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Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src- and Akt-dependent activation of eNOS

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Abstract

Objectives: An enhanced endothelial formation of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF), is thought to contribute to the protective effect of moderate consumption of red wine on coronary diseases. The present study has characterized endothelium-dependent relaxations to Concord grape juice (CGJ), a non-alcoholic rich source of grape-derived polyphenols, in the coronary artery.
Methods: Porcine coronary artery rings were suspended in organ chambers for the measurement of changes in isometric tension in the presence of indomethacin. NO formation was assessed by electron spin resonance spectroscopy, and the phosphorylation of Src, Akt and endothelial NO synthase (eNOS) by Western blot analysis in cultured endothelial cells.
Results: Endothelium-dependent relaxations to CGJ were slightly but significantly reduced by L-NA, not affected by charybdotoxin (CTX) plus apamin (APA, two inhibitors of EDHF-mediated responses) whereas the combination of L-NA, CTX plus APA reduced maximal relaxation to about 50%. In the presence of CTX plus APA, relaxations to CGJ were markedly reduced by the membrane permeant mimetic of superoxide dismutase (SOD), MnTMPyP, the membrane permeant analogue of catalase polyethyleneglycol-catalase (PEG-catalase), PP2, an inhibitor of Src kinase, and by wortmannin, an inhibitor of the PI3-kinase. CGJ stimulated the formation of reactive oxygen species and the N^{ω}-nitro-L-arginine-, PP2- and wortmannin-sensitive formation of NO in endothelial cells. The formation of NO was associated with a redox-sensitive and time-dependent phosphorylation of Src, Akt and eNOS.
Conclusions: CGJ induces endothelium-dependent relaxations of coronary arteries, which involve a NO-mediated component and also, to a minor extent, an EDHF-mediated component. In addition, CGJ-induced NO formation is due to the redox-sensitive activation of Src kinase with the subsequent PI3-kinase/Akt-dependent phosphorylation of eNOS.

Keywords: Endothelial function; Coronary disease; Endothelial factors; Nitric oxide

1. Introduction

Several epidemiological studies have indicated that regular intake of vegetables, fruit, and beverages such as red wine and green tea, is associated with a decreased global mortality due to a reduced number of cancer and coronary diseases [1–3]. The protective effect has been attributable, at least in part, to polyphenols [4,5]. Indeed, grape products such as red wine contain high levels of polyphenols, which are predominantly found in skins, seeds and stems. The protective effect of red wine polyphenols on the vascular system is thought to include their ability to prevent oxidation of low-density lipoproteins [6,7], platelet aggregation and adhesion [8,9], and smooth muscle cell migration and proliferation [10,11]. Alternatively, vascular protection might also be due to the direct action of polyphenols on endothelial cells resulting in an enhanced formation of nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF), two factors playing a major role in the control of

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vascular homeostasis [12,13]. Indeed, red wine polyphenols cause pronounced endothelium-dependent relaxations of isolated arteries, which are solely mediated by NO in the rat aorta but involve both NO and EDHF in porcine coronary arteries [14–16]. Besides red wines, grape juices, a non-alcoholic beverage, are an excellent alternative source of grape-derived polyphenols. Studies have shown that ingestion of purple grape juice improves flow-mediated vasodilatation, platelet function and platelet-dependent inflammatory responses in patients with coronary artery disease [7,17,18], and reduces blood pressure in moderately hypertensive patients [19]. In addition, consumption of purple grape juice also increased serum antioxidant capacity and protected LDL against oxidation in healthy subjects [20]. In order to better understand the protective effect of purple grape juice of the endothelial function, experiments were performed to determine whether purple grape juice induces endothelium-dependent relaxation of coronary arteries and, if so, to characterize the relaxing factors involved, and the signaling pathway leading to the formation of NO.

2. Methods

2.1. Reagents

Diethyldithiocarbamate sodium salt trihydrate (DETC), apamin, charybdotoxin, superoxide dismutase (SOD), polyethyleneglycol-SOD (PEG-SOD), catalase, PEG-catalase, indomethacin, bradykinin, hydroethidine, carboxyPTIO, and N^{ω}-nitro-L-arginine (L-NA) were from Sigma. 1*H*-[1,2,4] oxadiazolo[4,3a]quinoxalin-1-one (ODQ) was from Tocris, wortmannin, LY294002, and the superoxide dismutase (SOD) mimetic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) from Alexis Chemicals, U46619 (9,11-dideoxy-9α-methanoepoxy prostaglandin $F_2\alpha$) from Cayman Chemical, and PP2 (4-amino-5-(4-chlorophényl)-7-(*t*-butyl)pyrazolo [3,4-d]pyrimidine) from Calbiochem. Concord grape juice containing 2.3 g/l polyphenols (total phenolics: 2307 mg/l gallic acid equivalent; anthocyanins: 411 mg/l malvidin; proanthocyanidins: 509 mg/l catechin; potassium: 1460 mg/l) was provided by Welch Foods, Inc. (Concord, MA, USA).

2.2. Vascular reactivity studies

Left anterior descending coronary arteries (obtained from the local slaughterhouse) were cleaned of connective tissue and cut into rings (4–5 mm in length). As indicated the endothelium was removed by rubbing the intimal surface of rings with a pair of forceps. Rings were suspended in organ baths containing oxygenated (95% O_2 and 5% CO_2) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25, and D-glucose 11, pH 7.4, 37 °C) and the cyclooxygenase inhibitor indomethacin (10 μM), for the determination of changes in isometric tension. Following equilibration for 90 min under a resting tension of 5 g, rings were contracted with KCl (80 mM). After a 30-min washout period, rings were contracted with the thromboxane mimetic U46619 (1–60 nM) to about 80% of the maximal contraction before addition of bradykinin (0.3 μM) to check the presence of a functional endothelium. After washout and a 30-min equilibration period, rings were again contracted with U46619 before construction of a concentration–relaxation curve to CGJ. In some experiments, rings were exposed to an inhibitor for 30 min before the addition of U46619. In some experiments, rings were exposed to CGJ for 5 min before construction of a concentration–contraction curve to U46619.

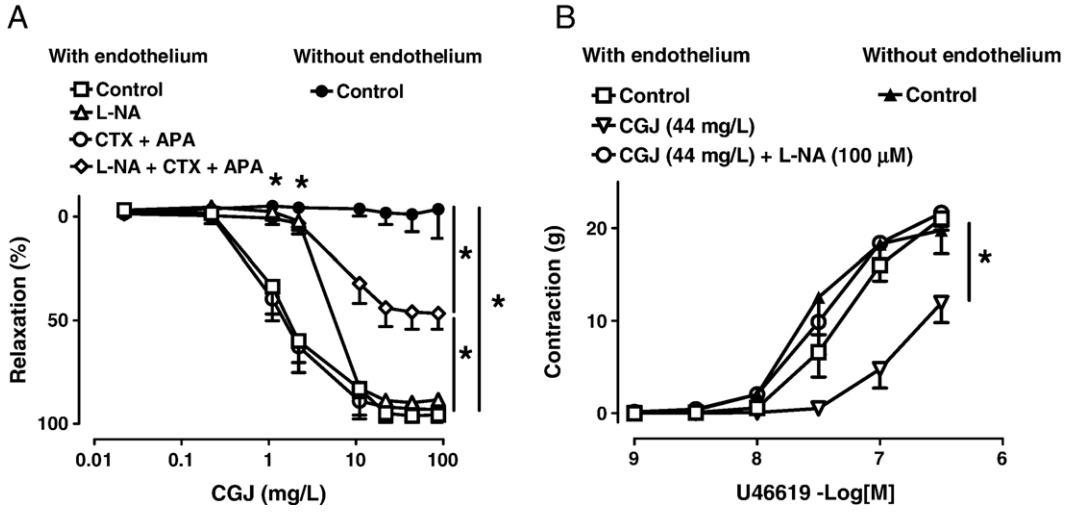

Fig. 1. Characterization of Concord grape juice (CGJ)-induced relaxations in porcine coronary artery rings (A). Intact and endothelium-denuded rings were contracted with U46619 before the addition of increasing concentrations of CGJ. Rings with endothelium were also incubated with either N^{ω}-nitro-L-arginine (L-NA, 100 μM, an inhibitor of eNOS), charybdotoxin (CTX, 100 nM) plus apamin (APA, 100 nM; two inhibitors of EDHF-mediated relaxations) or the combination of L-NA, CTX and APA for 30 min before addition of U46619. Effect of CGJ on contractile responses (B). As indicated intact rings were exposed to CGJ (44 mg/l) 5 min before the addition of increasing concentrations of U46619. Rings with endothelium were also incubated with L-NA 30 min before the addition of CGJ. Experiments were performed in the presence of indomethacin. $n=3$ to 8 different experiments. *$P<0.05$ versus control.

2.3. Cell culture

Porcine coronary artery segments were flushed with PBS without calcium to remove remaining blood. Thereafter, endothelial cells were isolated by collagenase treatment (type I, Worthington, 1 mg/ml for 12 min at 37 °C), and cultured in culture dishes containing medium MCDB 131 (Invitrogen) and 15% fetal calf serum supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 μg/ml), and L-glutamine (2 mM) (all from Cambrex), and grown for 48–72 h. All experiments were performed with confluent cultures of cells used at first passage. Cells were exposed to serum-free culture medium in the presence of 0.1% bovine serum albumin (QBiogene) for 6 h prior to treatment.

2.4. In situ detection of superoxide anions

The oxidative fluorescent dye hydroethidine was used to evaluate *in situ* production of superoxide anions by use of a method described by Miller et al. [21]. Hydroethidine is freely permeable to cells and in the presence of superoxide anions is oxidized to ethidium bromide, and trapped by intercalating with the DNA. Ethidium bromide is excited at 488 nm with an emission spectrum of 610 nm. Endothelial cells were rinsed in PBS and incubated in Hanks' balanced salt solution containing hydroethidine (10 μM) in a light-protected humidified chamber at 37 °C. After 30 min, cells were exposed to CGJ. Images were obtained with a Bio-Rad MRC-1024 laser scanning confocal microscope equipped with krypton/argon laser. Fluorescence was detected with a 605-nm long-pass filter.

2.5. Determination of NO formation

Determination of NO formation was assessed by electron spin resonance spectroscopy (ESR) after formation of [Fe(II)NO $(DETC)_2$], a paramagnetic DETC iron complex with NO, in cultured endothelial cells. The ESR methodology was used as reported previously with minor modifications [22]. Confluent cultures of endothelial cells (first passage, ~1 million cells per well) were washed twice with Hanks balanced salt solution (HBSS) buffered with 10 mM HEPES, and then they were incubated in a HBSS–HEPES solution in the presence of bovine serum albumin (20.5 mg/ml), 1.5 mM $CaCl_2$, 0.3 mM L-arginine and antioxidants or inhibitors as indicated for 30 min at 37 °C. Spin trap chemicals $FeSO_4$ (0.8 mM) and DETC (1.6 mM) were rapidly mixed to obtain a colloid form [Fe(II)(DETC)2], which was added to endothelial cells at a final concentration of 0.2 mM. After 5 min, the endothelial formation of NO was induced by addition of CGJ (44 mg/l) for 30 min. Thereafter, dishes were placed on ice, and the incubation medium was removed before addition of 0.2 ml of the HBSS–HEPES buffer. Cells were then scraped, and the cell suspension was collected in a calibrated tube. Tubes were rapidly frozen at 77 K for ESR measurements. ESR measurements were

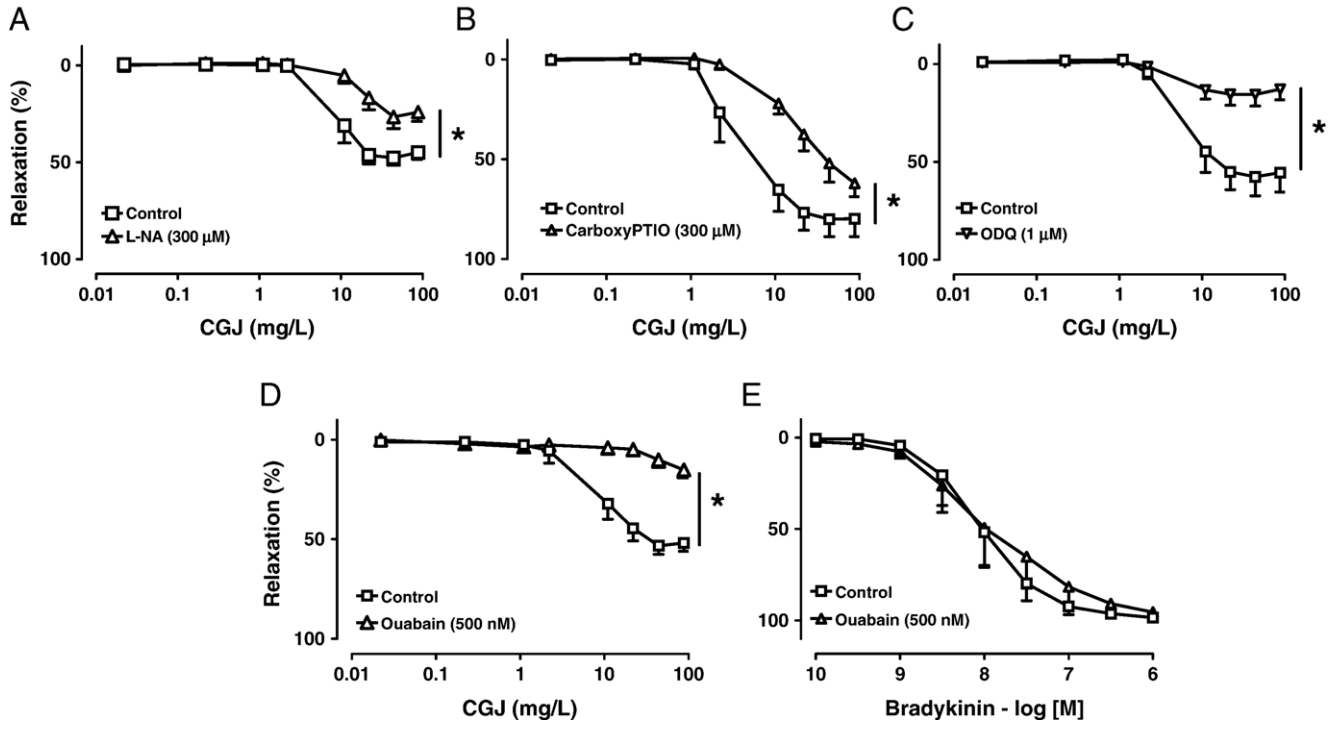

Fig. 2. Characterization of the L-NA (100 μM) resistant relaxation to CGJ in intact coronary artery rings. Rings were incubated with (A) L-NA (300 μM), (B) carboxyPTIO (300 μM, a NO scavenger), (C) ODQ (1 μM, an inhibitor of soluble guanylyl cyclase), and (D) ouabain (500 nM, an inhibitor of Na^+, K^+-ATPase) for 30 min before induction of contraction to U46619 and subsequent relaxation to CGJ. The effect of ouabain on relaxation to bradykinin is also shown (E). In A, B, C, and D experiments were performed in the presence of L-NA (100 μM), indomethacin, and charybdotoxin plus apamin and in E in the presence of indomethacin. $n=4$ to 6 different experiments. *$P<0.05$ versus control.

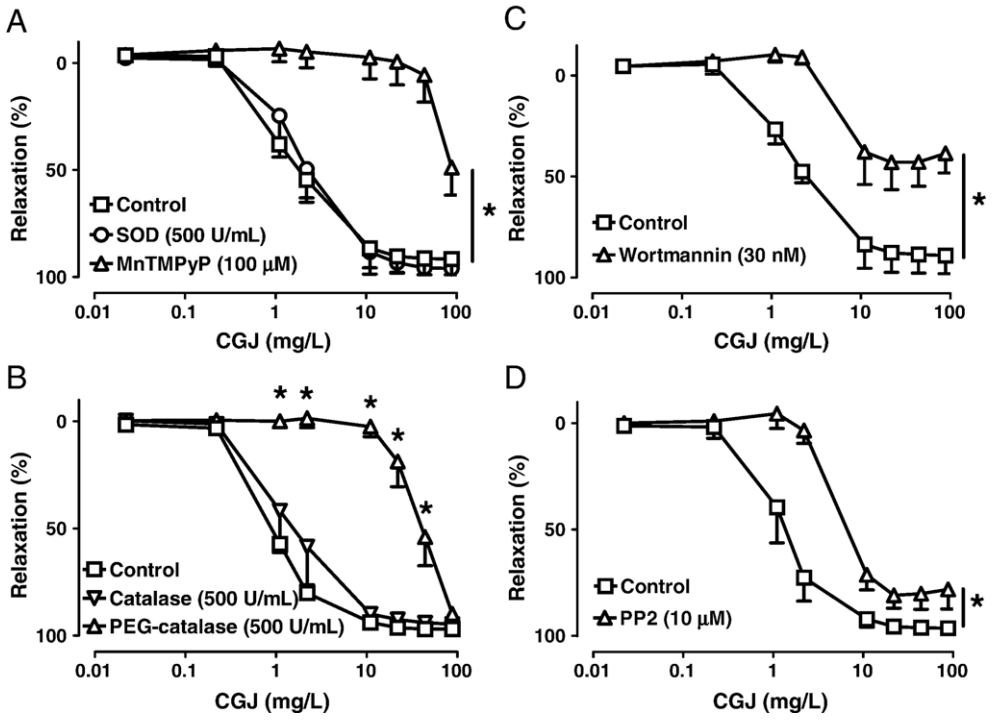

Fig. 3. Role of the redox-sensitive Src kinase and the PI3-kinase/Akt pathway in CGJ-induced endothelium-dependent relaxation. Coronary artery rings with endothelium were incubated with (A) MnTMPyP (100 μM), a cell permeable superoxide dismutase (SOD) mimetic, or native SOD, (B) the membrane permeant analogue of catalase polyethyleneglycol-catalase (PEG-catalase) (500 U/mL) or native catalase, (C) the PI3-kinase inhibitor, wortmannin (30 nM), and (D) the Src kinase inhibitor, PP2 (10 μM) for 30 min before contraction to U46619 and subsequent relaxation to CGJ. Experiments were performed in the presence of indomethacin, and charybdotoxin plus apamin. $n=4$ to 5 different experiments. *$P<0.05$ versus control.

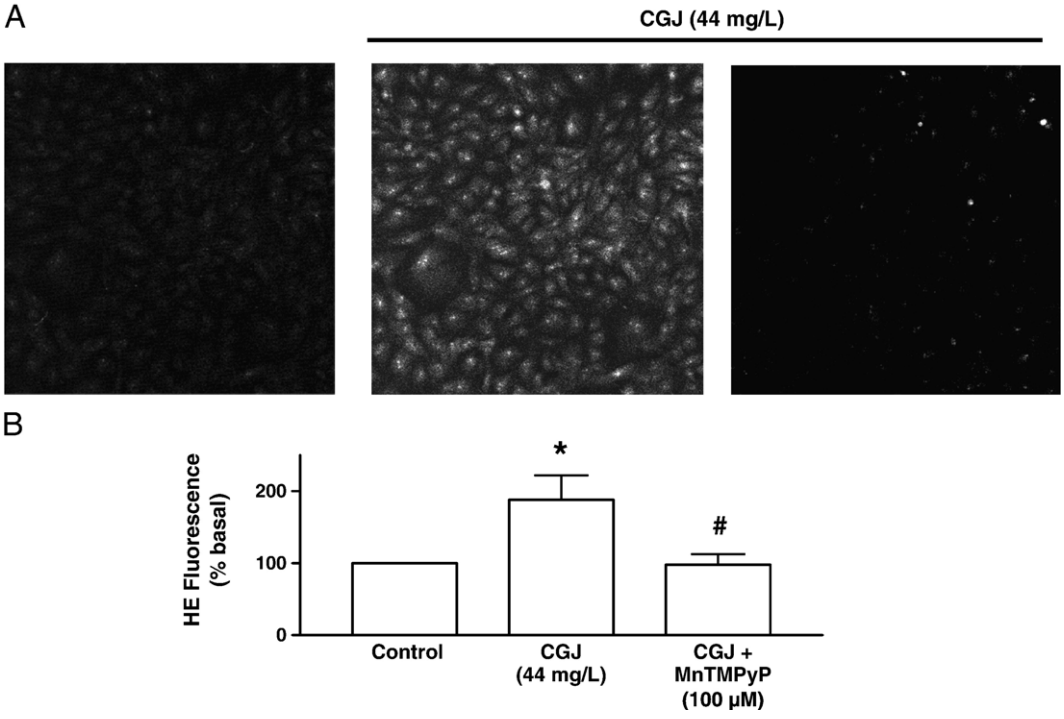

Fig. 4. CGJ-induced generation of ROS in cultured endothelial cells. Hydroethidine (HE)-loaded cells were stimulated with CGJ and, thereafter, the ethidium bromide fluorescence was monitored over 10 min using a confocal microscope. (A) Original pictures showing ethidium bromide fluorescence in unstimulated cells, and in cells stimulated with CGJ in the absence or presence of MnTMPyP (100 μM). (B) Corresponding cumulative data. $n=3$ to 4 different experiments. *$P<0.05$ versus control. #$P<0.05$ versus CGJ.

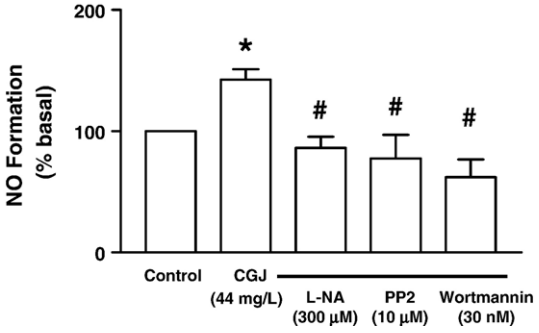

Fig. 5. CGJ stimulates the formation of NO in cultured endothelial cells as assessed by electron spin resonance spectroscopy. Cells were exposed to either solvent, L-NA, PP2 or wortmannin for 30 min before the addition of CGJ for 30 min. $n=3$ different experiments. *$P<0.05$ versus control. #$P<0.05$ versus CGJ.

performed on an MS100 spectrometer (Magnettech Ltd.) under the following conditions: temperature 77 K, microwave frequency 9.34 GHz, microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 1 mT. The third component of the ESR signal was used for relative comparison of the concentration of NO trapped in each sample.

2.6. Western blot analysis

After treatment, cells were washed twice with PBS and then lysed in extraction buffer (composition in mM: Tris/HCl 20 (pH 7.5; QBiogene), NaCl 150, Na_3VO_4 1, sodium pyrophosphate 10, NaF 20, okadaic acid 0.01 (Sigma), a tablet of protease inhibitor (Roche) and 1% Triton X-100 (QBiogen)). Total proteins (20 μg) were separated on 8% SDS-polyacrylamide (Sigma) gels at 70 V for 2.5 h. Separated proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Amersham) at 100 V for 120 min. Membranes were blocked with blocking buffer containing 3% bovine serum albumin for p-Akt and p-eNOS, and I-block for p-Src, Tris-buffered saline solution (Biorad) and 0.1% Tween 20 (Sigma) (TBS-T) for 1 h. For detection of phosphorylated proteins, membranes were incubated with the respective primary antibody (p-Src Tyr418, Biosource; p-Akt Ser473 and p-eNOS Ser1177, Cell Signaling Technology; dilution of 1:1000) overnight at 4 °C. After washing, membranes were incubated with the secondary antibody (peroxidase-labeled anti–rabbit IgG, dilution of 1:5000; Cell Signaling Technology) at room temperature for 60 min. Prestained markers (Invitrogen) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham). Ponceau staining was performed to verify the quality of the transfer and equal amounts of proteins in each lane.

2.7. Statistical analysis

Values are expressed as means±SEM. Statistical evaluation was performed with Student's t test for paired data or ANOVA followed by Fischer's protected least significant difference test where appropriate. Values of $P<0.05$ were considered statistically significant.

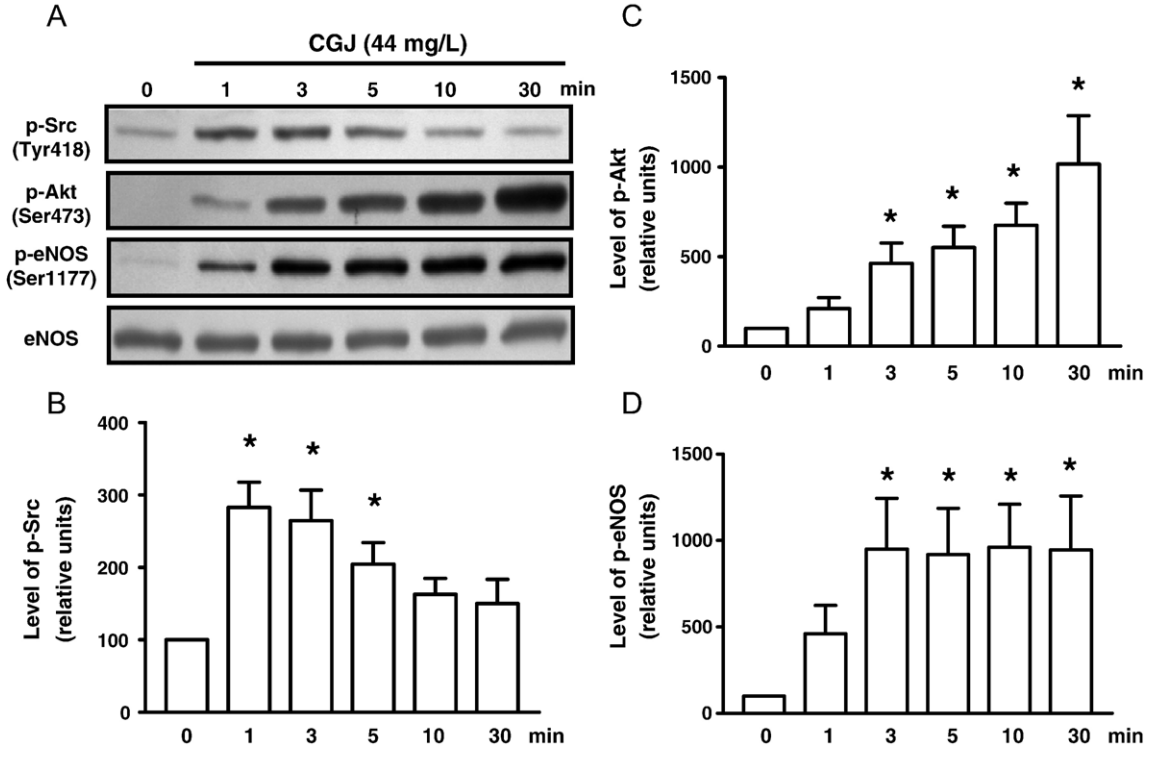

Fig. 6. CGJ caused a time-dependent phosphorylation of Src at Tyr418, Akt at Ser473 and eNOS at Ser1177 in endothelial cells. Cells were exposed to CGJ for the indicated times at 37 °C. Thereafter, the level of p-Src, p-Akt and p-eNOS was determined by Western blot analysis. (A) Representative immunoblots, and (B), (C), and (D) corresponding cumulative data. $n=3$ to 4 different experiments. *$P<0.05$ versus control.

3. Results

3.1. Concord grape juice induces endothelium-dependent relaxation and inhibition of contractile responses in coronary arteries

In the presence of indomethacin, CGJ caused concentration-dependent relaxations in coronary artery rings with endothelium but only minor ones in those without endothelium (Fig. 1A). Relaxations to CGJ were slightly but significantly reduced by L-NA (100 μM, a competitive inhibitor of NO synthase) without affecting the maximal relaxation, not affected by charybdotoxin plus apamin whereas the combination of L-NA, charybdotoxin plus apamin reduced maximal relaxation to about 50%. These findings indicate that CGJ induces endothelium-dependent relaxations of coronary arteries, which include a NO-mediated component, a modest EDHF-mediated component and also L-NA-, charybdotoxin plus apamin-resistant component. Next, experiments were performed to characterize the residual relaxation to CGJ. Increasing the concentration of L-NA from 100 to 300 μM further reduced relaxations to CGJ (Fig. 2A). They were also significantly reduced by a NO scavenger, carboxyPTIO and by an inhibitor of soluble guanylyl cyclase, ODQ (Fig. 2B and C). In addition, the Na^+, K^+-ATPase inhibitor ouabain markedly blunted relaxations to CGJ whereas those to bradykinin were not affected (Fig. 2D and E).

Next, the possibility that CGJ, besides inducing endothelium-dependent relaxations, also affects contractile responses was assessed. Exposure of coronary artery rings to CGJ 5 min prior to the addition of increasing concentrations of U46619 significantly blunted contractions in rings with endothelium but not in those without endothelium (Fig. 1B and data not shown). The inhibitory effect of CGJ was prevented by L-NA indicating that this effect is due to an enhanced endothelial formation of NO.

3.2. Role of reactive oxygen species (ROS), Src kinase and the PI3-kinase/Akt pathway in CGJ-induced relaxation

Previous studies have shown that red wine polyphenols-induced NO-mediated relaxations in coronary arteries are critically dependent on the redox-sensitive activation of the PI3-kinase/Akt pathway leading to the phosphorylation of eNOS [16]. Therefore, experiments were planned to determine whether this signaling pathway is also involved in CGJ-induced relaxations. In the presence of charybdotoxin plus apamin, NO mediated relaxations to CGJ were markedly reduced by the membrane permeant SOD mimetic MnTMPyP and by the cell permeable PEG-catalase whereas native SOD

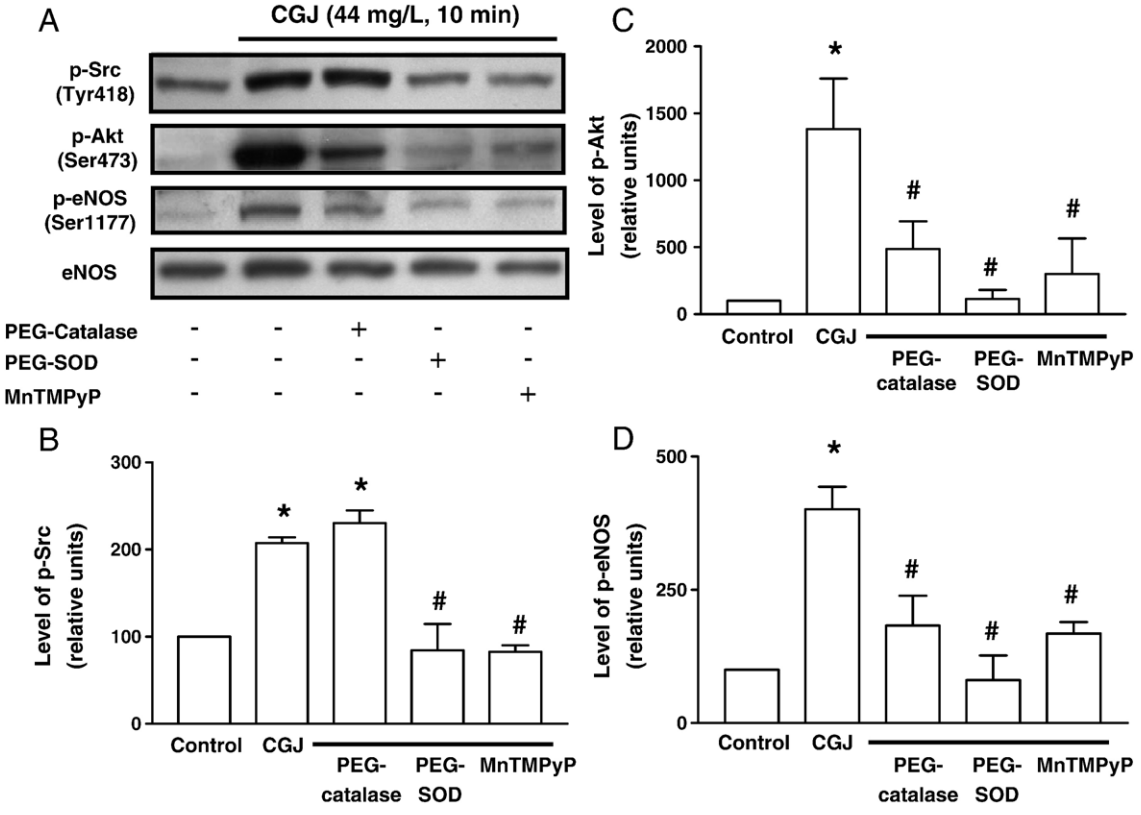

Fig. 7. Role of ROS in CGJ-induced phosphorylation of Src, Akt and eNOS in endothelial cells. Cells were incubated with either solvent, PEG-catalase (500 U/mL), PEG-SOD (500 U/mL) or MnTMPyP (100 μM) for 30 min before the addition of CGJ. The level of p-Src, p-Akt and p-eNOS was determined by Western blot analysis. (A) Representative immunoblots, and (B), (C), and (D) corresponding cumulative data. $n=3$ to 4 different experiments. *$P<0.05$ versus control. #$P<0.05$ versus CGJ.

and native catalase did not have such an effect (Fig. 3A, B). The PI3-kinase inhibitor, wortmannin also significantly reduced relaxations to CGJ (Fig. 3C). Since the Src family of kinases are redox-sensitive kinases [23] that have been shown to act as upstream activators of the PI3-kinase/Akt pathway [24], experiments were performed to determine the role of Src kinase in relaxations to CGJ. Inhibition of Src kinase with PP2 significantly reduced relaxations to CGJ (Fig. 3D). In contrast, relaxations to bradykinin were not affected by PP2 (relaxations to bradykinin 1 μM were 91.4±3.0 and 87.7±5.0% in the absence and presence of PP2, respectively; $n=6$).

3.3. CGJ causes the formation of ROS in endothelial cells

To provide further evidence that CGJ is able to cause an intracellular pro-oxidant response, the *in situ* formation of ROS was assessed in coronary artery endothelial cells using the redox-sensitive fluorescent probe hydroethidine. Exposure of cells to CGJ markedly increased the fluorescent signal, and MnTMPyP abolished this effect (Fig. 4).

3.4. CGJ stimulates the formation of NO in endothelial cells

Next, experiments were performed to provide direct evidence that CGJ induces NO formation in porcine coronary artery endothelial cells using electron spin resonance spectroscopy. Exposure of cells to CGJ induced about a 1.5-fold increase in the formation of NO (Fig. 5). The stimulatory effect was abolished by L-NA, PP2 and wortmannin (Fig. 5).

3.5. CGJ causes the redox-sensitive activation of Src with subsequent PI3-kinase/Akt-dependent phosphorylation of eNOS

To better characterize the signaling pathway involved in eNOS activation in response to CGJ, levels of p-Src, p-Akt and p-eNOS were assessed in endothelial cells by immunoblotting. Unstimulated endothelial cells had either no or only a low level of p-Src, p-Akt and p-eNOS (Fig. 6). CGJ increased within 1 min signals of p-Src, p-Akt and p-eNOS and, thereafter, these signals evolved differentially. Indeed, phosphorylation of Src was an early and transient event, which reached a peak value within 1 to 3 min and then returned to baseline at 10 min. In contrast, the level of p-Akt and p-eNOS was detected after 1 min and then increased steadily until 30 min (Fig. 6). CGJ-induced phophorylation of Src was abolished by PEG-SOD and MnTMPyP but not by PEG-catalase (Fig. 7A, B). In contrast, PEG-SOD, MnTMPyP and PEG-catalase prevented CGJ-induced phophorylation of Akt and eNOS (Fig. 7C, D). In addition, the PI3-kinase inhibitors wortmannin and LY294002

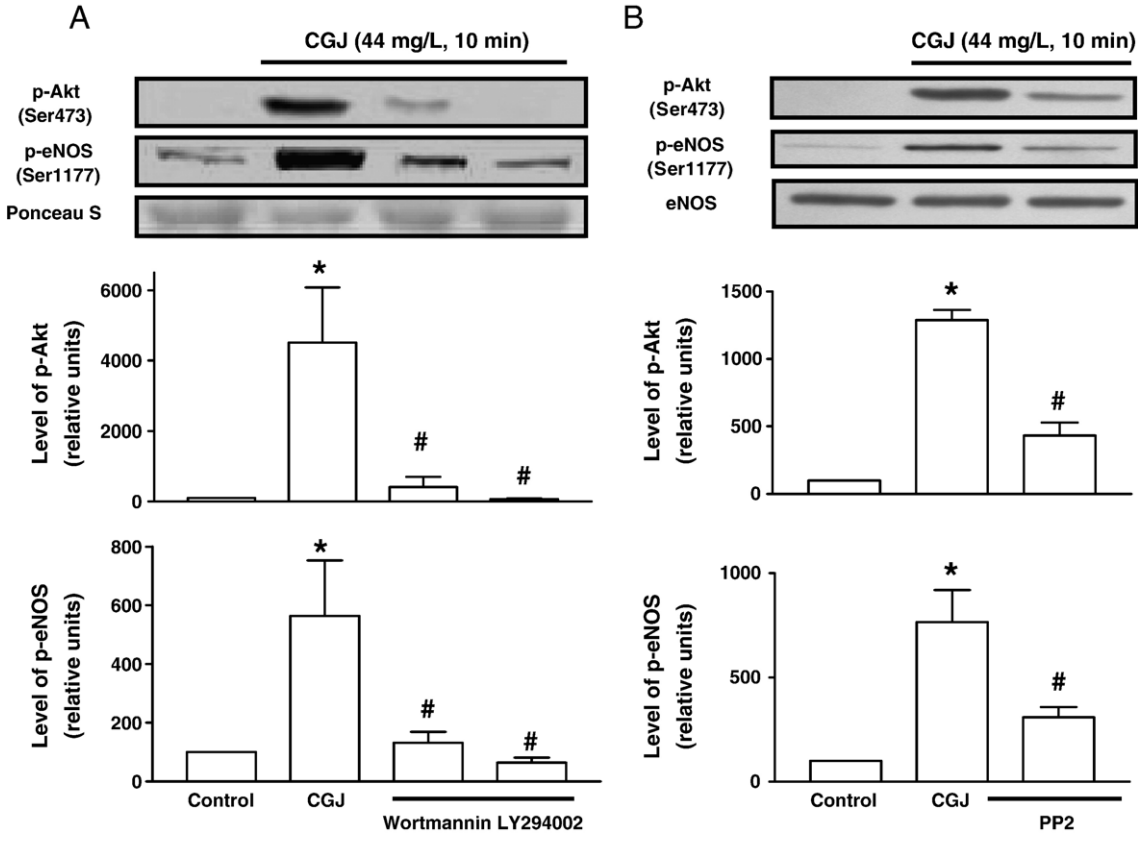

Fig. 8. Role of the PI3-kinase/Akt pathway and the Src kinase in CGJ-induced phosphorylation of Akt and eNOS in endothelial cells. Cells were incubated either with solvent, wortmannin (30 nM), LY294002 (30 μM), or PP2 (10 μM), for 30 min before the addition of CGJ. The level of p-Akt and p-eNOS was determined by Western blot analysis. Top, representative immunoblots, and bottom, corresponding cumulative data. $n=3$ to 4 different experiments. *$P<0.05$ versus control. #$P<0.05$ versus CGJ.

prevented phosphorylation of Akt and eNOS (Fig. 8A). These data indicate that ROS, especially superoxide anions, act as upstream mediators of Src kinase whereas both superoxide anions and hydrogen peroxide are involved in the activation of the PI3-kinase/Akt pathway leading to eNOS phosphorylation in response to CGJ. Moreover, CGJ-induced phophorylation of Akt and eNOS was abolished by the Src kinase inhibitor PP2, indicating that the redox-sensitive Src kinase is the major activator of the PI3-kinase/Akt pathway leading to eNOS activation (Fig. 8B).

4. Discussion

The present findings indicate that Concord grape juice is a powerful endothelium-dependent vasodilator of coronary arteries and that this effect involves several components. Indeed, relaxations to CGJ were slightly but significantly reduced by L-NA without affecting the maximal relaxation indicating a role for NO. Moreover, the present findings provide also direct evidence that CGJ activates eNOS as indicated by the L-NA-sensitive formation of NO and the enhanced phosphorylation of eNOS at Ser1177 in endothelial cells. Interestingly, CGJ induced a 1.5-fold increase in NO formation, which is similar to that induced by the physiological agonist bradykinin [16]. Although inhibition of EDHF-mediated responses with charybdotoxin plus apamin alone did not affect relaxations to CGJ, their combination with L-NA reduced maximal relaxation to about 50%, indicating that EDHF plays also a role, although only to some extent. Surprisingly, a substantial relaxation persisted in the presence of L-NA, charybdotoxin and apamin. In contrast to CGJ, the combination of L-NA, charybdotoxin and apamin abolished relaxations to a red wine extract in the coronary artery [16]. The persistent relaxation to CGJ was not due to the formation of relaxing prostanoids since all experiments were performed in the presence of indomethacin. In addition, it was not affected by the combination of a beta blocker (propranolol) and an alpha$_2$ blocker (yohimbine), and by an inhibitor of adenylyl cyclase (MDL12330), ruling out the involvement of endogenous catecholamines and the cyclic AMP relaxing pathway (data not shown). However, it was inhibited by increasing the concentration of L-NA, by a NO scavenger, carboxyPTIO and by an inhibitor of soluble guanylyl cyclase, ODQ. Thus, the ability of CGJ to stimulate the endothelial formation of NO persists in the presence of 100 μM of L-NA whereas no such effect is observed with a red wine extract [present findings and 16]. The possibility that CGJ is able to compete with L-NA at the eNOS synthase remains to be determined. In addition, relaxations to CGJ were also markedly reduced by ouabain whereas those to bradykinin were not affected. These findings suggest a role for the Na^+, K^+-ATPase in CGJ-induced but not in bradykinin-induced endothelial formation of NO. In addition, previous studies have indicated that red wine extracts, grape juices, grape skin and seed extracts cause endothelium-dependent relaxations in the rat aorta that are solely mediated by NO [14,15,25,26]. Altogether, the present findings indicate that CGJ causes potent endothelium-dependent relaxations involving predominantly NO and also to some extent EDHF in coronary arteries.

Red wine polyphenols-induced NO- and EDHF-mediated relaxations in coronary arteries are both redox-sensitive events involving the intracellular formation of ROS [16,27,28]. Therefore, the role of ROS in the NO-mediated CGJ-induced endothelium-dependent relaxation was assessed. In the presence of indomethacin and charybdotoxin plus apamin, both membrane permeant analogs of either SOD or catalase strongly reduced relaxations to CGJ whereas native SOD and native catalase were inactive indicating a key role of both intracellular superoxide anions and H_2O_2. In addition, PEG-SOD and PEG-catalase also prevented the CGJ-induced phosphorylation of eNOS at Ser1177. Moreover, direct evidence that CGJ is able to cause a modest MnTMPyP-sensitive pro-oxidant response in endothelial cells was obtained with the redox-sensitive fluorescent probe hydroethidine. An increased endothelial formation of superoxide anions has also been observed in response to red wine polyphenols [27]. Although the specific endothelial source of ROS remains to be determined, the redox-sensitive NO- and EDHF-mediated relaxations to red wine polyphenols were not affected by pharmacological inhibitors of mitochondrial function, xanthine oxidase, and cytochrome P450 ruling out these potential sources [16,27]. Moreover, the observation that NO-mediated relaxation to red wine polyphenols was similar in aortas from gp91phox knockout and wild-type mice is not consistent with a role of NADPH oxidase [16]. Potential sources include other types of ROS generating enzymes and the polyphenolic compound itself [29–31].

Numerous lines of evidence indicate that ROS act as key intracellular mediators activating redox-sensitive protein kinases to induce biological responses such as cell growth, survival, and apoptosis [32]. Moreover, the redox-sensitive PI3-kinase/Akt pathway has been shown to mediate the stimulatory effect of red wine polyphenols on both the endothelial formation of NO and EDHF [16,28]. Therefore, the role of the redox-sensitive PI3-kinase/Akt pathway on the endothelial formation of NO to CGJ was studied. The present findings indicate that CGJ caused activation of the PI3-kinase/Akt pathway in a redox-sensitive manner as indicated by the time-dependent phosphorylation of Akt at Tyr418, and its prevention by intracellular scavengers of ROS and by inhibitors of PI3-kinase in endothelial cells. Moreover, the PI3-kinase/Akt pathway mediates activation of eNOS since this response is abolished by wortmannin. The present findings also indicate that Src kinase, a redox-sensitive protein kinase, acts upstream of the PI3-kinase/Akt pathway since the Src kinase inhibitor PP2 prevented CGJ-induced phosphorylation of Akt in endothelial cells, and relaxations to CGJ. It is also consistent with the fact that CGJ caused an early and transient phosphorylation of Src that is followed by a delayed and more sustained phosphorylation of Akt and eNOS. In addition, CGJ-induced phosphorylation of Src was prevented selectively by intracellular scavengers of superoxide anions but not of catalase, whereas both types of scavengers prevented phosphorylation of Akt and eNOS. Altogether, these findings

suggest that the intracellular formation of superoxide anions is an early event, which triggers the cascade of events leading to an enhanced endothelial formation of NO by increasing the phosphorylation of eNOS via the Src/PI3-kinase/Akt pathway. Additionally activation of the PI3-kinase/Akt pathway might also result from the partial conversion of superoxide anions to H_2O_2, a potent activator of the PI3-kinase/Akt pathway [33].

Although polyphenols caused a pro-oxidant response in endothelial cells to activate signal transduction pathways such as the Src/PI3-kinase/Akt pathway, no such effect was observed in other types of vascular cells. Indeed, red wine polyphenols prevented growth factor-induced NADPH oxidase-dependent formation of ROS in vascular smooth muscle, and grape seed and skin extracts the stimulation-induced superoxide release in platelets [34,35]. These protective effects of polyphenols might be explained by their ability to scavenge superoxide anions, peroxyl radicals, hydroxyl radicals, and peroxynitrite [36–38]. Alternatively, the protective effect might also be due to their ability to inhibit the expression and the activity of pro-oxidant enzymes such as NADPH oxidase and xanthine oxidase, and to increase that of antioxidant enzymes such as catalase [39,40]. Altogether these findings suggest that the vasoprotective effect of polyphenols is due to their dual action on vascular cells: a moderate pro-oxidant action in endothelial cells to enhance vasoprotective mechanisms involving NO and EDHF, and an antioxidant action in vascular smooth muscle cells and platelets to keep them in a quiescent state.

In conclusion, the present findings indicate that Concord grape juice is a powerful endothelium-dependent vasodilator of coronary arteries by stimulating the endothelial formation of NO and EDHF. They further indicate that the intracellular formation of ROS in particular superoxide anions, is a crucial and early event leading to activation of eNOS via the Src/PI3-kinase/Akt pathway. Thus, the beneficial effect of CGJ consumption on endothelial and platelet function in patients with coronary heart disease [7,18] might be, in part, due to an enhancement of the formation of vasoprotective endothelial factors.

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