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

Indika EDIRISINGHE*, Britt BURTON-FREEMAN† and C. Tissa KAPPAGODA*

*Department of Internal Medicine, University of California Davis, Davis, CA 95616, U.S.A., and †Department of Nutrition, University of California Davis, Davis, CA 95616, U.S.A.

ABSTRACT

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 ± 1.0 %) were similar to those elicited by acetylcholine (64.2 ± 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.

Correspondence: Dr C. Tissa Kappagoda (email ctkappagoda@ucdavis.edu).

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 administrated 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, $MgCl_2$ 2.5 mmol/l $CaCl_2$, 22 mmol/l $NaHCO_3$, 1.2 mmol/l NaH_2PO_4 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 % O_2/5 % CO_2. 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 μmol/l)	Acetylcholine (10 μmol/l)	Acetylcholine (10 μmol/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 μmol/l). Some wells were treated with LY294002 (30 μmol/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 % CO_2 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 μg/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 Ser^{473}), phosphorylated eNOS (at Ser^{1177}), Akt and eNOS were detected after the membranes were incubated with the respective primary antibodies {rabbit anti-[phospho-eNOS (Ser^{1177})], anti-eNOS, anti-[phospho-Akt (Ser^{473})] 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 μmol/l) and the GSE (100 μmol/l), and ring 2 was treated sequentially with acetylcholine (10 μmol/l), methylated GSE and the GSE (both 100 μmol/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 ± 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.

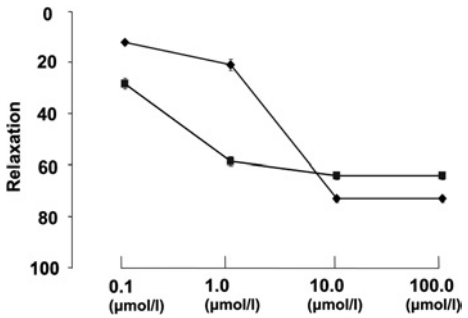

Figure 1 Dose-dependent relaxation of aortic rings induced by acetylcholine and the GSE

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

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}

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 Ser^{473}) and eNOS (on Ser^{1177}) *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 ERD 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).

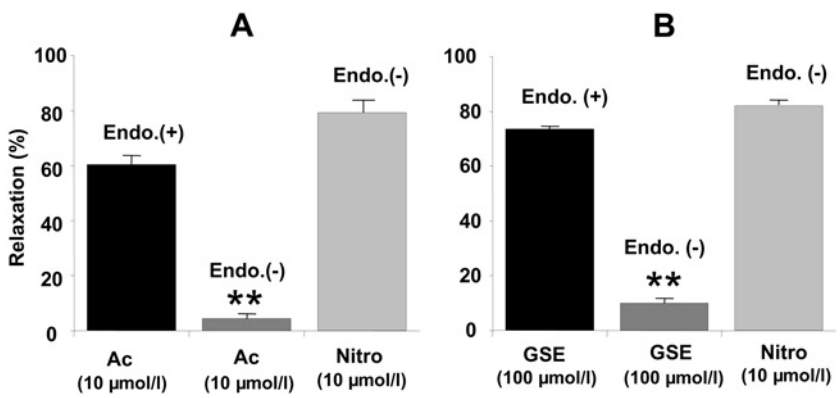

Figure 2 Effect of removal of endothelium on the maximum relaxation

(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.

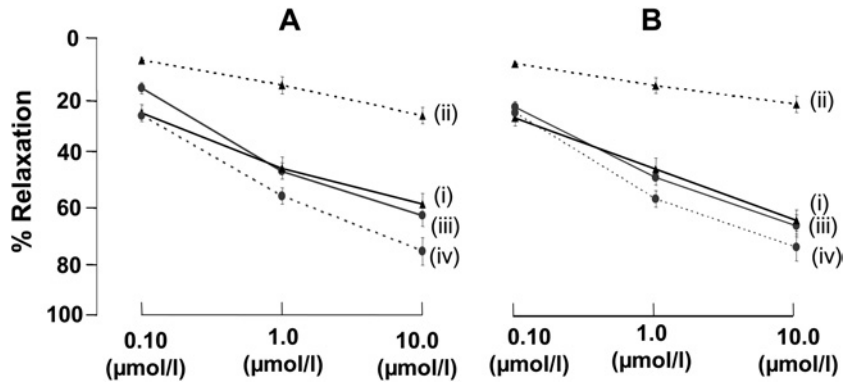

Figure 3 Dose–response curves evoked by the GSE after incubation with (A) wortmannin and (B) LY294002

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.

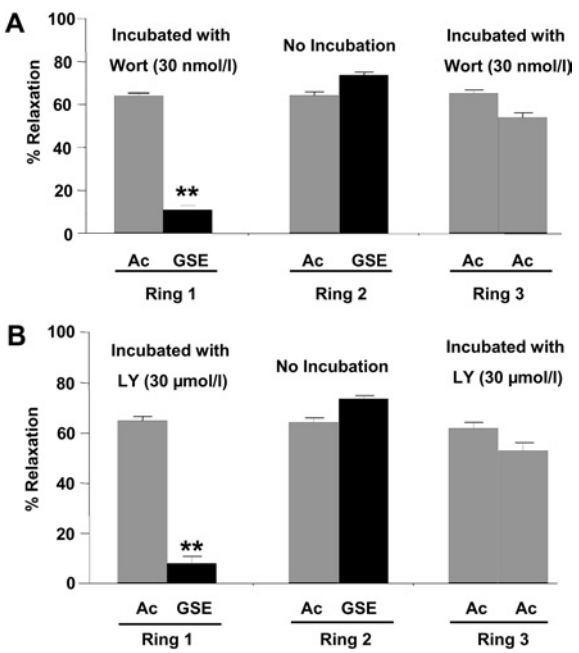

Figure 4 Effect of PI3K inhibitors on the maximum relaxation produced by GSE

(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 Ser^{1177} 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

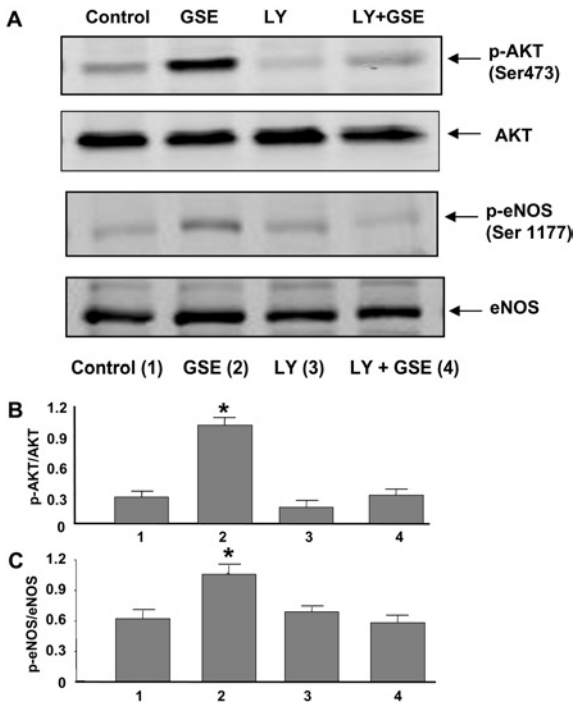

Figure 5 Effect of the GSE on eNOS and Akt phosphorylation

(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.

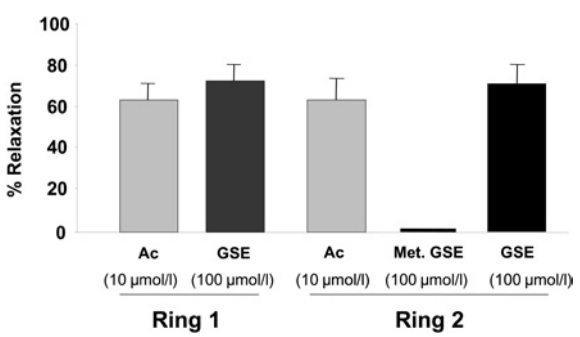

Figure 6 Effect of methylation of the GSE on EDR

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,

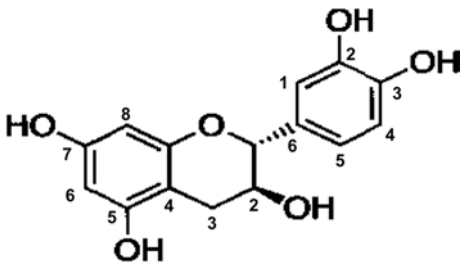

Figure 7 Basic structure of flavanoids

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 Ser^{1177} of eNOS and dephosphorylation at Thr^{495} 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 H_2O_2 [22,23]. It has been proposed that H_2O_2 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 H_2O_2

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.

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