JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Strawberry Extract Caused Endothelium-Dependent Relaxation through the Activation of PI3 Kinase/Akt

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Polyphenolic compounds are vasodilators and help to lower the risk of cardiovascular diseases. We hypothesized that a freeze-dried strawberry powder that is rich in polyphenolic compounds would cause an endothelium-dependent relaxation (EDR) through the activation of phosphatidylinositol-3 (PI3)-kinase/protein kinase B (Akt) in rabbit aorta. The powder was prepared by freeze drying a homogenate of ripe California strawberry fruits. An aqueous extract of strawberry powder was applied to rabbit aortic rings suspended in organ baths containing Krebs−Henseleit buffer maintained at 37 °C. In aortic rings precontacted with norepinephrine, the extract produced a dose-dependent relaxation. The maximum relaxations elicited by the extract (73.1 ± 1.0%) were similar to those elicited by acetylcholine (68.2 ± 1.5%) ($n = 14$ for each). The relaxation by strawberry extract was abolished by removal of the endothelium and by prior incubation with N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), confirming the essential role of endothelial nitric oxide synthase (eNOS). The responses to the strawberry were also abolished by incubation with wortmannin and LY294002, which are inhibitors of PI3 kinase. Using immunoblotting, we also demonstrated that the strawberry extract induced the phosphorylation of both Akt and eNOS in human umbilical vein endothelial cells (HUVECs) via PI3 kinase/Akt pathway. Taken together, our novel findings suggest that the EDR induced by the strawberry extract was mediated by activation of the PI3 kinase/Akt signaling pathway, resulting in phosphorylation of eNOS.

KEYWORDS: Bioactive phenols; PI3 kinase/Akt; eNOS; ROS; EDR; strawberry; anthocyanins

INTRODUCTION

Epidemiological studies have consistently demonstrated an association between the consumption of diets rich in fruits and vegetables and a lower risk for developing chronic diseases including cancer and atherosclerosis (*1*−*3*). It has been suggested that many of the health benefits from consuming these plant foods may come from bioactive compounds that are present in plants (*4*). These compounds vary widely in chemical structure and function and are grouped according to their chemical class. Phenolic compounds, including their subcategory, flavonoids, are present in all plants and have been studied extensively (*5*). They have been shown to induce endothelium-dependent relaxation (EDR) in a variety of in vitro experiments (*6, 7*). We have demonstrated recently that the EDR induced by a grape seed extract (GSE) is mediated by the activation of the phosphatidylinositol-3 (PI3) kinase/protein kinase B (Akt) signaling pathway (*7*).

Strawberries have been shown to be a rich source of phenolic compounds (*8, 9*). The phenolic compounds responsible for the red color in strawberry flesh, the anthocyanins, have been widely investigated and identified as glucosides of pelargonidin and cyanidin, with pelargonidin-3-glucoside as the major compound (*10*−*12*). Glucosides and glucuronides of quercetin and kaempferol have been identified as the main flavonols. Numerous studies have shown that strawberries have antioxidant effects that are mainly ascribed to their high content of phenolic compounds (*8, 13, 14*). Because the activation of the PI3 kinase/Akt signaling pathway (*7*) is redox sensitive, we hypothesized that an extract of freeze-dried strawberry powder that is rich in phenolic compounds would cause an EDR through activation of PI3 kinase/Akt, potentially due to the antioxidant action of the phenolic compounds. These studies were undertaken on both rabbit aortic rings and human umbilical vein endothelial cells (HUVEC).

MATERIALS AND METHODS

Study Design and Procedures. This study was approved by the Animal Use and Care Administrative Advisory Committee, University of California (Davis, CA). Unless otherwise stated, all chemicals were

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10.1021/jf801864t CCC: $40.75
Published on Web 09/25/2008

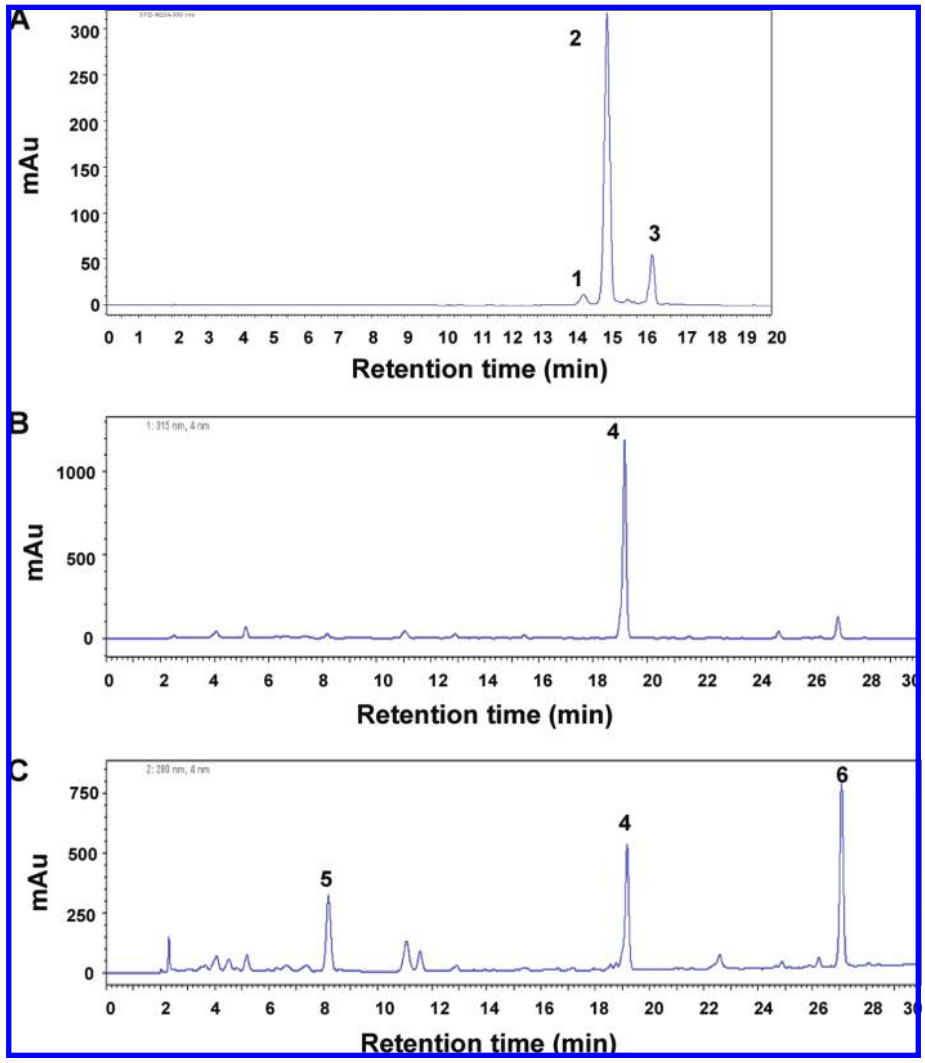

Figure 1. Analysis of strawberry extracts. (**A**) Wavelength (500 nm) chromatogram obtained from the analysis of the aqueous strawberry extract. Peaks: 1, cyanidin-3-*O*-glucoside; 2, pelargonidin-3-*O*-glucoside; and 3, pelargonidin-3-*O*-rutinoside. Chromatograms at (**B**) 315 and (**C**) 280 nm obtained from the analysis of the strawberry extract after concentration using solid phase extraction. Peaks: 4, 1-*O*-*p*-coumaroyl-D-glucopyranose; 5, unknown; and 6, *trans*-cinnamoyl-*O*-glucoside.

Table 1. Protocol for Testing the Effect of the PI3K Inhibitors on EDR Induced by the Strawberry Extract

steps	aortic ring # 1	aortic ring # 2	aortic 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 PI3/Akt blocker for 30 min	no incubation, KH buffer alone	incubate with PI3/Akt blocker for 30 min
4	dose−response curve with strawberry extract	dose−response curve with strawberry extract	dose−response curve with acetylcholine

of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO). Male New Zealand rabbits, weighing 3−3.5 kg, were sedated by intramuscular injection of acepromazine. After 5 min, a lethal dose of sodium pentobarbital (50 mg/kg, Abbott Laboratory, United States) 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 cold Krebs−Henseleit (KH) buffer (pH 7.4) containing 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) and placed in a dissecting tray filled with the same buffer. All surrounding connective tissues and fat were removed carefully.

The strawberry extract was prepared from a freeze-dried powder of California strawberries supplied by the California Strawberry Commission (CSC, Watsonville, CA). Freeze-dried strawberry powder was dissolved in KH buffer (100 mg/mL), vortexed for 10 min, and centrifuged at 5000*g* for 5 min, and the supernatant was used as the stock solution. The final concentrations of the extracts used in the organ bath ranged from 0.01 to 10 mg/mL. All of the samples were prepared fresh. The phenolic content of the strawberry extract (1 mg/mL) was measured using the Folin−Ciocalteu assay (*15*) and was found to be 1.15 ± 0.06 mM gallic acid units ($n = 5$). The major anthocyanins in the aqueous extract, pelargonidin-3-glucoside, and the two major phenolic compounds, 1-*O*-*p*-coumaroyl-D-glucopyranose and 1-*O*-cinnamoyl-D-glucopyranose, were identified using tandem mass spectrometry (*12, 16, 17*).

Sample Preparation for High-Performance Liquid Chromatography (HPLC) Analysis. The powder (1 g) was covered with water (10 mL) and then placed in a heated (50 °C) sonicator bath. After 30 min, the mixture was removed from the bath and then centrifuged for 15 min at 5000 rpm. The supernatant was carefully decanted, and an aliquot (5 mL) was mixed with cold acetone (40 mL). The resulting mixture was centrifuged (5000 rpm) at −10 °C for 15 min, and the supernatant was immediately decanted into a round-bottom flask and

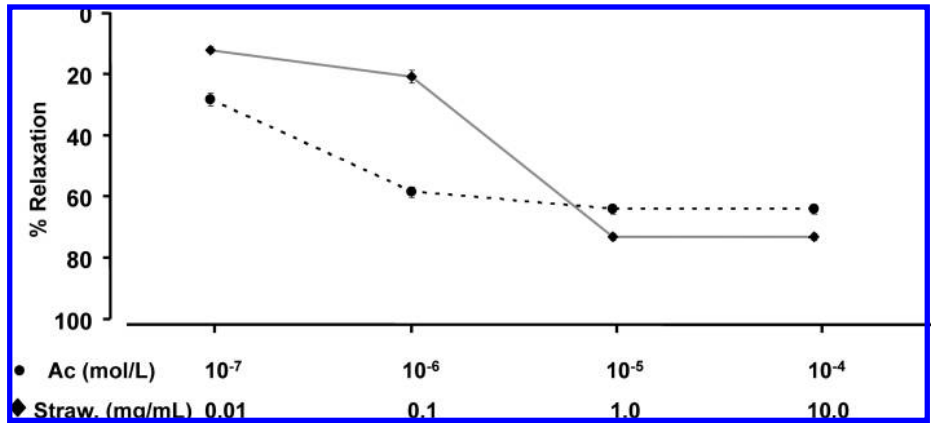

Figure 2. Strawberry extracts caused vasodilatation in a dose-dependent manner. Dose–response curves relating relaxation [percentage of contraction to norepinephrine (10^{-5} mol/L)] and concentration of agonist in organ bath. Dose–dependent relaxations were evoked by acetylcholine [Ac (●)] and strawberry extract [straw (◆)]. Each individual value is represented is a mean ± SEM ($n = 14$).

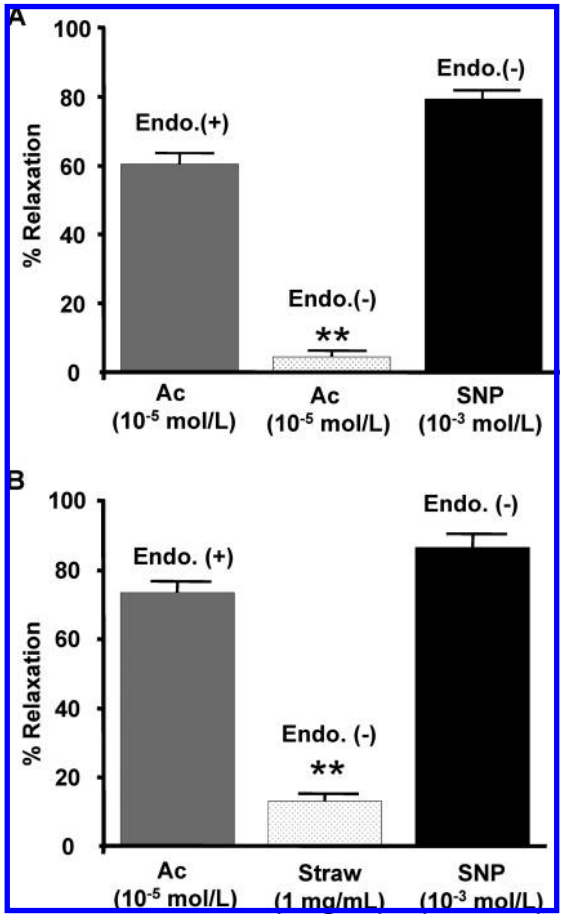

Figure 3. Strawberry extract-induced vasodilatation is an endothelium-dependent mechanism. Effect of removal of endothelium (Endo−) on the maximum relaxation induced by acetylcholine (Ac) (**A**) and strawberry extract (Straw) (**B**). Removal of endothelium abolished the responses elicited by Ac and Straw. The rings remained responsive to sodium nitroprusside (SNP), which is a nonendothelium-dependent relaxant of smooth muscle. Each value is represented as a mean ± SEM ($n = 4$). **$p < 0.01$, significant as compared to Ac (endo +) and SNP (endo−).

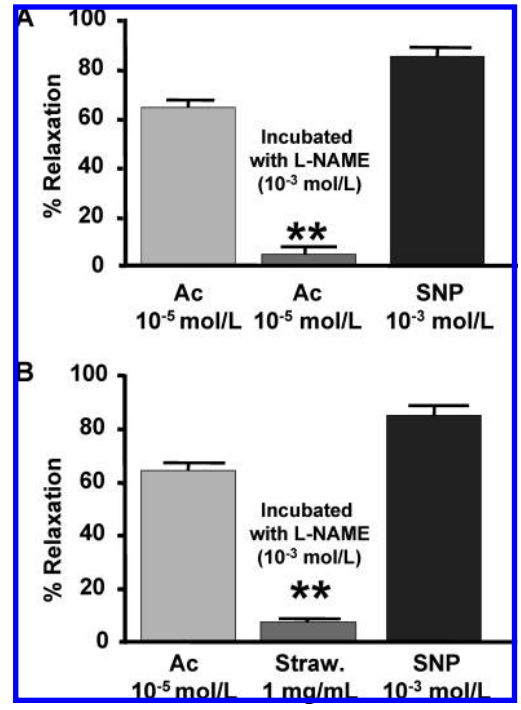

Figure 4. Strawberry extract induced vasodilatation through the activation of eNOS. The effect of L-NAME, a competitive inhibitor of eNOS, was tested in rabbit aortic rings. Incubation with L-NAME (10^{-3} mol/L) abolished EDR evoked by acetylcholine (Ac-10^{-3} mol/L) (**A**) and strawberry extract (Straw, 1 mg/mL) (**B**). Aortic rings remained responsive to sodium nitroprusside (SNP), which is a nonendothelium-dependent relaxant of smooth muscle, confirming that the incubation with L-NAME does not affect the relaxation property of smooth muscles. **$p < 0.01$, significant as compared to Ac- and SNP-induced relaxation ($n = 4$).

then concentrated (ca. 3−5 mL) under reduced pressure (200 mTorr). The concentrate was diluted with acetonitrile (50 mL), and the resulting mixture was evaporated to dryness under reduced pressure and with the assistance of a warm (50−55 °C) water bath. The resulting residue was dissolved in water (5 mL) and then extracted with hexane (5 mL). The top layer was removed, and an aliquot of the aqueous layer was subsequently analyzed for anthocyanins using the chromatographic conditions described below by monitoring the column effluent at 500 nm.

The aqueous layer (5 mL) was further processed for the analysis of phenolic compounds by adjusting its pH to ca. 2 with concentrated hydrochloric acid (30% w/w, 2−3 drops). The resulting acidified solution was then loaded onto a nonpolar polymeric solid phase extraction cartridge (Oasis HLB, 500 mg), which had previously been conditioned with acetonitrile (5 mL) and water (2 × 5 mL), and allowed to pass through the sorbent under the influence of gravity. Air was then drawn through the cartridge for a few minutes by applying a slight vacuum before elution of the extracted components in acetonitrile (10 mL). The solvent was evaporated under reduced pressure, and the

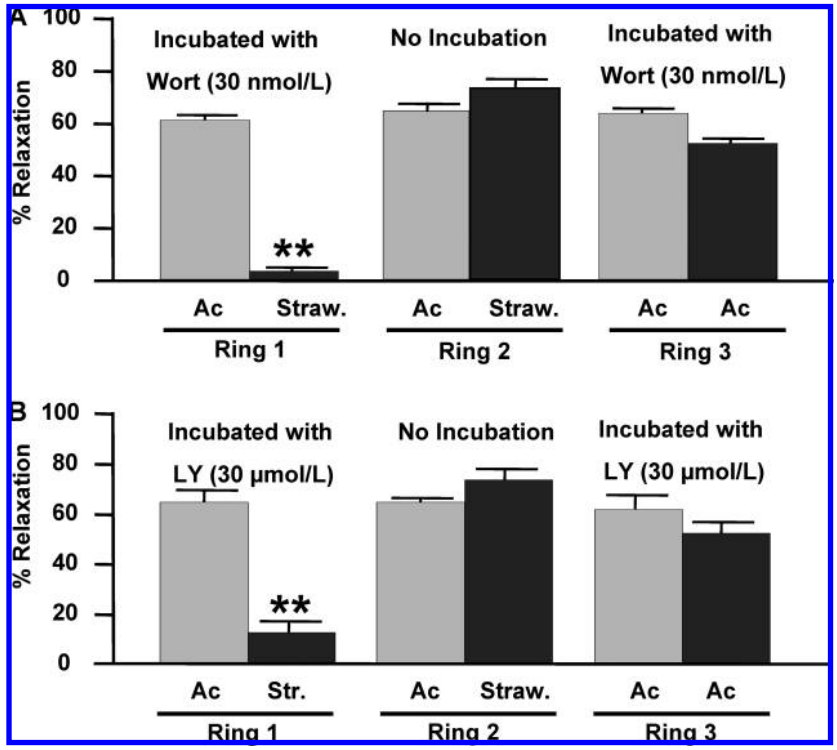

Figure 5. Strawberry extract caused vasodilatation through the activation PI3 kinase/Akt pathway in rabbit aorta. The effect of PI3 kinase/Akt blockers on the maximum relaxation produced by strawberry extract (Straw) was investigated as mentioned in the Materials and Methods. (**A**) All three rings responded to acetylcholine (Ac) initially. Ring #1, which was incubated with 30 nmol/L of Wortmannin (Wot) for 30 min and tested with Straw (1 mg/mL), showed a significantly attenuated relaxation (**$p < 0.01$). Ring #2, which was not incubated with Wot, showed a similar relaxation with the Straw. Ring #3, which was also incubated with Wot, showed no significant change in the responses Ac. (**B**) Corresponding findings with 30 μmol/L of LY294002 (LY) for Straw showing a significantly attenuated relaxation (**$p < 0.01$). All values are represented as means $\pm$ SEM ($n = 4$).

residue was dissolved in 50% aqueous methanol (1 mL). An aliquot (20 μL) of this extract was then analyzed for phenolic compounds by monitoring the effluents at 280 and 320 nm.

HPLC Conditions. The analysis of both anthocyanins and phenolic compounds was performed by partition chromatography on a reverse phase analytical column (4.6 mm × 250 mm, Synergi Hydro-RP, 4 μm, Phenomenex), which was maintained at 35 °C. The initial mobile phase composition was 0.025% (v/v) aqueous phosphoric acid and was maintained for 5 min before being changed to 1:1 0.025% aqueous phosphoric acid−methanol over a 50 min period, after which it was changed to 100% methanol during the next 25 min period. After 5 min, the mobile phase was returned to its initial composition, and the column was allowed to equilibrate under these conditions for 15 min before the next analysis. The flow through the column was 1.25 mL/min, and the effluent was passed through a photodiode array detector, which was set to scan over a wavelength range of 200−600 nm.

Calibration Curves. The amount of pelargonidin-3-*O*-glucoside in the strawberry extracts was determined using a four point calibration curve that was constructed by plotting the responses (area) at 500 nm of the standards against their corresponding concentrations, which were in the range of 10−100 μg/mL. In the case of the two cinnamoyl glucosides, because authentic standards of these two compounds were not commercially available, the corresponding phenolic acids were used to construct calibration curves. Thus, for 1-*O*-*p*-coumaroyl-D-glucopyranose, this was done by plotting the responses (area) at 315 nm of four *p*-coumaric acid standards against their corresponding concentrations. Similarly, a calibration curve for 1-*O*-cinnamoyl-D-glucopyranose was constructed by plotting the responses of four standards of *trans*-cinnamic acid at 280 nm.

Measurement of EDR. EDR was assessed as described previously (*7*). 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% oxygen and 5% carbon dioxide. A preload of 8 g was applied to the rings. The tissues were allowed to equilibrate for 60 min. The transducer was connected to a pen recorder (Gould −2400S recorder, Gould Inc., OH). The changes in tensions were also monitored using a Windaq computer program (2003 version, Dataq Instruments, OH).

After equilibration for 60 min at a preload of 8 g, the aortic rings were precontracted with norepinephrine (10 μmol/L). Acetylcholine was added in an incremental manner to achieve bath concentrations from 0.1 to 10 μmol/L to obtain dose−response curves for EDR. The relaxations were expressed as a percentage of the contraction induced by norepinephrine.

Strawberry Extract Induced Vasodilatation. After demonstration of the EDR evoked by acetylcholine, the rings were treated with increasing concentrations of strawberry extract (0.01−10 mg/mL final bath concentration) following precontraction with norepinephrine. In additional experiments, the effect of removing the endothelium on the relaxation evoked by acetylcholine and strawberry extract was examined to establish the endothelium-dependent nature of the vasodilatation. In these experiments, after demonstrating the absence of the relaxation, the rings were challenged with sodium nitroprusside to establish the ability of the aortic smooth muscle to relax. As a further control, the effect of incubation with N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; bath concentration, 1 mmol/L), a competitive inhibitor of nitric oxide synthase was examined to demonstrate the involvement of endothelial nitric oxide synthase (eNOS) in the relaxation of the rabbit aortic rings.

Effect of Blocking the PI3 Kinase/Akt Pathway on Vasodilatation Induced by Strawberry Extract. We and others have shown that the EDR evoked by polyphenolic compounds derived from grapes was abolished by blockers of the PI3 kinase/Akt signaling pathway (*6, 7*). In the present study, the effect of strawberry extract was examined after incubating the aortic rings with Wortmannin (30 nmol/L) and LY294002 (30 μmol/L) in KH buffer. Both Wortmannin and LY294002 are potent and specific PI3 kinase inhibitors. In testing the effect of each blocker, three aortic rings were tested simultaneously according to the sequence given in **Table 1**.

This protocol was based on a previous finding that prior exposure to GSE and other phenolic compounds (e.g., cocoa) attenuated the effect of subsequent exposure (*7*). Thus, it is not possible to expose a ring to the same extract twice, before and after exposure to the blocker, 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. Steps 3 and 4 established the effect of the blockers.

Ring 1 was used to examine the effect of the extract after incubation with the blocker. Ring 2 was used to demonstrate the response to the extract without prior exposure to a blocker. 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.

Analysis of Ascorbate Concentration in Strawberry Extract and the pH of the Organ Bath. Ascorbate and changes in the pH also induce vasodilatation (*18, 19*). Soluble pectins, sugars, organic acids, and ascorbate are present in strawberry and could potentially change the pH of the KH buffer in the organ bath. Therefore, we tested the ascorbate concentration and the pH changes in the fluid in the organ bath after the extract was added. The ascorbate concentration in the strawberry powder and organ bath was measured using HPLC as described by Sanchez Mata et al. (*20*). The pH was measured using a standard pH meter calibrated at 37 °C (Orion Three Star, Thermo Scientific, Beverly, MA).

Effect of PI3 Kinase Inhibitors on Strawberry Extract Induced Phosphorylation of eNOS and Akt in Vitro in HUVEC. HUVEC were grown in endothelial growth medium 2 (EGM-2) (Cambrex, United States) medium with 10% fetal bovine serum (FBS). Cells were grown to confluence (~90%) and starved for 6 h in serum-free medium before the cells were treated with strawberry extract (0.1 mg/mL). The pH of the EGM-2 culture media did not change (7.4) after the addition of strawberry extract (0.1 mg/mL). Some wells were treated with LY 294002 (30 μmol/L) or Wortmannin (30 nmol/L) for 30 min before exposure to strawberry extract. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 10 min. The reaction was stopped immediately by adding ice cold phosphate buffer solution (PBS; pH 7.4) and washed twice with the same PBS, and cell lysates were prepared in cell lysis buffer (Cell Signaling Technology, Danvers, MA). Total proteins (30 g) were separated on 7.5% sodium dodecyl sulfate−polyacrylamide gels. Separated proteins were transferred electrophoretically onto nitrocellulose membranes (Amersham-Pharmacia Biotech, Piscataway, NJ). Membranes were blocked with blocking buffer containing 5% nonfat milk in Tris-buffered saline solution and 0.1% Tween 20 (TBS-T) for 1 h. Phosphorylated Akt (Ser 473), phosphorylated eNOS (Ser 1177), Akt, and eNOS were detected after the membranes were incubated with the respective primary antibodies (rabbit anti-p-eNOS-Ser-1177, rabbit anti-eNOS, rabbit anti-p-Akt-Ser-473, and rabbit anti-Akt, Cell Signaling Technology, MA, dilution of 1:1000) overnight at 4 °C. Membranes were washed three times (10 min each) and incubated with the secondary antibody (peroxidase-labeled anti-rabbit IgG, 1:20000; Cell Signaling Technology) at room temperature for 60 min. Membranes were washed again three times (10 min each), and the specific protein bands were visualized using the enhanced chemiluminescence method (Amersham-Pharmacia Biotech). All four proteins were detected in the same blot, and the membranes were washed with stripping buffer (Pierce Biotechnology, Rockford, IL) for 30 min in 37 °C before it was incubated with the next primary antibody.

Statistical Analysis. Group data were expressed as means ± SEM. Comparisons between groups were compared using a paired *t* test or analysis of variance (ANOVA) depending on the number of groups being examined. Data were analyzed using Sigma Stat Version 3, 2003 (United States) statistical software. Statistical significance among treatments was determined as $p < 0.05$.

RESULTS

Quantitative Estimation of the Major Phenolic Compounds in the Aqueous Strawberry Extract. The extracted wavelength chromatogram at 500 nm that was obtained from the analysis of the aqueous strawberry extract indicated the presence of one major and two minor anthocyanins (**Figure 1A**). The chemical identity of the most abundant anthocyanin in the extract was shown to be pelargonidin-3-*O*-glucoside, and its concentration in the aqueous extract was estimated to be 1.15 ± 0.04 mg/g ($n = 4$). The minor anthocyanin components of strawberries at 500 nm wavelength chromatogram were identified as pelargonidin-3-*O*-rutinoside and cyanidin-3-*O*-glucoside.

At the wavelengths 315 and 280 nm, the analysis showed the presence of two major compounds in the extract (**Figure 1B**,**C**). These two compounds were identified as 1-*O*-*p*-coumaroyl-D-glucopyranose and *trans*-cinnamoyl-*O*-glucoside, and their concentrations were 0.37 and 0.22 mg/g of strawberry powder, respectively. The compounds that we identified are consistent with those reported by other investigators (*12, 16, 17*).

Strawberry Extract Caused EDR in Rabbit Aorta. Strawberry extract produced a dose-dependent relaxation of the aortic rings. The maximum relaxations observed were similar to those produced by acetylcholine (**Figure 2**). The major phenolic compounds present in the strawberry extract are pelargonidin-3-*O*-glucoside, coumaroyl- *O*-glucoside, and *trans*-cinnamoyl-*O*-glucoside, and their concentrations in the organ bath were approximately 2.6, 1.1, and 0.7 μmol/L, respectively. Removal of the endothelium abolished the responses evoked by acetylcholine and strawberry extract, confirming the obligatory role of endothelium (**Figure 3**). Incubation with L-NAME, a competitive eNOS inhibitor, also abolished the relaxation responses to acetylcholine and strawberry extract, suggesting that strawberry extract-induced vasodilatation is dependent on eNOS. However, thereafter, the aortic rings remained responsive to sodium nitroprusside, which is a nonendothelium-dependent relaxant of smooth muscle, confirming that the incubation with L-NAME does not affect the relaxation property of smooth muscles (**Figure 4**). These data confirmed that strawberry extract causes EDR in rings of rabbit aorta.

Ascorbate Levels in Strawberry Extract and pH Changes in Organ Bath. The ascorbate concentration in our freeze-dried strawberry powder was 2.3 ± 0.16 mg/g ($n = 3$) as measured by the method of Sanchez-Mata et al. (*20*). When the highest concentration of strawberry extract (1 mg/mL) was added to the organ bath at 37 °C, the pH dropped from 7.40 ± 0.00 to 7.34 ± 0.02 ($n = 5$), and the ascorbate concentration was 3.00 ± 0.03 μmol/L ($n = 3$).

Strawberry Extract Induced EDR through the Activation of PI3 Kinase/Akt. It is known that polyphenolic compounds derived from grapes induced EDR through PI3 kinase/Akt signaling pathway (*6, 7*). Therefore, we tested the effect of PI3 kinase inhibitors, Wortmannin and LY 294002, on strawberry extract-induced EDR. Incubation of aortic rings, which had been previously shown to be responsive to acetylcholine, with Wortmannin or LY 294002, significantly attenuated the relaxation induced by strawberry extract ($p < 0.01$). The responses evoked by acetylcholine were unaffected. The sequence described in the Materials and Methods was used in these experiments. The responses evoked by the highest concentration of strawberry extract (1 mg/mL) that showed the maximum relaxation in these experiments are summarized in **Figure 5**. It was also confirmed that acetylcholine-induced EDR was unaffected by PI3 kinase inhibitors. Therefore, it is apparent that

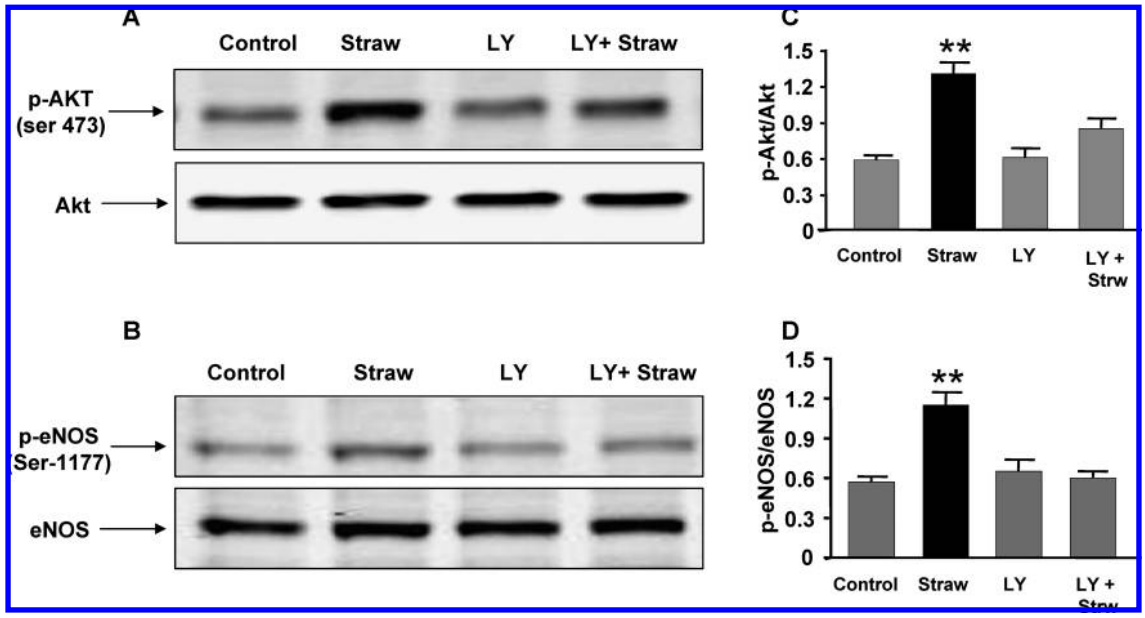

Figure 6. Strawberry extract activated Akt and eNOS through PI3 kinase in vitro in HUVEC. Strawberry extract (Straw)-induced Akt and eNOS phosphorylations were investigated in HUVECs. A PI3 kinase inhibitor, LY 294002 (LY), on Straw-induced Akt and eNOS phosphorylation was also investigated. Immunoblots showing the effect of strawberry extract on phosphorylation of Akt (p-Akt) (**A**) and eNOS (p-eNOS) (**B**). The blot in lane #1 (control) is the control showing the low concentration of phosphorylated Akt and eNOS. The blot in lane #2 (Straw) shows evidence of increased levels of phosphorylated Akt and eNOS after incubation with Straw (0.1 mg/mL) for 10 min. The effects observed in lane #2 are not evident in lanes 3 (LY alone) and 4 (LY + Straw). The histograms in both (**C**) and (**D**) shown are those obtained after quantification of the blots using densitometry ($n = 4$) for p-Akt and p-eNOS, respectively. The ordinates are the relative ratios of the phosphorylated and nonphosphorylated form of each enzyme. $^{**}p < 0.01$, significant as compared to control ($n = 4$).

prior exposure to a PI3 kinase inhibitor attenuated the EDR evoked by strawberry extract, suggesting that the EDR is mediated by activation of the PI3 kinase/Akt signaling pathway.

Strawberry Extract Phosphorylated Akt and eNOS Protein through the Activation of PI3 Kinase in HUVECs. It is known that EDR is caused by nitric oxide produced by the phosphorylation of eNOS (*21*). To further understand the mechanism of strawberry extract-induced EDR, we investigated the strawberry extract-induced phosphorylation of Akt (p-ser 473) and eNOS (p-ser 1177) in vitro in HUVECs. Strawberry extract-induced phosphorylation of Akt and eNOS was demonstrated by immunoblotting. Prior exposure to PI3 kinase inhibitor, LY 294002, abolished the phosphorylation of Akt and eNOS in HUVECs (**Figure 6**). These data also suggested that strawberry extract phosphorylates Akt and eNOS through the PI3 kinase pathway.

DISCUSSION

This investigation has shown for the first time that strawberry extract caused an EDR of the rabbit aorta. It is suggested that phenolic compounds present in the strawberry extract elicited EDR. The major phenolic compounds present in the aqueous strawberry extract are pelargonidin-3-*O*-glucoside, coumaroyl-*O*-glucoside, and *trans*-cinnamoyl-*O*-glucoside, and their concentrations in the organ bath at the maximum EDR were 2.6, 1.1, and 0.7 μmol/L, respectively. In our previous study, we have shown that a well-characterized GSE containing mixtures of oligomers and polymers of catechin and epicatechin caused EDR. The corresponding phenolic content that produced maximum relaxation by GSE was 3.92 $\pm$ 0.65 mmol/L of gallic acid units (*7*) as compared to the total phenolic content of strawberry extract of 1.15 $\pm$ 0.06 mmol/L of gallic acid units. Furthermore, the maximum relaxation induced by GSE (0.1 mg/mL, *7*) and the strawberry extracts (1 mg/mL) was not significantly different.

Possible Effect of Ascorbate and pH on EDR. Previous studies have shown that ascorbate also causes an EDR when applied over concentrations ranging from 1 to 300 μmol/L (*18*). In the experiments reported in this paper, the maximum concentration of ascorbate in the organ bath was found to be 3.00 $\pm$ 0.02 μmol/L. Although ascorbate is water-soluble, the concentration that we measured is only 23% of the total ascorbate present in the strawberry powder. This is due to the bicarbonates and CO_2 present in the KH buffer that are known to cause the degradation of ascorbate (*22*). Therefore, a small synergistic effect of polyphenolic compounds and ascorbate cannot be excluded. Thus, it is possible that this amount of ascorbate may have contributed approximately 10% to the maximum relaxation (73.1 $\pm$ 3.4%) caused by the strawberry extract (*18*).

Changes in pH have been shown to relax blood vessels by a mechanism that is independent of endothelium (*19*). The change in pH measured after adding the highest concentration of the strawberry extract was 0.06 $\pm$ 0.00. Such a change in pH is likely to cause a relaxation of approximately 5% (*18*). In the present study, after removal of the endothelial layer from aortic rings, we observed a residual relaxation of 12.8 $\pm$ 2.5% when the highest dose of the strawberry extract was added. Acetylcholine produced a relaxation of 4.3 $\pm$ 1.9% under the same circumstances. These differences, although small, are significant ($p < 0.05$) and may due to changes in pH.

Potential Mechanism of Action. We investigated whether the activation of EDR induced by strawberry extract was mediated by the PI3 kinase/Akt signaling pathway. PI3 kinase, which is a redox-sensitive protein kinase, appears to be activated by the redox sensitivity of phenols, leading to production of

nitric oxide (*6*). Akt is a serine/threonine protein kinase that is recruited to the endothelial cell membrane because of its binding to PI3 kinase-produced phosphoinositides. At the membrane, Akt is phosphorylated and activates eNOS (by phosphorylation at Ser 1177 in human), leading to the production of nitric oxide (*21*). In our previous study, we reported that GSE induced EDR through the activation of PI3 kinase/Akt signaling pathway (*7*). Recent studies performed in cell culture have also established that polyphenolic compounds affect the level of phosphorylation of Akt in a PI3 kinase-dependent manner, which in turn phosphorylates eNOS, resulting in an increased formation of nitric oxide (*7, 23*). In the present study, we observed that prior incubation with PI3 kinase blockers, Wortmannin and LY 294002, significantly attenuated the EDR induced by the strawberry extract. Furthermore, we showed that strawberry extracts induced concurrent phosphorylation of Akt and eNOS and that PI3 kinase inhibitors blocked the phosphorylation.

Potential Clinical Significance of Findings. Normal blood pressure is continuously regulated by the autonomic nervous system using an extensive network of receptors, hormones, and nerves. The endothelium is critical in this network for maintaining normal blood pressure. Likewise, endothelial dysfunction is recognized for its role in hypertension and the development of vascular disease. The consumption of fruits rich in polyphenolic compounds, including anthocyanin-rich formulations, has been found to lower blood pressure in humans, especially in those individuals having elevated levels (*24*−*26*). Several population-based studies have found an inverse relationship between flavonoid and polyphenolic intake and cardiovascular disease (*27, 28*). The effect of flavonoids and other plant-derived polyphenolic compounds on endothelial function has been a mechanism of major interest in recent years. The results of the present study support favorable effects of a strawberry extract on endothelial function. Evidence suggests that strawberry, an anthocyanin-rich fruit, maintains the functionality of the endothelium and can potentially have an important, clinically relevant impact on blood pressure and risk of cardiovascular disease (*29*). It has been reported that consumption of strawberry (200 g) containing 222 μmol of pelargonidin-3-glucoside could produce a pelargonidin-3-glucuronide concentration of 274 nmol/L in human blood (metabolite of pelargonidin-3-glucoside) (*30*). In the present study, we have observed EDR in response to strawberry extract over the concentration range 0.01−1 mg/mL. The corresponding pelargonidin-3-glucoside concentrations were 26 nmol/L to 2.6 μmol/L. Thus, our organ bath concentrations were biologically relevant to humans. However, further investigations are needed to validate the bioavailability and bioactivity of strawberry fruits in humans.

In summary, this study has shown that an aqueous extract of freeze-dried strawberry causes an EDR relaxation, which is mediated by nitric oxide produced by activation of eNOS. It is suggested that this effect of the strawberry extract is mediated by activation of the PI3 kinase/Akt signaling pathway, resulting in phosphorylation of eNOS.

ACKNOWLEDGMENT

Funding and strawberry powder were provided by the California Strawberry Commission. We acknowledge the technical contributions of Rima Juskelis who performed the strawberry analysis under the direction of Dr. Peter Varelis. We also acknowledge Dr. Alyson Mitchell and Dr. Eunmi Koh for helping us to determine ascorbate concentrations in strawberry powder and for their helpful suggestions.

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Received for review June 17, 2008. Revised manuscript received August 25, 2008. Accepted August 26, 2008.

JF801864T