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Redox-Sensitive Up-Regulation of eNOS by Purple Grape Juice in Endothelial Cells: Role of PI3-Kinase/Akt, p38 MAPK, JNK, FoxO1 and FoxO3a

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Abstract

The vascular protective effect of grape-derived polyphenols has been attributable, in part, to their direct action on blood vessels by stimulating the endothelial formation of nitric oxide (NO). The aim of the present study was to determine whether Concord grape juice (CGJ), which contains high levels of polyphenols, stimulates the expression of endothelial NO synthase (eNOS) in porcine coronary artery endothelial cells and, if so, to determine the signaling pathway involved. CGJ dose- and time-dependently increased eNOS mRNA and protein levels and this effect is associated with an increased formation of NO in endothelial cells. The stimulatory effect of CGJ on eNOS mRNA is not associated with an increased eNOS mRNA stability and inhibited by antioxidants such as MnTMPyP, PEG-catalase, and catalase, and by wortmannin (an inhibitor of PI3-kinase), SB 203580 (an inhibitor of p38 MAPK), and SP 600125 (an inhibitor of JNK). Moreover, CGJ induced the formation of reactive oxygen species (ROS) in endothelial cells and this effect is inhibited by MnTMPyP, PEG-catalase, and catalase. The CGJ-induced the phosphorylation of p38 MAPK and JNK kinases is abolished by MnTMPyP. CGJ induced phosphorylation of transcription factors FoxO1 and FoxO3a, which regulate negatively eNOS expression, and this effect is prevented by MnTMPyP, PEG-catalase, wortmannin, SB203580 and SP600125. Moreover, chromatin immunoprecipitation assay indicated that the FoxO3a protein is associated with the eNOS promoter in control cells and that CGJ induced its dissociation. Thus, the present study indicates that CGJ up-regulates the expression of eNOS mRNA and protein leading to an increased formation of NO in endothelial cells. The stimulatory effect of CGJ is a redox-sensitive event involving PI3-kinase/Akt, p38 MAPK and JNK pathways, and the inactivation of the FoxO transcription factors, FoxO1 and FoxO3a, thereby preventing their repression of the eNOS gene.

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Introduction

Several epidemiological studies have suggested that regular intake of polyphenolic rich meals including vegetables, fruits and beverages such as red wine and green tea, is associated with beneficial effects on the cardiovascular system [1,2,3]. The protective effect of polyphenols on the cardiovascular system has been attributable, at least in part, to their ability to prevent oxidation of low-density lipoproteins [4,5], platelet aggregation and adhesion [6,7], and smooth muscle cell migration and proliferation [8,9]. Moreover, vascular protection might also be due to the direct action of polyphenols on blood vessels by stimulating the formation of nitric oxide (NO), which is a potent vasodilator and inhibitor of platelet activation, in endothelial cells [3,10,11,12]. Indeed, red wine polyphenols have been shown to cause the redox-sensitive activation of the PI3-kinase/Akt pathway leading to the phosphorylation of eNOS at Ser 1177 and the formation of NO [10,12].

Grape-derived products such as red wine contain high levels of polyphenols, which are predominantly found in skins, seeds and stems. Besides red wines, grape juices, non-alcoholic beverages, are excellent alternative sources of grape-derived polyphenols. Previous studies have shown that ingestion of purple grape juice has protective effects on the vascular system by improving flow-mediated vasodilatation, platelet function and platelet-dependent inflammatory responses in patients with coronary artery disease [5,13,14], and by reducing blood pressure in moderately hypertensive patients [15]. In addition, consumption of purple grape juice increased serum antioxidant capacity and protected LDL against oxidation in healthy subjects [16]. In addition, we have shown that purple grape juice caused within seconds endothelium-dependent NO-mediated relaxations of coronary artery rings [12]. The signaling pathway leading to eNOS activation in response to grape juice is initiated by the intracellular formation of reactive oxygen species (ROS), in particular

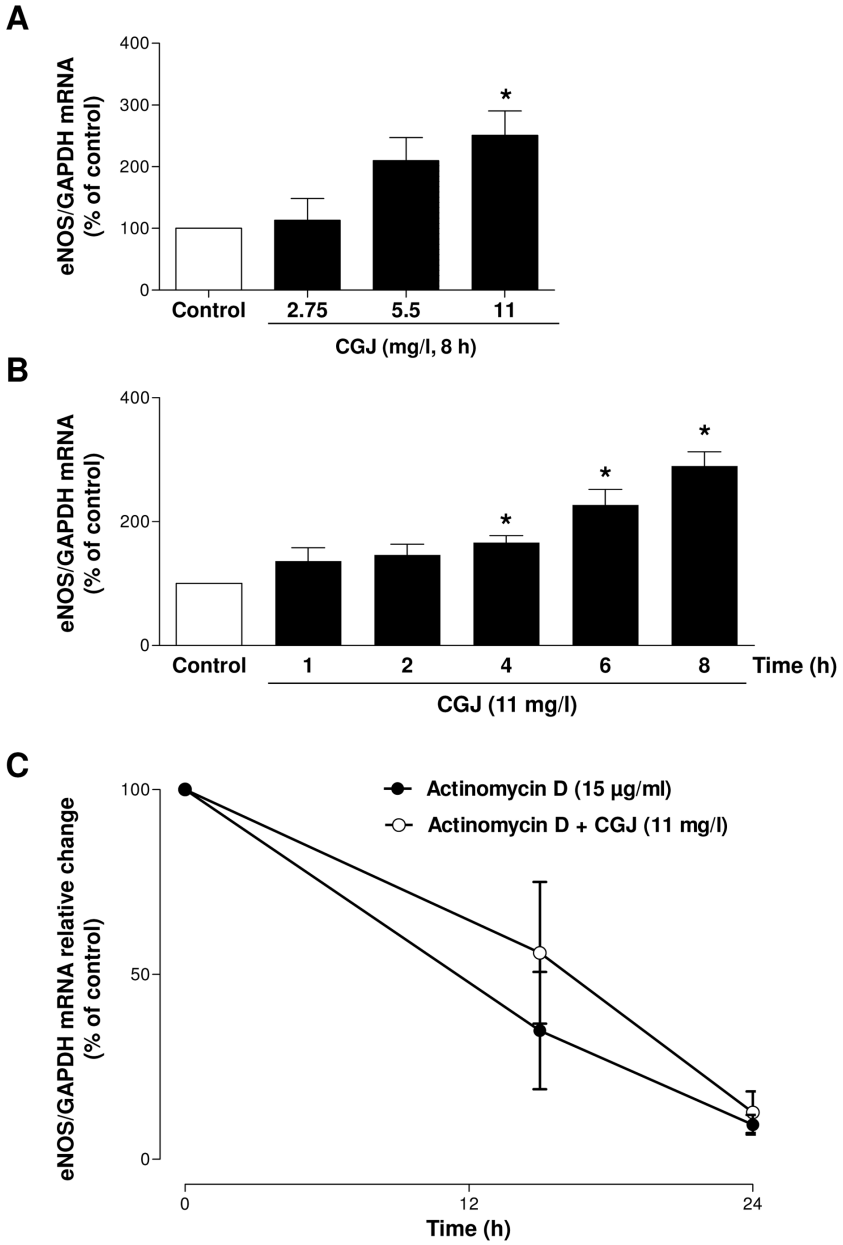

Figure 1. CGJ up-regulates eNOS mRNA expression in coronary artery endothelial cells. Endothelial cells were incubated either with different concentrations of CGJ for 8 hours (A) or CGJ (11 mg/l) in a time-dependent manner until 8 hours (B). Then total mRNA was extracted and transcripted into cDNA. The level of eNOS mRNA was determined using RT-PCR analysis. n = 6 different experiments. CGJ did not affect eNOS mRNA stability (C). Actinomycin (15 µg/mL), an inhibitor of transcription, was added to endothelial cells in the presence or absence of CGJ (11 mg/l). n = 4 different experiments *$P<0.05$ versus control.
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superoxide anions, which activate the Src/PI3-kinase/Akt pathway leading to the phosphorylation of eNOS at Ser 1177 [12].

Besides causing a rapid activation of eNOS, polyphenols might also induce a more sustained formation of NO by up-regulating the expression of eNOS in endothelial cells. Indeed, red wine, resveratrol and an artichoke leaf extracts caused a 2-fold up-regulation of eNOS mRNA and protein levels resulting in an increased formation of NO [17,18,19]. Since previous publications have shown that ROS especially hydrogen peroxide (H_2O_2) are able to induce the expression of eNOS [20,21], the aim of the present study was to determine whether grape juice (CGJ) stimulates the expression of eNOS in coronary artery endothelial cells via a redox-sensitive mechanism and, if so, to determine the signaling pathway involved.

Methods and Materials

Chemicals

Superoxide dismutase (SOD), catalase, polyethyleneglycol-catalase (PEG-catalase), N^{ω}-nitro-L-arginine (L-NA), SP 600125, actinomycin D and dihydroethidine were from Sigma (St. Louis, MO). Wortmannin, PD98059, SB203580 and the SOD mimetic Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) were from Alexis Chemicals and PP2 (4-amino-5-(4-chlorophenyl)-7-(t-

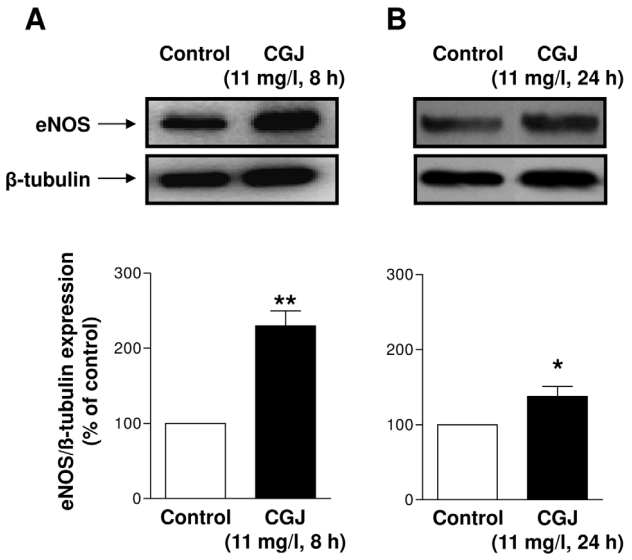

Figure 2. CGJ up-regulates eNOS protein level in endothelial cells. Endothelial cells were stimulated with CGJ for 8 (A) and 24 hours (B) and then the proteins were extracted. The level of eNOS protein was determined by Western blot analysis (top) and by densitometric analysis (bottom). n = 9 different experiments. *$P<0.05$ versus control.
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butyl)pyrazolo[3,4-d]pyrimidine) from Calbiochem. Concord grape juice (CGJ 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).

Culture of Coronary Artery Endothelial Cells

Pig hearts were collected from the local slaughterhouse. Left circumflex coronary arteries were excised, cleaned of loose connective tissue and 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 minutes 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 hours. All experiments were performed with confluent cultures of endothelial 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 hours prior to treatment and subsequent determination of the phosphorylation level of p38 MAPK, JNK, FoxO1 and FoxO3a.

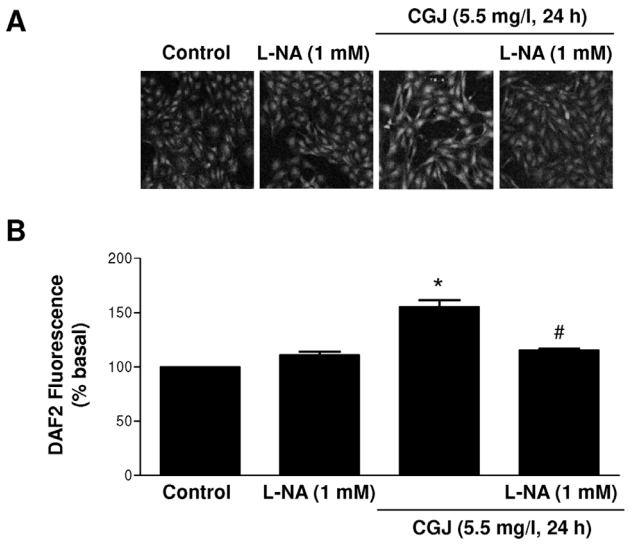

Figure 3. CGJ stimulates the endothelial formation NO. NO formation was assessed using the fluorescent NO-sensitive probe diaminofluorescein-2 diacetate (DAF-2DA) by confocal microscope. n = 3 different experiments. *$P<0.05$ versus control. $^{\#}P<0.05$ versus CGJ treatment.
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In situ Detection of NO

The formation of NO was assessed in endothelial cells using the fluorescent probe 4,5-diaminofluorescein-diacetate (DAF2-DA). They were exposed to serum-free culture medium in the presence of 0.1% bovine serum albumin for 6 hours prior to treatment. Endothelial cells were treated with CGJ (5.5 mg/l) for 24 hours and then they were exposed to DAF2-DA (1 μM) for 20 minutes at 37°C, in the absence or presence of N^{ω}-nitro-L-arginine (L-NA; 1 mM). Thereafter, DAF2 fluorescence was determined by confocal microscope (1024 MRC; Bio-Rad, Hercules, CA) with a 10x epifluorescence objective (Nikon, Tokyo, Japan). After excitation at 488 nm with a Krypton/Argon laser, the emission signal was recorded with a Zeiss 565–610 nm filter. Images were analyzed using Photoshop software.

Determination of the Cellular Formation of ROS

The oxidative fluorescent dye dihydroethidine was used to evaluate the *in situ* formation of ROS. Porcine coronary artery endothelial cells were cultured in Lab-Tek chamber slides (Thermo Fisher Scientific) until they reached 70–80% of confluence. To determine the nature of ROS, cells were incubated either with MnTMPyP (100 μM), SOD (500 U/ml), catalase (500 U/ml), or PEG-catalase (500 U/ml) for 30 minutes at 37°C before the addition of dihydroethidine (10 μM) for 30 minutes. Then, cells were challenged with or without CGJ (11 mg/l) for 30 minutes. Chamber slides were then washed three times with PBS, mounted in Vectashield and cover-slipped. Images were obtained with a Leica SP2 UV DM IRBE laser scanning confocal microscope. Quantification of staining levels was performed using FIJI GPL v2 software.

Western Blot Analysis

After treatment, endothelial 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 (25 μg) were separated on 6–10% SDS-polyacrylamide (Sigma) gels at 100 V for 2 hours. Separated proteins were transferred electrophoretically onto polyvinylidine difluoride membranes (Amersham) at 100 V for 120 minutes. Membranes were blocked with blocking buffer containing 5% bovine serum albumin, Tris-buffered saline solution (Biorad) and 0.1% Tween 20 (Sigma) (TBS-T) for 1 hour. For detection of phosphorylated proteins, membranes were incubated with the respective primary antibody (p–p38 MAPK Thr186/Tyr182, p-JNK Thr183/Tyr185, p-FoxO1 Thr24/FoxO3a Thr32) and for total eNOS, membranes were incubated with a primary antibody directed against eNOS (Cell Signaling Technology; dilution of 1:1,000) overnight at 4°C. Detection of β-tubulin protein was used for normalization and

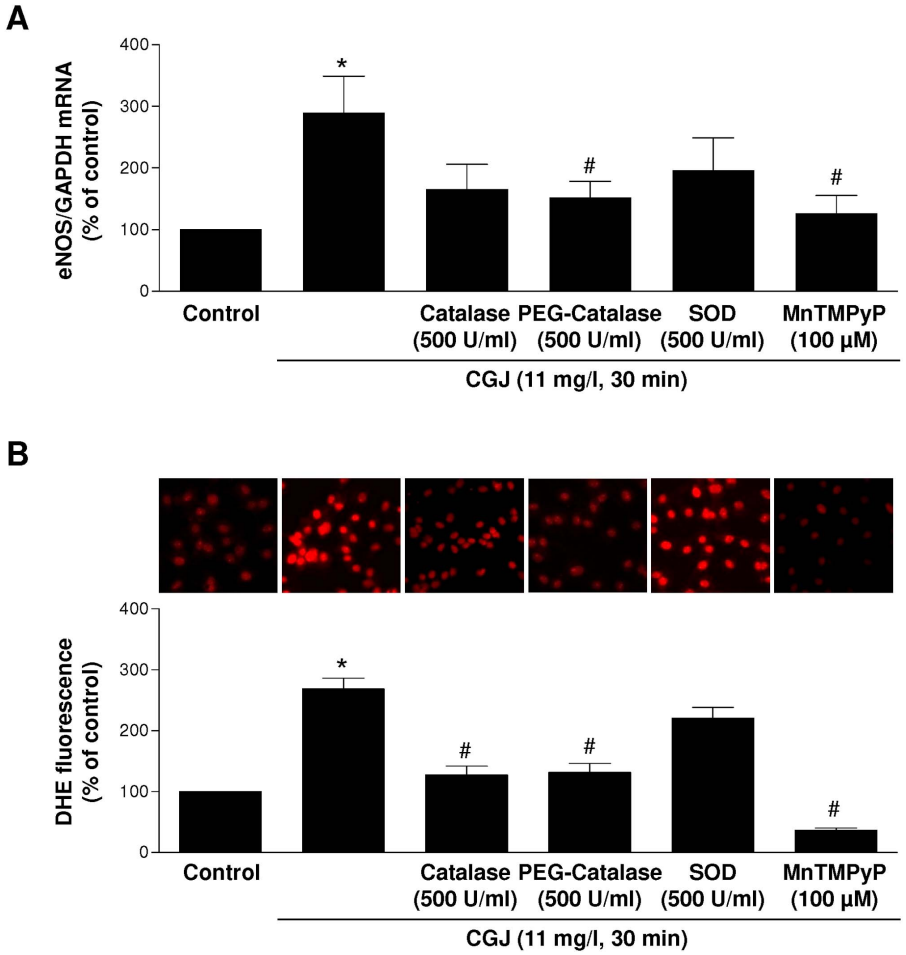

Figure 4. CGJ induces a redox-sensitive up-regulation of eNOS mRNA. Endothelial cells were exposed to either solvent or CGJ for 6 hours at 37°C. Catalase (500 U/ml), PEG-catalase (500 U/ml), SOD (500 U/ml) and MnTMPyP (100 µM), were added to cells 30 minutes before the addition of CGJ and then total mRNA was extracted. RT-PCR analysis was performed to detect the level of mRNA coding for eNOS (A). n = 6 different experiments. CGJ induces the formation of ROS in endothelial cells (B). *$P<0.05$ versus control. #$P<0.05$ versus CGJ treatment.
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quantification. After washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (diluted to 1:20,000 for anti-mouse antibody and 1:5,000 for anti-rabbit antibody, Cell Signaling Technology) at room temperature for 60 minutes. Prestained markers (Invitrogen) were used

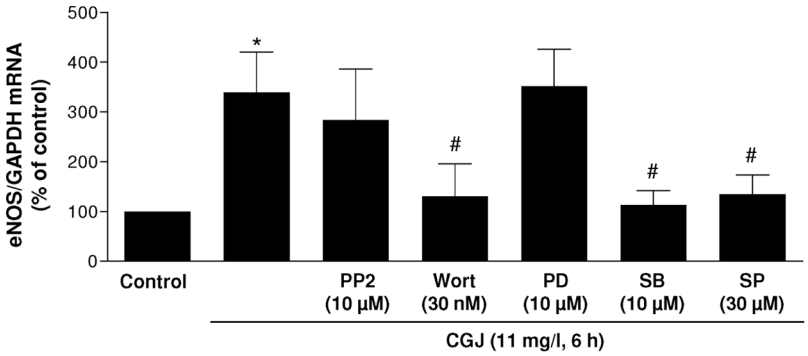

Figure 5. PI3-kinase, p38 MAPK and JNK mediate the redox-sensitive CGJ-increased expression of eNOS. Endothelial cells were exposed to CGJ for 6 hours at 37°C with either solvent, PP2 (10 µM, an inhibitor of Src), wortmannin (30 nM, an inhibitor of PI3K), PD 098059 (10 µM, an inhibitor of ERK 1/2), SB 203580 (10 µM, an inhibitor of p38 MAPK) and SP 600125 (30 µM, an inhibitor of JNK) for 30 minutes before the addition of CGJ and then total mRNA was extracted. RT-PCR analysis was performed to detect the level of mRNA coding for eNOS. n = 5 to 10 different experiments. *$P<0.05$ versus control. #$P<0.05$ versus CGJ treatment.
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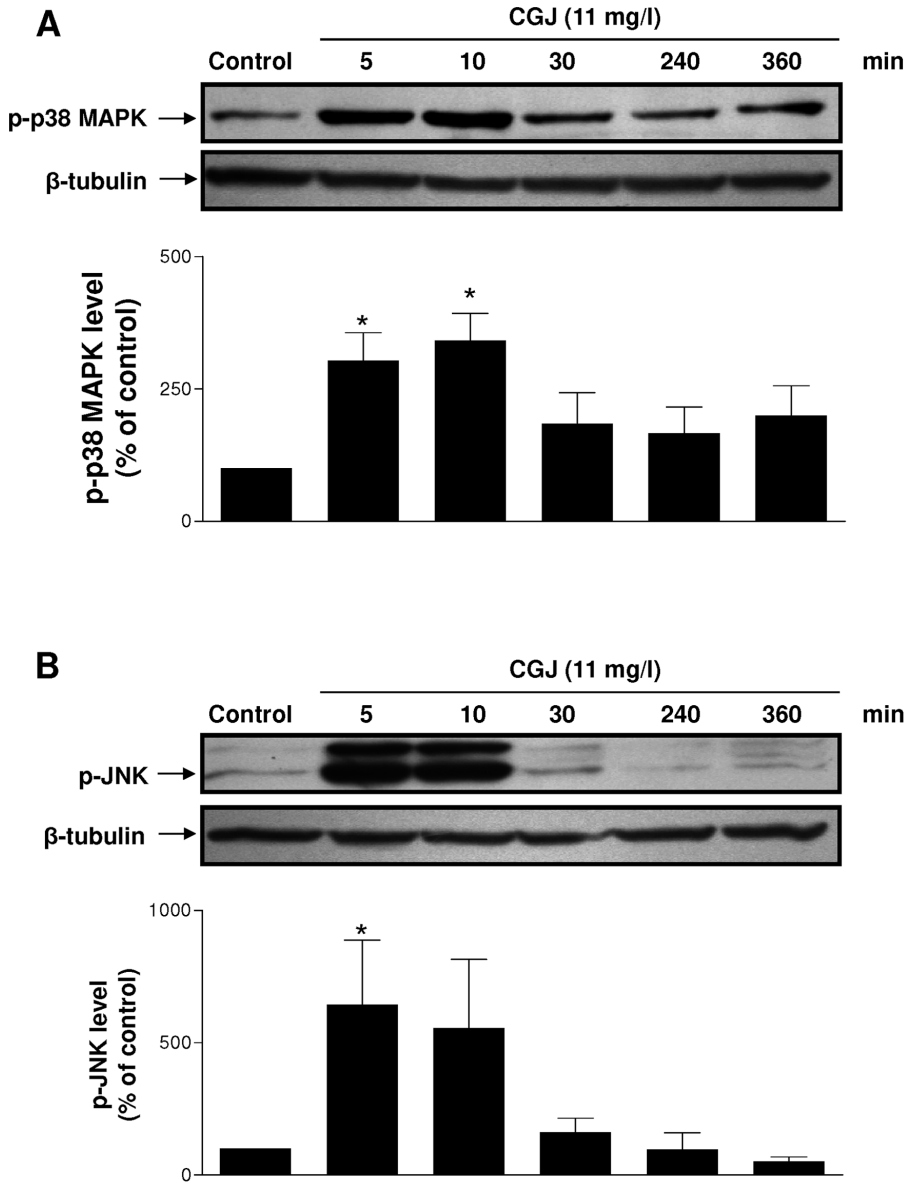

Figure 6. CGJ causes a time-dependent phosphorylation of p38 MAPK at Thr186/Tyr182 and JNK at Thr183/Tyr185 in endothelial cells. Cells were exposed to CGJ for the indicated times at 37°C. Thereafter, the level of p–p38 MAPK (A) and p-JNK (B) was determined by Western blot analysis. Top, representative immunoblots, and bottom, corresponding cumulative data. n = 5 different experiments. * $P<0.05$ versus control.
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for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (Amersham).

Real-Time PCR

Total RNA was isolated from endothelial cells using RNeasy Micro kit (Qiagen, Courtaboeuf, France). cDNA was synthesized from total RNA using iScript cDNA Synthesis kit (Bio-Rad, Marnesla-Coquette, France), and PCR amplification was performed using IQ SYBR Green Supermix (Bio-Rad). The specific primers were as follows: eNOS sense, 5′-AGCGGCTGCATGACATTGAG-3′, eNOS antisense, 5′-GTCGCCGCAGACAAACATGT-3′. The control housekeeping gene was porcine GAPDH. Relative quantitation was determined by standard $2^{(-\Delta\Delta CT)}$ calculations.

Chromatin Immunoprecipitation (ChIP) assay

Coronary artery endothelial cells (approximately 1.5×10^7) were cross-linked for 10 minutes at room temperature by adding 37% formaldehyde to the culture medium. The fixed cells were lysed and the chromatin was isolated using ChIP-IT^{TM} Express Enzymatic (Active Motif, Carlsbad, California, USA). For ChIP, sheared chromatin was incubated with rabbit polyclonal to FoxO3a-ChIP Grade (Abcam, Cambridge, UK). The following steps were performed according to the manufacture's instructions. The isolated precipitated DNA was analysed by PCR amplification of approximately 215-bp fragment of the human eNOS promoter (forward, 5′-CGGAGCAGGTGATAGAAGCTAGG-3′ and reverse, 5′-GCTTCCCTGGAGTCTTGTGTAAGG-3′.

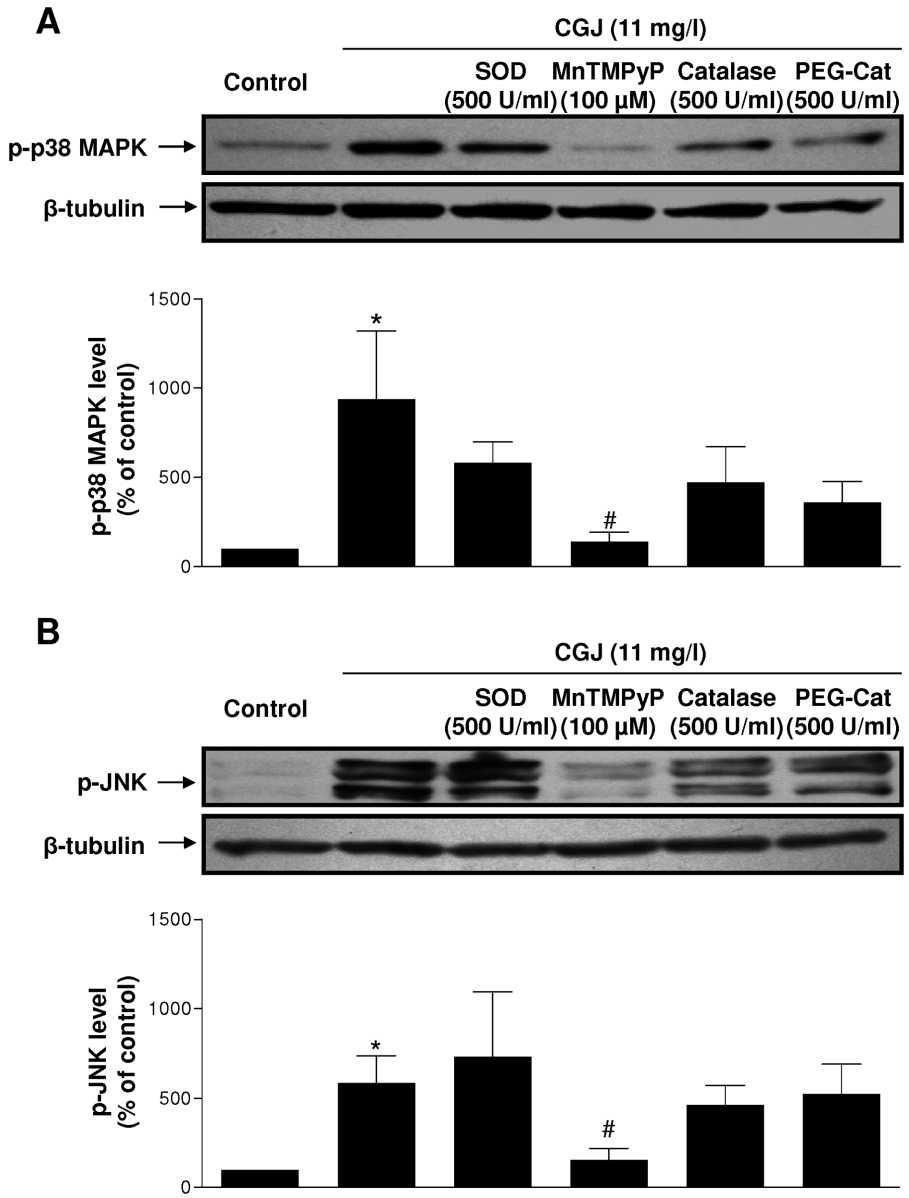

Figure 7. Role of reactive oxygen species in the CGJ-induced phosphorylation of p38 MAPK and JNK in endothelial cells. Cells were incubated either with solvent, SOD (500 U/ml), MnTMPyP (100 μM), catalase (500 U/m) and PEG-catalase (500 U/ml) for 30 minutes before the addition of CGJ for 5 minutes. The level of p–p38 MAPK (A) and p-JNK (B) was determined by Western blot analysis. Top, representative immunoblots, and bottom, corresponding cumulative data. n = 5 to 6 different experiments. * $P<0.05$ versus control. # $P<0.05$ versus CGJ treatment.
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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.

Results

Concord grape juice up-regulates eNOS mRNA level in endothelial cells

To determine the effect of long-term treatment of endothelial cells with CGJ, eNOS mRNA levels were determined as a function of concentration and time using RT-PCR As shown in Fig. 1A, treating endothelial cells with CGJ for 8 hours induced a concentration-dependent increase of the eNOS mRNA level, which reached significance at 11 mg/l. Thereafter, this concentration was used to study the time course of the stimulatory effect of CGJ on eNOS mRNA level (Fig. 1B) CGJ induced a time-dependent increase of the eNOS mRNA level, which reached significance at 4 hours and, thereafter, it increased steadily up to, at least, 8 hours. To determine whether this effect is due to an increased stability of eNOS mRNA, cells were exposed to actinomycin D, an inhibitor of transcription, in the absence and presence of CGJ for 15 and 24 hours. CGJ did not affect the time-dependent decrease of eNOS mRNA indicating that the stimulatory effect of CGJ is not due to an increased stability of eNOS mRNA (Fig. 1C).

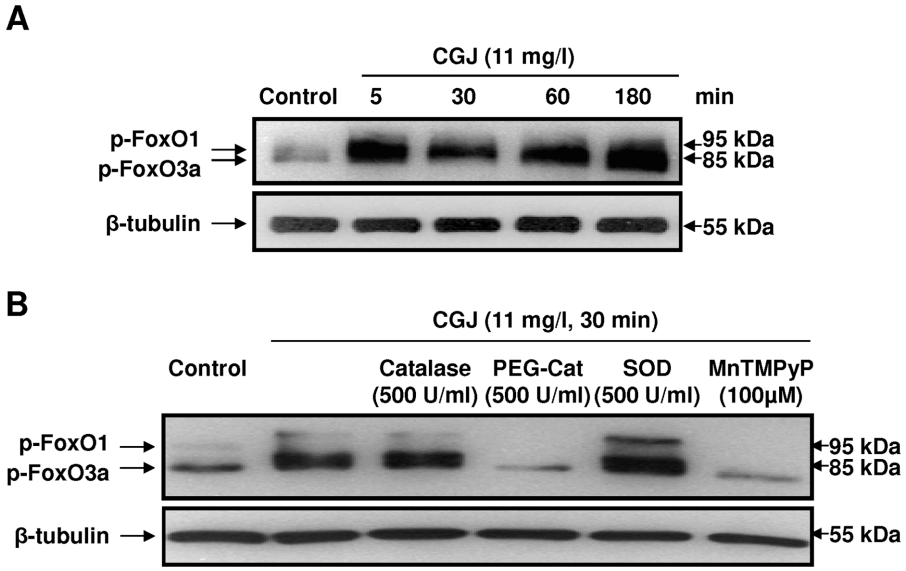

Figure 8. CGJ induces a redox-sensitive phosphorylation of FoxO1 and FoxO3a. Endothelial cells were exposed to CGJ for the indicated times at 37°C. The level of p-FoxO1 and p-FoxO3a was determined by Western blot analysis (A). Role of ROS in the CGJ-induced phosphorylation of FoxO1 and FoxO3a in endothelial cells (B). Cells were incubated either with solvent, SOD (500 U/ml), MnTMPyP (100 μM), catalase (500 U/ml) or PEG-catalase (500 U/ml) for 30 minutes before the addition of CGJ for 30 minutes.
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Concord grape juice up-regulates eNOS protein leading to an enhanced formation of NO in endothelial cells

Next, Western blot analysis was performed to verify that the increased eNOS mRNA level induced by CGJ leads to an increased eNOS protein level. After an 8-hour treatment period, CGJ (11 mg/l) significantly increased the eNOS protein level compared to control cells, and this effect persisted up to 24 hours (Fig. 2). In order to determine that the CGJ-induced expression of eNOS is associated with an enhanced formation of NO, endothelial cells were exposed to a fluorescent probe known to detect NO, DAF2-DA. As shown in Fig. 3, the fluorescence signal was significantly higher after a 24-hour treatment period of endothelial cells with CGJ. Pre-treatment of endothelial cells with the competitive inhibitor of eNOS, L-NA, prevented the stimulatory effect of CGJ (Fig. 3). These data indicate that CGJ increased the eNOS-derived NO formation in endothelial cells.

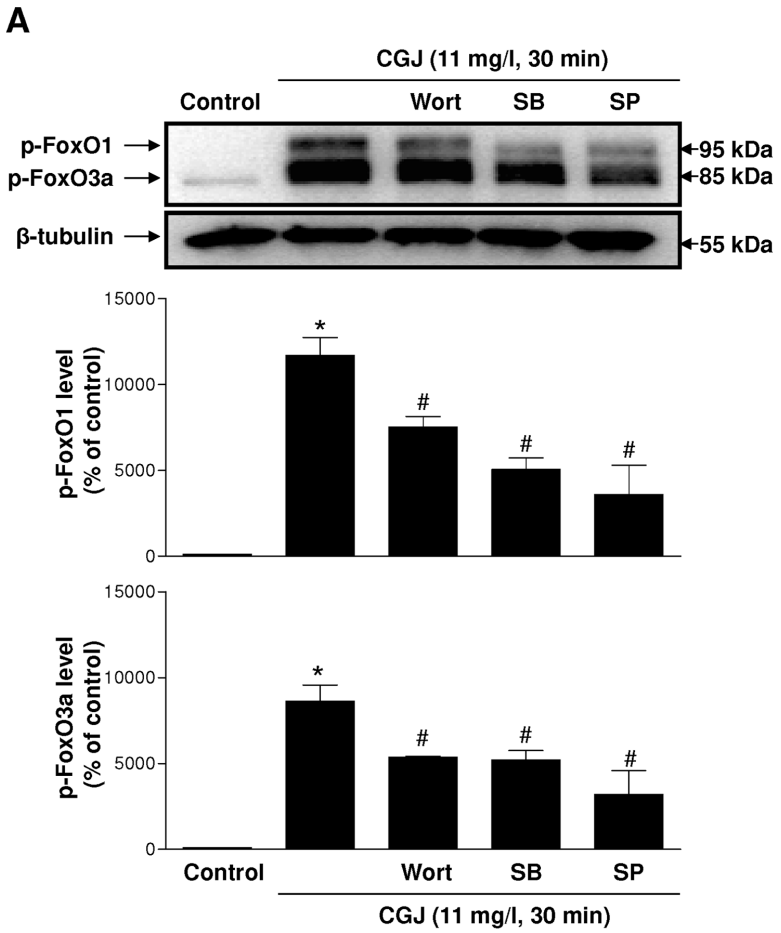
CGJ induces a redox-sensitive expression of eNOS mRNA in endothelial cells

Previous studies have shown that ROS are able to stimulate the expression of eNOS in endothelial cells [20,21]. Moreover, we have previously shown that CGJ induces the formation of ROS in coronary artery endothelial cells leading acutely to eNOS activation [12]. Therefore, we performed experiments to determine the role of ROS in the up-regulation of eNOS induced by CGJ. Modulators of ROS strongly inhibited the expression of eNOS induced by CGJ. Indeed as shown in Fig. 4A, membrane-permeant analogs of either SOD (MnTMPyP) or catalase (PEG-catalase) significantly prevented the increased eNOS mRNA level induced by CGJ. Although native SOD and catalase reduced CGJ-induced eNOS expression, this effect did not reach statistical significance (Fig. 4A). In addition, MnTMPyP or PEG-catalase alone affected little eNOS mRNA levels in control endothelial cells (eNOS mRNA level were 104.9±14.1% and 107.3±18.5% compared to control cells, n = 3). Thus, these findings indicate a major role of intracellular ROS and especially superoxide anions and H_2O_2 in the signaling pathway leading to the expression of eNOS in response to CGJ.

Direct evidence that CGJ stimulates the formation of ROS in endothelial cells was obtained using the redox-sensitive probe DHE (Fig. 4B). CGJ increased about two-fold the DHE fluorescence signal, and this effect was abolished by MnTMPyP, and significantly reduced by catalase and PEG-catalase, and not affected by SOD (Fig. 4B). Since ROS are well-known to activate redox-sensitive kinases in endothelial cells to induce biological responses such as cell growth, survival and apoptosis [22], experiments were performed to determine the role of Src-kinase using PP2, PI3-kinase using wortmannin, ERK1/2 (Extracellular signal-regulated kinase 1 and 2) using PD 098059, p38 MAPK using SB 203580, and JNK using SP 600125. As shown in Fig. 5, wortmannin, SB 203580 and SP 600125 significantly prevented the CGJ-induced expression of eNOS mRNA whereas PP2 and PD 098059 were without effect. In addition, the inhibitors alone affected little the basal eNOS mRNA expression level in endothelial cells (values were 94.2±10.2% and 85.0±2.9% for wortmannin and SP 600125, respectively, n = 3). Thus, these findings suggest a key role of PI3 kinase, p38 MAPK and JNK in the signal transduction pathway leading to eNOS expression in response to CGJ.

CGJ causes the redox-sensitive activation of p38 MAPK and JNK

Unstimulated endothelial cells had either no or only a low level of p–p38 MAPK and p-JNK (Fig. 6 A,B). CGJ increased within 5 minutes signals of p–p38 MAPK and p-JNK, which reached a peak value within 5 to 10 minutes and then returned to baseline at 30 minutes. CGJ-induced phophorylation of p38 MAPK and JNK was abolished by MnTMPyP and not significantly reduced by native SOD, PEG-catalase and native catalase (Fig. 7 A,B). These data indicate that ROS, especially superoxide anions, act as intracellular upstream mediators of p38 MAPK and JNK leading to eNOS expression in response to CGJ.



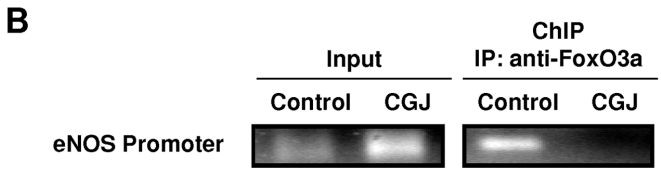


Figure 9. PI3-kinase, p38 MAPK and JNK mediate the redox-sensitive CGJ-induced phosphorylation of FoxO1 and FoxO3a leading to the FoxO3a dissociation from the eNOS gene promoter. Endothelial cells were incubated with wortmannin (30 nM), SB 203580 (10 μM) and SP 600125 (30 μM) for 30 minutes before the addition of CGJ for 30 minutes. The level of p-FoxO1 and p-FoxO3a protein (A) was determined by Western blot analysis (top) and by densitometric analysis (bottom). n = 4 different experiments. $^{*}P<0.05$ versus control, $^{\#}P<0.05$ versus CGJ treatment. Endothelial cells were exposed to CGJ for 30 minutes and then chromatin-bound DNA was immunoprecipitated with an antibody against FoxO3a (B). Immunoprecipitated DNA was analyzed by PCR using primers to amplify a fragment of the human eNOS promoter as mentioned in methods and materials. n = 2 different experiments.
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CGJ induced the inactivation of FoxO1 and FoxO3a

It has been shown that activation of the PI3-kinase pathway leads to an Akt-dependent inactivation of FoxO transcription factors resulting in a reduced DNA binding such as to the eNOS promoter [23,24,25]. To determine whether CGJ inactivates FoxO transcription factors, we studied the effect of CGJ on the phosphorylation of FoxO1 and FoxO3a. Exposure the endothelial cells to CGJ induced phosphorylation of the transcription factors FoxO1 and FoxO3a at 5 minutes and this effect persisted at least until three hours (Fig. 8A). Both MnTMPyP and PEG-catalase prevented the phosphorylation of FoxO1 and FoxO3a induced by CGJ (Fig. 8B) whereas native SOD and catalase had only minor effects (Fig. 8B). In addition, the CGJ-induced phosphorylation of FoxO1 and FoxO3a was significantly prevented by wortmannin, SB 203580 and SP 600125 (Fig. 9A). Chromatin immunoprecipitation (ChIP) assay showed that FoxO3a binds to the eNOS promoter and that CGJ targeted this interaction leading to FoxO3a dissociation from the eNOS promoter (Fig. 9B). Thus, these findings indicate an important role of intracellular ROS, p38 MAPK, JNK and PI3-kinase in the signal transduction pathway leading to phosphorylation of FoxO1 and FoxO3a in response to CGJ. Moreover they further support a key role of FoxO3a phosphorylation in the CGJ-induced eNOS gene activation.

Discussion

The present findings indicate that CGJ stimulates the expression of eNOS both at the mRNA and protein level and that this effect is associated with a sustained formation of NO in endothelial cells. They further indicate that the stimulatory effect of CGJ on eNOS expression is initiated by a moderate pro-oxidant event involving superoxide anions and hydrogen peroxide, which regulate eNOS expression through activation of several kinases including PI-3-kinase, p38 MAPK and JNK. The PI3-kinase, p38 MAPK and the JNK pathways are involved in the phosphorylation of the transcription factors FoxO1 and FoxO3a thereby reducing their repressor effect on the expression of the eNOS gene.

Chronic intake of grape-derived polyphenols has been shown to induce vasoprotective effects in both humans and animals. Indeed, intake of wine, red wine without alcohol and purple grape juice improved flow-mediated vasodilatation of the brachial artery in healthy subjects and in subjects with coronary artery diseases [5,26,27]. Chronic intake of grape-derived polyphenols also prevented hypertension and improved endothelial dysfunction in several experimental models of hypertension [28,29,30]. The beneficial effects of grape-derived polyphenols involve, at least in part, their ability to enhance acutely the endothelial formation of NO and endothelium-derived hyperpolarizing factor, two major vasoprotecting factors [31,32,33,34]. The grape-derived polyphenols-induced endothelial formation of NO is mediated by the phosphorylation of Ser 1177 of eNOS via the Src kinase/PI3-kinase/Akt pathway [12,31]. Surprisingly, reactive oxygen species including superoxide anions and hydrogen peroxide act as upstream mediators of the Src kinase/PI3-kinase/Akt pathway [12,31,35]. Although the endothelial source of reactive oxygen species remains to be determined, the redox-sensitive NO- and EDHF (endothelium-derived hyperpolarizing factor)-mediated relaxations to grape-derived polyphenols are not affected by pharmacological inhibitors of the mitochondrial respiration chain, xanthine oxidase, and cytochromes P450, and NO-mediated relaxations persisted in NADPH oxidase gp91phox knockout mice ruling out these potential sources [10,36]. Alternatively, the polyphenolic structure itself might provide the oxidative activator signal since the structure can undergo redox cycling leading to the formation of superoxide anions [37]. Besides grape-derived polyphenols, a redox-sensitive pathway involving the Src kinase/PI3-kinase/Akt pathway mediates also activation of eNOS in response to the major green tea polyphenol epigallocatechin-3-gallate [35,38].

In addition to acutely stimulating the endothelial formation of NO, the vasoprotective effect of grape-derived polyphenols may also involve their ability to increase the expression level of eNOS mRNA and protein with a subsequent sustained formation of NO [17,18,19,39,40]. The present findings indicate that the CGJ induced an up-regulation of eNOS mRNA already after 4 hours and that this effect results in a 1.5-fold increased formation of NO after a 24 hour-incubation period. The stimulatory effect of CGJ is not due to an increased stability of eNOS mRNA.

Previous studies have indicated that red wine and resveratrol increased the activity of the eNOS promoter and also stabilized to some extent eNOS mRNA [18,19,39]. Altogether, these findings indicate that grape-derived polyphenols-induced up-regulation of eNOS expression involves transcriptional and possibly also post-transcriptional mechanisms. The present investigations further indicate that the stimulatory effect of grape-derived polyphenols on eNOS expression is critically dependent on a redox-sensitive event. Indeed, the CGJ-induced expression of eNOS mRNA is associated with the formation of cellular ROS and it is markedly reduced by membrane permeant analogs of superoxide dismutase and catalase indicating a key role of intracellular superoxide anions and hydrogen peroxide. Moreover, direct evidence that grape-derived polyphenols stimulate the intracellular formation of superoxide anions and hydrogen peroxide has also been obtained previously in both native and cultured endothelial cells but not in the vascular smooth muscle using redox-sensitive fluorescent probes [36,41]. Previous studies have also shown that hydrogen peroxide caused a time- and concentration-dependent up-regulation of eNOS in endothelial cells through both transcriptional and post-transcriptional mechanisms [21]. Altogether, the present findings in conjunction with those previous ones indicate that both the acute activation of eNOS and its subsequent increased expression in response to grape-derived polyphenols are controlled by a pro-oxidant event in endothelial cells involving intracellular superoxide anions and hydrogen peroxide. A role for intracellular hydrogen peroxide has also been suggested in oscillatory shear stress-induced up-regulation of eNOS mRNA and NO formation in endothelial cells since both of these responses were markedly reduced by PEG-catalase [42].

The fact that reactive oxygen species act as important endogenous signaling molecules modulating gene expression in endothelial cells through activation of redox-sensitive intracellular targets such as protein kinases including Src kinase, PI3-kinase, ERK1/2, p38 MAPK and JNK prompted experiments to determine their role in the CGJ-induced expression of eNOS [21,43,44,45]. The present findings indicate that the stimulatory effect of CGJ is abolished by a selective inhibitor of either PI3-kinase, p38 MAPK or JNK whereas inhibition of Src kinase or ERK1/2 was without effect. Moreover, CGJ induced within 5 minutes the phosphorylation of p38 MAPK and JNK, both of these responses were transient and returned to baseline within 30 minutes. Intracellular superoxide anions have an important role in the CGJ-induced phosphorylation of p38 MAPK and JNK since both responses are abolished by MnTMPyP and not significantly affected by native superoxide dismutase, catalase and PEG-catalase. In addition, we have previously shown that CGJ also causes within minutes the PI3-kinase-dependent phosphorylation of Akt and that this effect is dependent on intracellular superoxide anions and hydrogen peroxide [12]. Thus, CGJ induces expression of eNOS in endothelial cells resulting in a sustained formation of NO through the redox-sensitive activation of several intracellular signaling pathways involving PI3-kinase/Akt, p38 MAPK and JNK.

Previous studies have indicated that the eNOS promoter region contains putative binding sites for redox-sensitive transcription factors, including FoxO1 and FoxO3a, activator protein-1 (AP-1), Sp1, and antioxidant-responsive elements [23,46,47,48]. Indeed, FoxO1 and FoxO3a have been shown to bind to the eNOS gene promoter and to repress eNOS expression [23]. The present findings indicate that the FoxO3a protein is associated with the eNOS promoter in control endothelial cells and that CGJ induced its dissociation most likely following the phosphorylation of FoxO3a leading to its exclusion from the nucleus into the cytoplasm. Thus, the CGJ-induced phosphorylation of FoxO1 and FoxO3a appears to be an important event leading to eNOS expression.

In addition, recent findings suggest that the stimulatory effect of grape-derived polyphenols on eNOS expression is also observed *in vivo* since intake of red wine polyphenols in the drinking water (150 mg/kg/day) during 3 weeks is associated with a significant 1.6-fold up-regulation of the eNOS protein level in the rat aorta [49].

In conclusion, the present findings indicate that CGJ caused an up-regulation of eNOS resulting in a sustained formation of NO and that this effect is critically dependent on the intracellular formation of superoxide anions and hydrogen peroxide. They further indicate that the stimulatory effect on eNOS expression involves several redox-sensitive kinases including PI3-kinase, p38 MAPK, JNK and the transcription factors FoxO1 and FoxO3a. Thus, the dual ability of grape-derived polyphenols to acutely enhance the endothelial formation of NO by changing the phosphorylation level of eNOS and to cause a more sustained endothelial formation of NO following up-regulation of the eNOS protein might contribute to explain its protective effect on the vascular system.

Author Contributions

Conceived and designed the experiments: MA EA VBS. Performed the experiments: MA EA SR JHK. Analyzed the data: SR SVFM CB. Wrote the paper: MA EA VBS.

References

1. Keli SO, Hertog MG, Feskens EJ, Kromhout D (1996) Dietary flavonoids, antioxidant vitamins, and incidence of stroke: the Zutphen study. Arch Intern Med 156: 637–642.
2. Renaud S, de Lorgeril M (1992) Wine, alcohol, platelets, and the French paradox for coronary heart disease. Lancet 339: 1523–1526.
3. Stoclet JC, Chataigneau T, Ndiaye M, Oak MH, El Bedoui J, et al. (2004) Vascular protection by dietary polyphenols. Eur J Pharmacol 500: 299–313.
4. Frankel EN, Kanner J, German JB, Parks E, Kinsella JE (1993) Inhibition of oxidation of human low-density lipoprotein by phenolic substances in red wine. Lancet 341: 454–457.
5. Stein JH, Keevil JG, Wiebe DA, Aeschlimann S, Folts JD (1999) Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. Circulation 100: 1050–1055.
6. Freedman JE, Parker C 3rd, Li L, Perlman JA, Frei B, et al. (2001) Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. Circulation 103: 2792–2798.
7. Wollny T, Aiello L, Di Tommaso D, Bellavia V, Rotilio D, et al. (1999) Modulation of haemostatic function and prevention of experimental thrombosis by red wine in rats: a role for increased nitric oxide production. Br J Pharmacol 127: 747–755.
8. Iijima K, Yoshizumi M, Hashimoto M, Kim S, Eto M, et al. (2000) Red wine polyphenols inhibit proliferation of vascular smooth muscle cells and down-regulate expression of cyclin A gene. Circulation 101: 805–811.
9. Iijima K, Yoshizumi M, Hashimoto M, Akishita M, Kozaki K, et al. (2002) Red wine polyphenols inhibit vascular smooth muscle cell migration through two distinct signaling pathways. Circulation 105: 2404–2410.
10. Ndiaye M, Chataigneau M, Lobysheva I, Chataigneau T, Schini-Kerth VB (2005) Red wine polyphenol-induced, endothelium-dependent NO-mediated relaxation is due to the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of endothelial NO-synthase in the isolated porcine coronary artery. Faseb J 19: 455–457.
11. Mombouli JV, Vanhoutte PM (1999) Endothelial dysfunction: from physiology to therapy. J Mol Cell Cardiol 31: 61–74.
12. Anselm E, Chataigneau M, Ndiaye M, Chataigneau T, Schini-Kerth VB (2007) Grape juice causes endothelium-dependent relaxation via a redox-sensitive Src- and Akt-dependent activation of eNOS. Cardiovasc Res 73: 404–413.
13. Chou EJ, Keevil JG, Aeschlimann S, Wiebe DA, Folts JD, et al. (2001) Effect of ingestion of purple grape juice on endothelial function in patients with coronary heart disease. Am J Cardiol 88: 553–555.
14. Albers AR, Varghese S, Vitseva O, Vita JA, Freedman JE (2004) The antiinflammatory effects of purple grape juice consumption in subjects with stable coronary artery disease. Arterioscler Thromb Vasc Biol 24: e179–180.
15. Park YK, Kim JS, Kang MH (2004) Concord grape juice supplementation reduces blood pressure in Korean hypertensive men: double-blind, placebo controlled intervention trial. Biofactors 22: 145–147.
16. O'Byrne DJ, Devaraj S, Grundy SM, Jialal I (2002) Comparison of the antioxidant effects of Concord grape juice flavonoids alpha-tocopherol on markers of oxidative stress in healthy adults. Am J Clin Nutr 76: 1367–1374.
17. Li H, Xia N, Brausch I, Yao Y, Forstermann U (2004) Flavonoids from artichoke (Cynara scolymus L.) up-regulate endothelial-type nitric-oxide synthase gene expression in human endothelial cells. J Pharmacol Exp Ther 310: 926–932.
18. Leikert JF, Rathel TR, Wohlfart P, Cheynier V, Vollmar AM, et al. (2002) Red wine polyphenols enhance endothelial nitric oxide synthase expression and subsequent nitric oxide release from endothelial cells. Circulation 106: 1614–1617.
19. Wallerath T, Deckert G, Ternes T, Anderson H, Li H, et al. (2002) Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. Circulation 106: 1652–1658.
20. Cai H, Davis ME, Drummond GR, Harrison DG (2001) Induction of endothelial NO synthase by hydrogen peroxide via a Ca(2+)/calmodulin-dependent protein kinase II/janus kinase 2-dependent pathway. Arterioscler Thromb Vasc Biol 21: 1571–1576.
21. Drummond GR, Cai H, Davis ME, Ramasamy S, Harrison DG (2000) Transcriptional and posttranscriptional regulation of endothelial nitric oxide synthase expression by hydrogen peroxide. Circ Res 86: 347–354.
22. Irani K (2000) Oxidant signaling in vascular cell growth, death, and survival: a review of the roles of reactive oxygen species in smooth muscle and endothelial cell mitogenic and apoptotic signaling. Circ Res 87: 179–183.
23. Potente M, Urbich C, Sasaki K, Hofmann WK, Heeschen C, et al. (2005) Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J Clin Invest 115: 2382–2392.
24. Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, et al. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96: 857–868.
25. Kops GJ, de Ruiter ND, De Vries-Smits AM, Powell DR, Bos JL, et al. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B. Nature 398: 630–634.
26. Whelan AP, Sutherland WH, McCormick MP, Yeoman DJ, de Jong SA, et al. (2004) Effects of white and red wine on endothelial function in subjects with coronary artery disease. Intern Med J 34: 224–228.
27. Hashimoto M, Kim S, Eto M, Iijima K, Ako J, et al. (2001) Effect of acute intake of red wine on flow-mediated vasodilatation of the brachial artery. Am J Cardiol 88: 1457–1460, A1459.
28. Sarr M, Chataigneau M, Martins S, Schott C, El Bedoui J, et al. (2006) Red wine polyphenols prevent angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase. Cardiovasc Res 71: 794–802.
29. Soares De Moura R, Costa Viana FS, Souza MA, Kovary K, Guedes DC, et al. (2002) Antihypertensive, vasodilator and antioxidant effects of a vinifera grape skin extract. J Pharm Pharmacol 54: 1515–1520.
30. Diebolt M, Bucher B, Andriantsitohaina R (2001) Wine polyphenols decrease blood pressure, improve NO vasodilatation, and induce gene expression. Hypertension 38: 159–165.
31. Ndiaye M, Chataigneau T, Chataigneau M, Schini-Kerth VB (2004) Red wine polyphenols induce EDHF-mediated relaxations in porcine coronary arteries through the redox-sensitive activation of the PI3-kinase/Akt pathway. Br J Pharmacol 142: 1131–1136.
32. Andriambeloson E, Kleschyov AL, Muller B, Beretz A, Stoclet JC, et al. (1997) Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. Br J Pharmacol 120: 1053–1058.
33. Fitzpatrick DF, Fleming RC, Bing B, Maggi DA, O'Malley RM (2000) Isolation and characterization of endothelium-dependent vasorelaxing compounds from grape seeds. J Agric Food Chem 48: 6384–6390.
34. Fitzpatrick DF, Hirschfield SL, Coffey RG (1993) Endothelium-dependent vasorelaxing activity of wine and other grape products. Am J Physiol 265: H774–778.
35. Kim JA, Formoso G, Li Y, Potenza MA, Marasciulo FL, et al. (2007) Epigallocatechin gallate, a green tea polyphenol, mediates NO-dependent vasodilation using signaling pathways in vascular endothelium requiring reactive oxygen species and Fyn. J Biol Chem 282: 13736–13745.
36. Ndiaye M, Chataigneau T, Andriantsitohaina R, Stoclet JC, Schini-Kerth VB (2003) Red wine polyphenols cause endothelium-dependent EDHF-mediated relaxations in porcine coronary arteries via a redox-sensitive mechanism. Biochem Biophys Res Commun 310: 371–377.
37. Akagawa M, Shigemitsu T, Suyama K (2003) Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. Biosci Biotechnol Biochem 67: 2632–2640.
38. Lorenz M, Wessler S, Follmann E, Michaelis W, Dusterhoft T, et al. (2004) A constituent of green tea, epigallocatechin-3-gallate, activates endothelial nitric oxide synthase by a phosphatidylinositol-3-OH-kinase-, cAMP-dependent protein kinase-, and Akt-dependent pathway and leads to endothelial-dependent vasorelaxation. J Biol Chem 279: 6190–6195.
39. Wallerath T, Poleo D, Li H, Forstermann U (2003) Red wine increases the expression of human endothelial nitric oxide synthase: a mechanism that may contribute to its beneficial cardiovascular effects. J Am Coll Cardiol 41: 471–478.
40. Wallerath T, Li H, Godtel-Ambrust U, Schwarz PM, Forstermann U (2005) A blend of polyphenolic compounds explains the stimulatory effect of red wine on human endothelial NO synthase. Nitric Oxide 12: 97–104.
41. Madeira SVF, Anselm E, Chataigneau M, Chataigneau T, Soares de Moura R, et al. (2008) Grape skin polyphenols-induced eNOS activation in porcine coronary arteries. J Vasc Res. In press.

42. Cai H, McNally JS, Weber M, Harrison DG (2004) Oscillatory shear stress upregulation of endothelial nitric oxide synthase requires intracellular hydrogen peroxide and CaMKII. J Mol Cell Cardiol 37: 121–125.
43. Ullrich V, Bachschmid M (2000) Superoxide as a messenger of endothelial function. Biochem Biophys Res Commun 278: 1–8.
44. Wolin MS (2000) Interactions of oxidants with vascular signaling systems. Arterioscler Thromb Vasc Biol 20: 1430–1442.
45. Finkel T (1999) Signal transduction by reactive oxygen species in non-phagocytic cells. J Leukoc Biol 65: 337–340.
46. Robinson GL, Cordle SR, Henderson E, Weil PA, Teitelman G, et al. (1994) Isolation and characterization of a novel transcription factor that binds to and activates insulin control element-mediated expression. Mol Cell Biol 14: 6704–6714.
47. Venema RC, Nishida K, Alexander RW, Harrison DG, Murphy TJ (1994) Organization of the bovine gene encoding the endothelial nitric oxide synthase. Biochim Biophys Acta 1218: 413–420.
48. Teichert AM, Karantzoulis-Fegaras F, Wang Y, Mawji IA, Bei X, et al. (1998) Characterization of the murine endothelial nitric oxide synthase promoter. Biochim Biophys Acta 1443: 352–357.
49. Walter A, Etienne-Selloum N, Sarr M, Kane MO, Beretz A, et al. (2008) Angiotensin II Induces the Vascular Expression of VEGF and MMP-2 in vivo: Preventive Effect of Red Wine Polyphenols. J Vasc Res 45: 386–394.