in the complete blood counts.

In 5 subjects who received the placebo capsules, there was no increase in total plasma catechin concentrations after 90 minutes (2.2 ± 3 vs 3.2 ± 3 ng/L, $P = .7$, paired t test). The corresponding values in the subjects who received 300 mg of the extract were 2.0 ± 4 and 22.0 ± 22.8 ng/L, respectively. This increase was significant ($P = .032$, Whitney-Mann).

4. Discussion

The findings of this randomized controlled trial indicate that GSE when administered at a dose of either 300 or 150 mg/d reduced both systolic and diastolic blood pressures

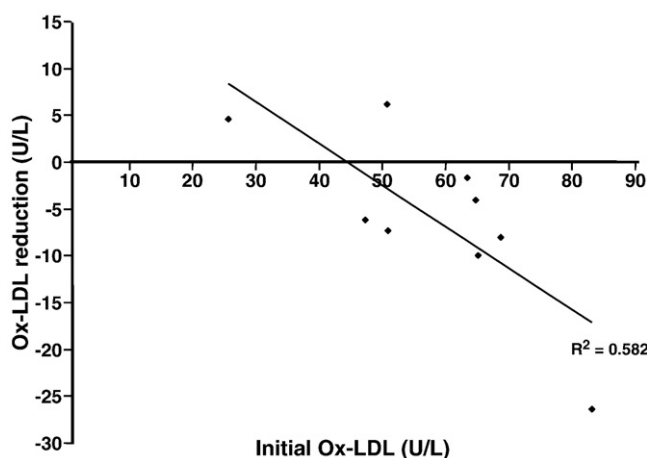


Fig. 2. Change in Ox-LDL levels plotted against the baseline Ox-LDL levels in plasma. Administration of GSE appeared to reduce the concentration of Ox-LDL in plasma in subjects with metabolic syndrome. This effect was particularly evident in those who received 300 mg/d of GSE. There was a significant positive correlation between the change in the concentration of Ox-LDL and the baseline Ox-LDL levels ($n = 9$, $P < .05$).

in subjects with the metabolic syndrome as defined by the National Cholesterol Education Program Adult Treatment Panel III. All the blood pressures were obtained using ambulatory devices over 12-hour periods as opposed to a single clinic blood pressure measurement. These findings are consistent with several related observations.

We have demonstrated previously that the GSE used in the present study caused an EDR in the rabbit aorta [3]. It was shown also that, in human umbilical vein endothelial cells, eNOS was activated by GSE via the PI3 kinase/Akt pathway [3]. Wallerath et al [4] observed that GSE up-regulated eNOS in human umbilical vein endothelial cells. Based on these findings, it is suggested that the lowering of blood pressure in human subjects with metabolic syndrome after administration of the GSE is mediated by an eNOS-associated mechanism.

Grape seed extract used in the current study is known to have high content of polyphenolic compounds (94%). It is made up principally of catechin units and has an average polymerization of 2.4. The other unusual feature of this extract is the absence of terminal gallate units (see archived information in Edirisinghe et al [3]). The present study has

Table 3
Changes in serum lipids

	Placebo		150 mg/d		300 mg/d	
	Initial levels	After 1 mo	Initial levels	After 1 mo	Initial levels	After 1 mo
Total cholesterol (mg/dL)	199 \pm 12	197 \pm 11	216 \pm 9	209 \pm 10	194 \pm 18	196 \pm 15
HDL cholesterol (mg/dL)	51 \pm 2	49 \pm 3	51 \pm 4	48 \pm 4	44 \pm 3	45 \pm 3
LDL cholesterol (mg/dL)	125 \pm 10	124 \pm 9	147 \pm 5	135 \pm 10	126 \pm 15	128 \pm 14
Triglycerides (mg/dL)	179 \pm 21	183 \pm 19	176 \pm 15	178 \pm 15	178 \pm 18	176 \pm 15
Ox-LDL (U/L)	58 \pm 4	60 \pm 4	60 \pm 6	57 \pm 7	62 \pm 7	58 \pm 8

There is no significant difference before and after GSE treatments.

also shown that the extract is absorbed into the systemic circulation. Previous studies undertaken in humans have not shown a reduction in blood pressure. Some of these discrepancies may in part be due to differences in the composition of the extract [13].

The metabolic syndrome is an important public health problem affecting Western societies. In the United States, the prevalence of the condition is estimated to be 39% [14]. It is an important risk factor for the development of both coronary artery disease [15] and type 2 diabetes mellitus [16]. One of the associated features of the syndrome is the state of oxidative stress that is present in these individuals. One manifestation of this state is the increase in Ox-LDL in plasma that is a “precursor” to the development of atherosclerosis [17,18]. We observed that GSE at a dose of 300 mg/d reduced Ox-LDL, particularly when the Ox-LDL concentrations were high. It is suggested that this observation may point toward a potential therapeutic benefit from the extract. The observation in this article relating to blood pressure in humans is consistent with recent observations in spontaneously hypertensive rats treated with red wine polyphenols [6]. However, it was suggested that the mechanism involved was an enhancement in the bioactivity of nitric oxide through a reduction in oxidative stress.

In summary, the present study has demonstrated that an extract of grape seed lowers blood pressure in subjects with the metabolic syndrome. We have shown that the phenolic compounds in the extract are absorbed and that its antioxidant properties could reduce the concentration of Ox-LDL in plasma.

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References

[1] Cishek MB, Galloway MT, Karim M, et al. Effect of red wine on endothelium-dependent relaxation in rabbits. *Clin Sci (Lond)* 1997;97:507-11.
 [2] De Lorgeril M, Salen P, Paillard F, et al. Mediterranean diet and the French paradox: two distinct biogeographic concepts for one

consolidated scientific theory on the role of nutrition in coronary artery disease. *Cardiovasc Res* 2002;54:503-15.
 [3] Edirisinghe I, Burton-Freeman B, Kappagoda T. The mechanism of the endothelium dependent relaxation evoked by a grape seed extract. *Clin Sci (Lond)* 2008;114:331-7.
 [4] Wallerath T, Li H, Gödtel-Ambrust U, et al. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation* 2002;106:1652-8.
 [5] Ndiaye M, Chataigneau M, Lobysheva I, et al. Red wine polyphenol-induced, endothelium-dependent NO-mediated relaxation is due to the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of endothelial NO-synthase in the isolated porcine coronary artery. *FASEB J* 2004;19:455-7.
 [6] López-Sepúlveda R, Jiménez R, Romero M, et al. Wine polyphenols improve endothelial function in large vessels of female spontaneously hypertensive rats. *Hypertension* 2008;51:1088-95.
 [7] Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 2001;285:2486-97.
 [8] Chobanian AV, Bakris GL, Black HR, et al. Seventh report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure. *Hypertension* 2003;42:1206-52.
 [9] Suzuki T, Hirata K, Elkind MS, et al. Metabolic syndrome, endothelial dysfunction, and risk of cardiovascular events: the Northern Manhattan Study (NOMAS). *Am Heart J* 2008;156:405-10.
 [10] Anter E, Keaney Jr JF. Oxidative stress, antioxidants, and endothelial function. *Curr Med Chem* 2004;11:1093-104.
 [11] Leifert WR, Abeywardena MY. Cardioprotective actions of grape polyphenols. *Nutr Res* 2008;28:729-37.
 [12] Gu L, House SE, Rooney LW, et al. Sorghum extrusion increases bioavailability of catechins in weanling pigs. *J Agric Food Chem* 2008;56:1283-8.
 [13] Ward NC, Hodgson JM, Croft KD, et al. The combination of vitamin C and grape-seed polyphenols increases blood pressure: a randomized, double-blind, placebo-controlled trial. *J Hypertens* 2005;23:427-34.
 [14] Grundy SM, Brewer Jr HB, Cleeman Jr JI, et al. American Heart Association, National Heart, Lung, and Blood Institute. Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation* 2004;109:433-8.
 [15] Malik S, Wong ND, Franklin SS, et al. Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults. *Circulation* 2004;110:1245-50.
 [16] Laaksonen DE, Lakka HM, Niskanen LK, et al. Metabolic syndrome and development of diabetes mellitus: application and validation of recently suggested definitions of the metabolic syndrome in a prospective cohort study. *Am J Epidemiol* 2002;156:1070-7.
 [17] Sigurdardottir V, Fagerberg B, Hulthe J. Circulating oxidized low-density lipoprotein (LDL) is associated with risk factors of the metabolic syndrome and LDL size in clinically healthy 58 year old men (AIR study). *J Internal Med* 2002;252:440-7.
 [18] Holvoet P, Vanhaecke J, Janssens S, et al. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation* 1998;98:1487-94.