Published in final edited form as:

Free Radic Biol Med. 2010 August 1; 49(3): 339–347. doi:10.1016/j.freeradbiomed.2010.04.022.

DIETARY FLAVONOID QUERCETIN STIMULATES VASORELAXATION IN AORTIC VESSELS

Nicholas K.H. Khoo^{1,9,*}, C. Roger White^{2,7}, Lucas Pozzo-Miller³, Fen Zhou¹, Chad Constance¹, Takafumi Inoue⁸, Rakesh P. Patel^{1,5}, and Dale A. Parks^{1,4,6,7}

¹ Department of Anesthesiology, University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA

² Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA

³ Department of Neurobiology, University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA

⁴ Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA

⁵ Department of Pathology, University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA

⁶ Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA

⁷ Department of Physiology & Biophysics, University of Alabama at Birmingham, Birmingham, AL 35294-2172, USA

⁸ Department of Life Science and Medical Bioscience, Faculty of Science and Engineering, Waseda University, Tokyo 162-8480, Japan

⁹ Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh, Pennsylvania, 15261, USA

Abstract

Considerable epidemiological evidence indicates that dietary consumption of moderate levels of polyphenols decreases both the incidence of cardiovascular disease and the mortality associated with myocardial infarction. Molecular mechanisms of this cardiovascular protection remain uncertain but can involve changes in rates of nitric oxide (NO) generation by endothelial nitric oxide synthase (eNOS). We examined the vascular responses to quercetin using a combination of biochemical and vessel function criteria. Quercetin treatment for 30 min enhanced relaxation of rat aortic ring segments. Moreover, the addition of L-NAME (100 μ M) or charybdotoxin (ChTx) blocked quercetin-mediated vasorelaxation thus demonstrating the effect was partially dependent on NOS and endothelium-derived hyperpolarizing factor (EDHF). Additionally, bovine aortic endothelial cells (BAEC) treated with quercetin showed a rapid increase of intracellular Ca^{2+} concentrations as well as a dose- and time-dependent stimulation of eNOS phosphorylation with a concomitant increase in NO production. These results demonstrate that quercetin-mediated stimulation of eNOS phosphorylation increases NO bioavailability in endothelial cells and can thus play a role in the vascular protective effects associated with improved endothelial cell function.

* Address correspondence to: Nicholas K.H. Khoo, Ph.D., Department of Pharmacology & Chemical Biology, E1314 BST, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA; Tel: 412-648-9671; Fax: 412-648-2229; nkhoopitt.edu.

Keywords

nitric oxide synthase; endothelium; cardioprotection; eNOS; heart; catalase; endothelium-derived hyperpolarizing factor; phosphorylation; aortic ring segments; H₂O₂; fura-2; microfluorometric Ca²⁺ imaging

Introduction

The endothelium plays an important role in maintaining vascular homeostasis. Oxidative stress has been implicated in numerous vascular diseases. Cardiovascular risk factors, such as hypertension, diabetes and smoking, alter the redox state in the vessel and can lead to endothelial dysfunction resulting in atherosclerosis. Endothelial dysfunction and the loss of nitric oxide (NO) production are early manifestations and hallmarks of vascular disorders. The increase in oxidative stress, due to endothelial dysfunction and lack of NO bioavailability, alters several physiological functions such as leukocyte adhesion, platelet aggregation, and blood flow in the endothelium [1].

Consumption of flavonoids found in fruits, vegetables, and beverages such as tea and wine are inversely correlated with mortality from coronary heart disease [2–4]. The Rotterdam study demonstrated an inverse relationship between flavonoid intake and occurrence of myocardial infarction [5]. The cardiovascular benefits of flavonoid-rich foods have been attributed to their antioxidant effects (inhibiting the oxidation of low density lipoproteins (LDL) [6]), NO-mediated vasodilatory effects, reducing platelet aggregation [7], and activating Nrf2-induced phase II detoxification through antioxidant response element (ARE)-mediated gene expression [8–11].

Epidemiological studies have suggested an inverse correlation between the incidence and mortality rates from coronary heart disease and light to moderate alcohol consumption (1–2 drinks/day), in particular red wine. Several studies have shown the beneficial effects of moderate alcohol consumption have been attributed to the alcohol content [12–14]. Special attention has focused on the French Paradox - a lower incidence of cardiovascular mortality among the Mediterranean population in association with red wine consumption despite having a diet high in saturated fat intake. Moreover, studies demonstrate the beneficial effects of red wine on the vasculature are attributed to the high polyphenol content found in red wines [11, 15,16]. In particular, red wine polyphenols (RWPs) as well as individual polyphenolic compounds found in red wine, such as quercetin, resveratrol, and catechin, attenuated the progression of atherosclerotic lesions [14,17,18]. Pioneering *ex vivo* studies suggested that flavonoids, in particular red wine polyphenols, may favorably affect endothelial function by stimulating endothelium-dependent relaxation through NO/NOS-dependent pathway [19–21]. Moreover, these effects were partially attenuated with PI3K inhibitors (Wortmannin and LY294002) [22]. These data indicate that dietary polyphenols may play a role in the cardiovascular protection by improving endothelial cell function.

Quercetin is the most prevalent flavonoid in the human diet and abundantly found in apples, onions, berries, and red wines. A recent study demonstrated that low to moderate oral supplementation of quercetin (50, 100, or 150 mg/day) for two weeks increased plasma quercetin concentrations dose-dependently in healthy individuals. The highest dose of 150 mg/day increased plasma quercetin concentrations over 570% with a median concentration of 0.38 µM and a maximal concentration observed at 1.3 µM [23]. Quercetin is part of the flavone family which contains a double bond in the central aromatic ring. It is a polyphenol that has been shown to engage in redox cycling reactions in cellular systems thereby endowing both antioxidant and prooxidant (by generating H₂O₂) properties [24,25]. The effects of quercetin

on vessel function are not clear and specifically whether the proposed generation of H₂O₂ can modulate eNOS activity and vessel relaxation. This study provides new insight in the ability of quercetin to improve endothelial function by inducing vasorelaxation through an eNOS phosphorylation process blocked by an increase in catalase activity, Ca²⁺-mediated eNOS-dependent and -independent pathways. These results demonstrate that quercetin-mediated stimulation of eNOS phosphorylation increases NO bioavailability in endothelial cells and can thus play a role in the vascular protective effects associated with improved endothelial cell function.

Experimental procedures

Biochemicals

All chemicals were obtained from Sigma (St. Louis, MO), unless specified. Quercetin was obtained from Acros Organics (Morris Plains, NJ). Monoclonal eNOS and polyclonal phospho-eNOS (Ser¹¹⁷⁷) antibodies were obtained from Transduction Laboratories (Lexington, KY) and Cell Signaling Technology (Beverly, MA), respectively. The secondary antibody was obtained from Amersham-Pharmacia Biotech (Piscataway, NJ). Ad.catalase and Ad.empty were obtained from the Gene Transfer Vector Core (University of Iowa; Iowa City, IA).

Animals

Ten-week-old Sprague-Dawley rats ranging from 300–350g body weight were obtained from Charles River Laboratories, Inc (Wilmington, MA) and were maintained at constant humidity (60 ± 5%), temperature (24 ± 1°C), and light cycle (6AM to 6 PM). All rats were fed a standard rat pellet diet (Ralston Purina Diet) ad libitum. All animal protocols were approved by the Animal Review Board of the University of Alabama at Birmingham. All animals were anesthetized prior to surgical procedures with ketamine and Rompun (10 and 1.5 mg/100 g body weight, respectively).

Quercetin preparation

Quercetin was made fresh daily, dissolved in DMSO (> 24 mg/mL), and the exact concentration was obtained using UV spectroscopy (extinction coefficient; $\epsilon_{\text{mM}} = 0.026$; Abs, 377 nm).

Vessel reactivity studies

The assessment of vascular function was conducted as previously described [26]. In brief, the aorta was excised, cleaned of fat and excess tissue, cut into individual ring segments (2–3 mm width), and suspended from a force-displacement transducer in a water-jacketed tissue bath containing bicarbonate-buffered, Krebs-Henseleit buffer (all of the following in mM concentrations-118 NaCl, 4.6 KCl, 27.2 NaHCO₃, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.75 CaCl₂, 0.03 Na₂EDTA, and 11.1 glucose). The buffer was maintained at 37 and aerated with 95% O₂/5% CO₂. Isometric tension was then measured in isolated aortic ring segments (a passive load of 2g was applied to all ring segments and maintained throughout the experiment). At the beginning of each experiment, ring segments were depolarized with 70 mM potassium chloride (KCl) to determine maximal contractile capacity of the vessel. Rings were then thoroughly washed and allowed to equilibrate.

In subsequent experiments, ring segments were pretreated with or without L-NAME for 30 min followed by quercetin or vehicle (DMSO) treatment for 30 min. Ring segments were submaximally contracted (40% of KCl response) with phenylephrine (PE), $\sim 3 \times 10^{-8}$ to 10^{-7} M. When tension development reached a plateau, acetylcholine (ACh), 10^{-9} to 3×10^{-6} M was added cumulatively to the bath to stimulate endothelium-dependent relaxation via calcium-dependent NO formation in response to ligand receptor interactions. In other experiments, ring

segments were treated (as indicated above) and contractile responses were tested by the cumulative addition of PE. Dose response profiles under different experimental conditions were analyzed and tested for differences in relaxation and contraction parameters. In a subset of studies, sodium nitroprusside (SNP, 5 μ M) was added to elicit residual EC-independent relaxation and ensure the ability of the rings to respond to NO. In some experiments, vessels were denuded to confirm endothelial-dependence of the enhanced response to Ach.

For hyperpolarization experiments, ring segments were depolarized with 70 mM KCl twice to achieve maximal contractile capacity of the vessels. Rings were then thoroughly washed and allowed to equilibrate before the addition of 50 nM charybdotoxin (ChTx) for 30 min. Immediately following ChTx, the rings were contracted with another dose of 70 mM KCl (achieved maximal contractile capacity) and then 5 μ M quercetin or equivalent DMSO was added to the vessel bath for 30 min in intact and denuded vessels. At the end of the experiments, acetylcholine (Ach) dose responses were performed to ensure maximal relaxation. Data was collected for all experiments and downloaded to an IBM PC for later analysis using Workbench for Windows (v.3) software.

cGMP analysis

Rat thoracic aortas were excised, cleaned, cut into ring segments, weighed, placed in Krebs buffer and bubbled continuously with 95% O₂/5% CO₂. The ring segments were incubated with 5 μ M quercetin for 60 min, immediately flash frozen in liquid nitrogen, and stored at -80° C until analysis. The cGMP values were determined using the cGMP enzyme immunoassay assay from Cayman Chemical Company (Ann Arbor, MI) according to the manufacturer's specifications. Additionally, aortic ring segments were also incubated with 25 μ M BAPTA/AM for 30 min prior to the addition of quercetin. Diethylenetriamine/NO (DETA/NO), a nitric oxide donor was used as a positive control. Protein concentrations were used for normalization and determined by the BCA protein assay kit (Pierce, Rockford, IL). Data were expressed as pmol/mg protein.

Cell Culture

Bovine aortic endothelial cells (BAECs) were isolated from descending thoracic aortas and maintained in Medium 199 (Cellgro; Herndon, VA) containing 5% fetal bovine serum (Hyclone; Logan, UT), 5% fetal calf serum (Hyclone), 10 μ M thymidine (Gibco; Carlsbad, CA), and Antibiotic Antimycotic Solution (Cellgro). Cells were monitored visually for typical cobblestone morphology indicative of endothelial cells by staining for von Willebrand factor expression and were not passaged for more than eight cycles.

Western blotting

Confluent BAECs were treated with quercetin for 15–120 min. After treatment, cells were rinsed twice with ice cold phosphate buffer saline (PBS) and homogenized in lysis buffer consisting of 1% Triton X-100, 10 mM NaF, 1 mM vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin in Tris-buffer saline at pH 7.5. The protein concentration was determined by using BCA protein assay kit. Protein was denatured by boiling, separated on a 7.5 % SDS-PAGE gel, and transferred to nitrocellulose. Membranes were then incubated overnight in blocking buffer containing anti-phospho-eNOS (Ser¹¹⁷⁷) at a dilution of 1:1000, washed with TTBS, incubated with horseradish peroxidase-conjugated antibody and washed extensively. Immunoreactive bands were visualized using chemiluminescence (Pierce).

Assessment of NO production

NO was detected by chemiluminescence following the reduction of NO_2^- with KI and CuSO_4 , according to manufacturer's instructions (Sievers). BAECs were grown to confluency in six-well plates in 10% fetal bovine serum medium. The medium was removed and cells were washed twice with HBSS at 37°C prior to incubation with 0.75 ml of HBSS containing 25 μM L-arginine plus the addition of quercetin or vehicle (DMSO) for 30–120 min. Following treatment, the HBSS plus treatment was collected from each well and centrifuged at $1,000 \times g$ for 10 min. The resulting supernatant was then injected (50 μl via Hamilton syringe) into the Sievers nitric oxide analyzer [27]. Each treatment was performed in triplicate and normalized to cell protein content. A serial dilution of nitrite standards were prepared for each experiment using a freshly prepared NaNO_2 solution. Background levels of NO_2^- concentrations were determined using samples of dH_2O and HBSS containing L-arginine.

Adenovirus gene transfer

Adenoviruses were purchased from the University of Iowa Gene Transfer Vector Core Facility. *AdCMVCatalase* (*AdCat*), *AdCMVLacZ* (*AdLacZ*), *Adempty* were manufactured at the University of Iowa's Vector Core Facility by inserting catalase and *LacZ* genes into the E1 region of an Ad5 E1/partial E3-deleted replication-deficient adenoviral vector [28]. Briefly, BAECs were infected with the *AdCat* at the indicated multiplicity of infection (MOI) for 24 h after which the infection medium was removed and replaced with fresh medium for an additional 24 h. *AdLacZ* or *Adempty* (25, 50 MOI) were used as a control for adenovirus infection.

Catalase enzymatic activity

Catalase activity was determined as described previously [29]. Briefly, 200 μg of total cell lysate was added to 30 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.8), and the disappearance of H_2O_2 was measured at 240 nm for 60 sec at 8 sec intervals.

Calcium measurements

Microfluorometric imaging of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) was performed on BAECs cultured on glass coverslips until confluence. Briefly, BAECs were rinsed with HBSS twice and then loaded with 5 μM fura-2/AM (Molecular Probes; Carlsbad, CA) for 15 min. After three washes with HBSS, coverslips were placed in a imaging chamber mounted on a fixed-stage upright microscope (Zeiss Axioskop FS; Oberkochen, Germany) and continuously perfused (2 mL/min) with HBSS at room temperature. Fura-2 was alternatively excited at 360 and 380 nm using a monochromator (Polychrome-IV, TILL Photonics; Munich, Germany), and its emission (>510 nm) filtered and detected with a frame-transfer cooled CCD camera (Quantix-57, Photometrics; Tucson, AZ). After BAECs were equilibrated (~ 10 min), time-lapse series of fura-2 image pairs were acquired every 2 sec before and after bath application of DMSO (vehicle), 2.5 or 5 μM quercetin. Background-subtracted fluorescence intensity measurements were obtained within regions-of-interest (ROIs) defined over BAEC cell bodies. The average ratio of 360nm and 380nm fluorescence within each ROI was used as an estimate of intracellular Ca^{2+} concentration, as these two parameters are directly proportional to each other [30]. Synchronized image acquisition and monochromator control was performed using custom-written software (TIWorkBench, written by T.I.) on a G4 Macintosh computer (Apple). Each imaging session had 10–20 cells per experiment, and results were derived from at least seven independent experiments.

Results

Quercetin reduces vasoconstrictor sensitivity in rat aortic ring segments by an NO-dependent mechanism

Quercetin (5 and 10 μM for 30 min) attenuated phenylephrine (PE)-induced contraction in rat aortic vessels compared to vehicle control (DMSO). Line graphs represent concentration-response curves of aortic ring segments to PE following the incubation of aortic ring segments with 5 and 10 μM quercetin for 30 min (Fig 1A). There was no distinguishable difference in contractile response between 5 and 10 μM quercetin at 30 min, between untreated and vehicle treated (DMSO), and longer treatments (up to 120 min) did not alter the response significantly (not shown). Therefore the aortic ring segments were incubated with 5 μM quercetin for 30 min in all subsequent experiments. In Figure 1B, the contractile response of a normal rat aorta (filled squares) was altered by pretreating the vessels with NOS inhibitor, L-NAME, the contractile curve shifted to the left indicating the basal NOS activity in the rat aorta (open squares). Quercetin treatment (filled triangle) for 30 minutes shifted the contractile response to the right indicating more PE was needed to produce the same contractile response compared to the vehicle control or L-NAME treated vessels. The quercetin response was completely blocked by pretreating the ring segments with L-NAME (100 μM) for 30 min (open circles). Quercetin treatment for 30 min had a significant vasorelaxant effect in response to acetylcholine (Ach). Aortic ring segments treated with quercetin or DMSO for 30 min showed a dose-dependent relaxation in response to acetylcholine (Ach) with an ED_{50} of 1.97 ± 1.2 or 7.96 ± 1.4 , respectively (Fig 1C).

Quercetin stimulates eNOS phosphorylation at Ser¹¹⁷⁹

Several eNOS phosphorylation sites have been identified such as Ser¹¹⁷⁹, Thr⁴⁹⁷, and Ser¹¹⁶ that regulate eNOS activity. Short-term treatment (30 or 60 min) of quercetin (5 and 10 μM) stimulates phosphorylation of eNOS at Ser¹¹⁷⁹ in a dose-dependent manner in BAECs while the total eNOS protein levels remain unchanged (Figure 2A). Lower concentrations of quercetin (1 and 2.5 μM) stimulated the phosphorylation of eNOS at Ser¹¹⁷⁹, albeit not significant (not shown). Quercetin treatment did not alter Thr⁴⁹⁷ and Ser¹¹⁶ levels (not shown).

Quercetin increases NO_2^- accumulation in BAECs

The ability of quercetin to stimulate eNOS phosphorylation at Ser¹¹⁷⁹ led to an increase in NO production over time in BAECs. The media was collected and analyzed for NO_2^- levels (indicative of eNOS-derived NO production) after treating cells with 5 μM quercetin for 30, 60, and 120 min. This NO_2^- accumulation in the media was normalized for cell protein content and reported as a percent over the DMSO control (basal $\text{NO}_2^- = 1.04 \pm 0.09$ nmoles/mg protein). No changes in NO_2^- accumulation were observed for untreated or DMSO-treated cells at the same sampling times (not shown). Exposure of cells to quercetin increased NO_2^- accumulation 1.3-, 1.5-, and 1.8-fold over DMSO control at 30, 60, and 120 min, respectively (Fig. 2B). Calcium ionophore was used as a positive control.

Quercetin increases cGMP in aortic ring segments

The downstream effects of NO were tested by incubating rat aortic ring segments with 5 μM quercetin for 60 min. Quercetin increased cGMP levels and this effect was inhibited by treating the aortic ring segments with 25 μM BAPTA/AM for 30 min prior to the quercetin treatment. Diethylenetriamine/NO (D, DETA/NO), a nitric oxide donor, was used as a positive control (Fig 2C).

Quercetin increases intracellular Ca^{2+} levels in BAECs

Previous studies demonstrated that polyphenols found in red wine elevate intracellular Ca^{2+} levels [31], and such increases enhance endothelial NO production [32]. Indeed, application of quercetin to BAECs increased the intracellular concentration of free calcium in BAECs, as determined by fura2/AM imaging (left panel, Fig 3A). These fura-2 signals peaked within the first 2 min of exposure to quercetin, and remained elevated until the removal of Ca^{2+} from the extracellular solution (not shown). On the other hand, vehicle controls using DMSO did not affect fura-2 signals (right panel, Fig 3A). The activation of eNOS by Ach or shear stress is accompanied by an increase in intracellular Ca^{2+} and a subsequent release of NO [33]. To test this, BAECs were pretreated with the Ca^{2+} chelator BAPTA/AM (25 μM for 30 min) and then stimulated with quercetin for 30 min. Fig 3B demonstrates that chelation of Ca^{2+} by BAPTA attenuates quercetin-stimulated eNOS phosphorylation at Ser¹¹⁷⁹. Moreover, excised rat aortic ring segments incubated with quercetin increased cGMP and this effect was blocked by the pretreatment of BAPTA/AM (Fig 3B). Rat aortic ring segments were incubated with BAPTA/AM or DMSO for 30 min followed by quercetin treatment (5 μM) for 1 h.

Overexpression of catalase by adenovirus blocks quercetin-induced eNOS phosphorylation

Quercetin has been shown to generate H_2O_2 via semi-quinone radical [34]. Rat aortic ring segments treated with 1, 10, or 50 μM H_2O_2 for 30 min markedly reduced the responses to PE (not shown) and consistent with previous findings [35,36]. Additionally, H_2O_2 has been shown to phosphorylate eNOS at Ser¹¹⁷⁹ in BAECs via PI3K-dependent signaling pathway [36]. Therefore, BAECs were transduced with adenovirus containing catalase (Ad.catalase) and incubated for 24 h to allow catalase protein and activity to increase significantly (Fig 4A). Overexpression of catalase dramatically diminished the basal phosphorylation levels of eNOS. More importantly, quercetin treatment (5 μM) for 30 min failed to stimulate eNOS phosphorylation at Ser¹¹⁷⁹ (Fig 4B) with densitometric analysis (Fig 4C).

Endothelium derived hyperpolarization factor (EDHF) may also be responsible for quercetin-mediated vasorelaxation

Quercetin treatment significantly induced endothelium-dependent relaxation compared to vehicle control in intact vessels (Fig 5A) but not in denuded vessels (Fig 5B). Pretreatment with charybdotoxin (ChTx), which blocks large- conductance (BK_{Ca})- and intermediate-conductance (IK_{Ca})- Ca^{2+} -activated K^{+} -channels, significantly attenuated the quercetin-mediated response in intact vessels (Fig 5A). ChTx did not have any effect on basal vessel tone. At the end of the experiments, acetylcholine (Ach) dose responses were performed to elicit maximal endothelium-dependent relaxation. There was no significant difference between quercetin and quercetin plus Ach with the pretreatment of ChTx or without (not shown). There were no significant differences in the denuded vessel for all of the treatments. As a control, an Ach dose response was performed to verify the endothelium was completely denuded (Fig 5B).

Discussion

This study reveals that quercetin induced vasorelaxation and increased cGMP levels in rat aortic vessels. Moreover, *in vitro* experiments indicated that quercetin treatment rapidly increased Ca^{2+} , stimulated eNOS phosphorylation at Ser¹¹⁷⁹ resulting in NO generation in BAECs. Quercetin-stimulated eNOS phosphorylation was completely blocked by increasing cellular catalase enzymatic activity with the transduction of Ad.Cat.

Investigation into the underlying mechanism(s) revealed that quercetin-induced vasorelaxation could be regulated, in part, by the formation of an oxidant. More studies are needed to verify these findings; however cell culture experiments provided some insight. The transduction of

catalase activity via Ad.Cat ameliorated quercetin stimulated eNOS phosphorylation in BAECs.

New evidence supports that flavonoids, such as quercetin, not only have antioxidant capabilities but more importantly may act as signaling molecules [37]. In fact, it has been shown that quercetin can act as an antioxidant leading to the formation of quinones and prooxidants [38]. Furthermore, quercetin can be metabolized to a quinone/quinone methide metabolite such as o-semiquinone and o-quinone [39–41]. O-semiquinone has the capability to generate $O_2^{\bullet-}$ in cell culture [42] whereas o-quinone depletes glutathione (GSH) in the presence of excess ascorbate [41,42]. The depletion of GSH and the generation $O_2^{\bullet-}$ through these quercetin metabolites suggest a prooxidant effect. Moreover, these quinone/quinone methide metabolites may trigger signaling pathways [37,43]. In this context, quercetin and its cellular metabolites act more like signaling molecules than prooxidants that cause cell death per se. The formation of quinone/quinone methide metabolites may lead to a low level production of oxidants, which may in turn activate certain signaling cascades.

By acting as a sensor as well as a signaling molecule, H_2O_2 is an important regulator of signal transduction. An additional mechanism of action of quercetin then is through regulating H_2O_2 levels. H_2O_2 regulates endothelial cell function such as proliferation, inflammatory responses, apoptosis, and endothelium-dependent vasorelaxation [44]. The generation of H_2O_2 to act as a signaling molecule is tightly regulated and depends mainly on location of production and the surrounding antioxidant enzymatic activity. The dismutation of $O_2^{\bullet-}$ can occur spontaneously or catalytically by SOD generating H_2O_2 . Interestingly, endogenously produced H_2O_2 is more effective in eliciting a signaling response compared to exogenously added H_2O_2 [45]. Moreover, minute intracellular fluxes in H_2O_2 concentrations may rapidly alter cell signaling responses in the endothelium resulting in endothelium-derived vasorelaxation.

Vascular endothelial cells have the capacity to release H_2O_2 [46]. Endothelium-derived H_2O_2 stimulates EDHF. The endothelium produces and releases several vasoactive mediators involved in vessel tone, such as NO, prostacyclins, and EDHF. RWPs have been shown to induce EDHF-dependent vasorelaxation in porcine coronary arteries [22]. Herein, quercetin stimulates vasodilation in intact vessels which is significantly attenuated by the pretreatment of ChTx. Quercetin does not stimulate relaxation significantly in denuded vessels (Fig 5). These results demonstrate that quercetin stimulates endothelium-dependent vasorelaxation. The inhibition of BK_{Ca} , IK_{Ca} , and some voltage-dependent K^+ -channels with ChTx blunts the response suggesting EDHF playing a role in quercetin-induced vasorelaxation.

Studies demonstrated that grape seed extracts (which are high in flavonoids and used to make red wine) and RWPs, stimulate endothelium-dependent relaxation through NO/NOS-dependent pathways by increasing cGMP levels in rat aortic vessels [19–21,47]. The precise mechanism still remains to be elucidated. Additional studies demonstrated that RWP-induced vasorelaxation was actually impaired by the preincubation of porcine vessels with PEG-SOD or SOD mimetic (MnTMPyP). Surprisingly, PEG-catalase (but not native catalase) also impaired RWP-induced vasorelaxation suggesting that RWP potentially generate H_2O_2 or peroxide-like molecules [22,48]. Additionally, RWPs stimulate vasorelaxation in small mesenteric arteries of rats is endothelium- and NOS-dependent. These effects were inhibited by SOD plus catalase or using the NADPH oxidase inhibitor DPI suggesting that RWP generate oxidants. In the presence of Ca^{2+} , RWPs induced superoxide production [49]. The subsequent formation of H_2O_2 or the peroxide-like molecule may indeed mediate a signaling response resulting in RWP-induced vasorelaxation.

Exogenous H₂O₂ mediates vasorelaxation in aortas and is blunted by catalase but not SOD [50]. H₂O₂ treatment increases eNOS activity and NO bioavailability in primary cultured endothelial cells through the phosphorylation of eNOS at Ser¹¹⁷⁹ and the concomitant dephosphorylation at Thr⁴⁹⁵. The phosphorylation of eNOS at Ser¹¹⁷⁹, and not Thr⁴⁹⁵, is mediated by Ca²⁺ and the PI3K/Akt pathway [36]. Additionally, H₂O₂ dose-dependently increases Ca²⁺ in endothelial cells [51]. The removal of Ca²⁺ from buffers and/or aorta rings pretreated with BAPTA/AM significantly reduced H₂O₂-mediated vasorelaxation [35].

Other studies have shown that cellular responses to RWPs, such as resveratrol, are mediated through redox-sensitive pathways such as p38 MAPK, ERK1/2, PI3K [48,52,53]. Resveratrol can increase eNOS mRNA, protein, and promoter activity resulting in an increase eNOS activity and NO [54,55]. Regardless of these mechanism(s), the physiological relevance cannot be overlooked. Herein, quercetin stimulated vasorelaxation in rat aortic vessels through the phosphorylation of eNOS at ser¹¹⁷⁹. Quercetin treatment did not alter Thr⁴⁹⁷ and Ser¹¹⁶ levels (not shown) suggesting that the quercetin-mediated NO production may be due to the stimulation of eNOS phosphorylation at Ser¹¹⁷⁹. Several studies have shown that PI3K inhibitors block agonist-stimulated eNOS phosphorylation. At higher concentrations, quercetin has been shown to partially block the PI3K pathway [56]. Moreover, pretreatment of the PI3K pathway inhibitor wortmannin (100 nM) or LY294002 (10 μM) for 30 min failed to attenuate quercetin-stimulated eNOS phosphorylation at Ser¹¹⁷⁹ suggesting that this process is not mediated by the PI3K pathway. Moreover, quercetin did not significantly increase eNOS mRNA or protein (not shown).

Flavonoid and/or RWP studies report vasorelaxation through a NO synthase (NOS)- and PI3K-dependent pathway [19,21,22,47], although the precise mechanism(s) still remains unclear. Black tea polyphenols (BTP) induced eNOS phosphorylation at Ser¹¹⁷⁷ and dephosphorylation of Thr⁴⁹⁵, increased cGMP levels, and enhanced conversion of L-[³H] arginine to L-[³H] citrulline in porcine aortic endothelial cells (PAECs) within 5 min [57]. The BTP-induced cGMP and conversion of arginine to citrulline was blocked by L-nitro-arginine methyl ester (L-NAME), the PI3K inhibitor LY294002, and a dominant negative p38 MAP kinase (MAPK) α suggesting that this effect is NOS, PI3K, and p38 MAPK dependent. Additionally, RWP-induced vasorelaxation was ablated by PI3K inhibitors (wortmannin and LY294002) and not affected by ERK1/2 (PD98059) nor p38 MAPK (SB203580) inhibitors [48]. These studies are highly suggestive that polyphenols mainly activate the redox-sensitive PI3K pathway inducing endothelium-dependent NO-mediate relaxation through polyphenol-stimulated eNOS phosphorylation.

Quercetin stimulates eNOS phosphorylation at ser¹¹⁷⁹ and perhaps sensitizes eNOS to lower concentrations of Ca²⁺. Moreover, quercetin increases the intracellular concentration of Ca²⁺, whereas Ca²⁺ chelation with BAPTA/AM inhibits quercetin-stimulated phosphorylation of Ser¹¹⁷⁹ in BAEC (Fig 3). In cultured endothelial cells, quercetin-mediated increases in Ca²⁺ may be responsible for the stimulation of eNOS phosphorylation at ser¹¹⁷⁹ eventually resulting in NO production. Not to diminish the fact that polyphenols are also widely accepted antioxidants and can scavenge ROS and reactive nitrogen species (RNS) such as O₂^{•-} and peroxynitrite (ONOO⁻) [58]. BTPs and RWPs can inhibit lipid peroxidation and increase the antioxidant capacity in human plasma *in vitro* [59,60]. Additionally, lipid radicals serve as a sink for NO [61,62] suggesting that polyphenolic antioxidant properties lead to an increase in NO bioavailability by reducing ROS and RNS, which are known sinks for NO consumption. This may help explain the inverse relationship between flavonoid intake and cardiovascular risk.

In summary, endothelial dysfunction is often associated with the lower NO bioavailability. We have shown that quercetin improves endothelial function by stimulating endothelium-

dependent vasorelaxation *ex vivo*, eNOS phosphorylation *in vitro* thereby resulting in an increase in NO bioavailability. This suggests that NO bioavailability may play an important role in the quercetin-dependent attenuation of vascular dysfunction through endothelial dependent NO producing enzymes and EDHF. These findings serve to further elucidate potential mechanisms regarding the epidemiological evidence from several cohort studies demonstrating an inverse correlation with dietary intake of flavonoids and CVD.

Acknowledgments

This study has been supported in part by the National Institutes of Health AA12649 and AA/DK11589 and HL70071 (to D.A.P), American Heart Association #0525330B (to N.K.H.K) and The Alcohol Beverage Foundation and The Wine Institute.

Abbreviations

Ach	acetylcholine
AdCat	AdCMVCatalase
AdLacZ	AdCMVLacZ
Adempty	empty cassette
ARE	antioxidant responsive element
BAECs	bovine aortic endothelial cells
BTP	black tea polyphenol
ChTx	charybdotoxin
CVD	cardiovascular diseases
DCF	dichlorofluorescein
DETA	diethylenetriamine
EDHF	endothelium-derived hyperpolarizing factor
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
GSH	glutathione
H ₂ O ₂	hydrogen peroxide
HBSS	Hank's buffered salt solution
KCl	potassium chloride
L-NAME	L-nitro-arginine methyl ester
LDL	low-density lipoprotein
MOI	multiplicity of infection
NO	nitric oxide
NO ₂ ⁻	nitrite
NOA	nitric oxide analyzer
O ₂ ^{•-}	superoxide
p38	p38 mitogen-activated protein kinase
PAGE	polyacrylamide gel electrophoresis

PAECs	porcine aortic endothelial cells
PBS	phosphate buffer saline
PE	phenylephrine
PI3K	phosphoinositide 3-kinases
RNS	reactive nitrogen species
ROIs	regions-of-interest
ROS	reactive oxygen species
RWP	red wine polyphenol
SDS	sodium dodecyl sulfate
TTBS	tween 20 tris-buffered saline

References

1. Thomas SR, Witting PK, Drummond GR. Redox control of endothelial function and dysfunction: molecular mechanisms and therapeutic opportunities. *Antioxid Redox Signal* 2008;10:1713–1765. [PubMed: 18707220]
2. Vita JA. Tea consumption and cardiovascular disease: effects on endothelial function. *J Nutr* 2003;133:3293S–3297S. [PubMed: 14519828]
3. Duffy SJ, Keaney JF Jr, Holbrook M, Gokce N, Swerdloff PL, Frei B, Vita JA. Short- and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease. *Circulation* 2001;104:151–156. [PubMed: 11447078]
4. Renaud S, de Lorgeril M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* 1992;339:1523–1526. [PubMed: 1351198]
5. Geleijnse JM, Launer LJ, van der Kuip DAM, Hofman A, Witteman JCM. Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam Study. *Am J Clin Nutr* 2002;75:880–886. [PubMed: 11976162]
6. de Whalley CV, Rankin SM, Hoult JR, Jessup W, Leake DS. Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochem Pharmacol* 1990;39:1743–1750. [PubMed: 2344371]
7. Pignatelli P, Pulcinelli FM, Celestini A, Lenti L, Ghiselli A, Gazzaniga PP, Violi F. The flavonoids quercetin and catechin synergistically inhibit platelet function by antagonizing the intracellular production of hydrogen peroxide. *Am J Clin Nutr* 2000;72:1150–1155. [PubMed: 11063442]
8. Nijveldt RJ, van Nood E, van Hoorn DEC, Boelens PG, van Norren K, van Leeuwen PAM. Flavonoids: a review of probable mechanisms of action and potential applications. *Am J Clin Nutr* 2001;74:418–425. [PubMed: 11566638]
9. Rasmussen SE, Frederiksen H, Struntze KK, Poulsen L. Dietary proanthocyanidins. Occurrence, dietary intake, bioavailability, and protection against cardiovascular disease. *Mol Nutr Food Res*. 2005
10. Oak MH, El Bedoui J, Schini-Kerth VB. Antiangiogenic properties of natural polyphenols from red wine and green tea. *J Nutr Biochem* 2005;16:1–8. [PubMed: 15629234]
11. Mann GE, Rowlands DJ, Li FYL, de Winter P, Siow RCM. Activation of endothelial nitric oxide synthase by dietary isoflavones: Role of NO in Nrf2-mediated antioxidant gene expression. *Cardiovasc Res* 2007;75:261–274. [PubMed: 17498676]
12. Abou-Agag LH, Khoo NK, Binsack R, White CR, rley-Usmar V, Grenett HE, Booyse FM, Digerness SB, Zhou F, Parks DA. Evidence of cardiovascular protection by moderate alcohol: role of nitric oxide. *Free Radic Biol Med* 2005;39:540–548. [PubMed: 16043025]
13. Ralay RH, Diebolt M, Schott C, Andriantsitohaina R. Polyphenolic compounds from Cognac induce vasorelaxation in vitro and decrease post-ischaemic cardiac infarction after an oral administration. *Fundam Clin Pharmacol* 2004;18:331–338. [PubMed: 15147285]

14. Vinson JA, Teufel K, Wu N. Red wine, dealcoholized red wine, and especially grape juice, inhibit atherosclerosis in a hamster model. *Atherosclerosis* 2001;156:67–72. [PubMed: 11368998]
15. Cordova AC, Jackson LS, Berke-Schlessel DW, Sumpio BE. The cardiovascular protective effect of red wine. *J Am Coll Surg* 2005;200:428–439. [PubMed: 15737855]
16. Dell'Agli M, Busciana A, Bosisio E. Vascular effects of wine polyphenols. *Cardiovasc Res* 2004;63:593–602. [PubMed: 15306214]
17. Stocker R, O'Halloran RA. Dealcoholized red wine decreases atherosclerosis in apolipoprotein E gene-deficient mice independently of inhibition of lipid peroxidation in the artery wall. *Am J Clin Nutr* 2004;79:123–130. [PubMed: 14684408]
18. Hayek T, Fuhrman B, Vaya J, Rosenblat M, Belinky P, Coleman R, Elis A, Aviram M. Reduced progression of atherosclerosis in apolipoprotein E-deficient mice following consumption of red wine, or its polyphenols quercetin or catechin, is associated with reduced susceptibility of LDL to oxidation and aggregation. *Arterioscler Thromb Vasc Biol* 1997;17:2744–2752. [PubMed: 9409251]
19. Fitzpatrick DF, Hirschfield SL, Ricci T, Jantzen P, Coffey RG. Endothelium-dependent vasorelaxation caused by various plant extracts. *J Cardiovasc Pharmacol* 1995;26:90–95. [PubMed: 7564372]
20. Benito S, Lopez D, Saiz MP, Buxaderas S, Sanchez J, Puig-Parellada P, Mitjavila MT. A flavonoid-rich diet increases nitric oxide production in rat aorta. *Br J Pharmacol* 2002;135:910–916. [PubMed: 11861318]
21. Andriambeloson E, Kleschyov AL, Muller B, Beretz A, Stoclet JC, Andriantsitohaina R. Nitric oxide production and endothelium-dependent vasorelaxation induced by wine polyphenols in rat aorta. *Br J Pharmacol* 1997;120:1053–1058. [PubMed: 9134217]
22. Ndiaye M, Chataigneau T, Chataigneau M, Schini-Kerth VB. 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* 2004;142:1131–1136. [PubMed: 15249422]
23. Egert S, Wolfram S, Bosy-Westphal A, Boesch-Saadatmandi C, Wagner AE, Frank J, Rimbach G, Mueller MJ. Daily Quercetin Supplementation Dose-Dependently Increases Plasma Quercetin Concentrations in Healthy Humans. *J Nutr* 2008;138:1615–1621. [PubMed: 18716159]
24. Gliszczynska-Swiglo A, van der WH, de Haan L, Tyrakowska B, Aarts JM, Rietjens IM. The role of quinone reductase (NQO1) and quinone chemistry in quercetin cytotoxicity. *Toxicol In Vitro* 2003;17:423–431. [PubMed: 12849725]
25. Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of flavonoids: structure-activity relationships. *Free Radic Biol Med* 1997;22:749–760. [PubMed: 9119242]
26. Zhang C, Patel R, Eiserich JP, Zhou F, Kelpke S, Ma W, Parks DA, Darley-Usmar V, White CR. Endothelial dysfunction is induced by proinflammatory oxidant hypochlorous acid. *Am J Physiol Heart Circ Physiol* 2001;281:H1469–H1475. [PubMed: 11557534]
27. Kershaw EE, Schupp M, Guan H-P, Gardner NP, Lazar MA, Flier JS. PPAR{gamma} regulates adipose triglyceride lipase in adipocytes in vitro and in vivo. *Am J Physiol Endocrinol Metab* 2007;293:E1736–1745. [PubMed: 17848638]
28. Lam EWN, Zwacka R, Seftor EA, Nieva DRC, Davidson BL, Engelhardt JF, Hendrix MJC, Oberley LW. Effects of antioxidant enzyme overexpression on the invasive phenotype of hamster cheek pouch carcinoma cells. *Free Radic Biol Med* 1999;27:572–579. [PubMed: 10490277]
29. Preuss M, Girmun GD, Darby CJ, Khoo N, Spector AA, Robbins ME. Role of antioxidant enzyme expression in the selective cytotoxic response of glioma cells to gamma-linolenic acid supplementation. *Free Radic Biol Med* 2000;28:1143–1156. [PubMed: 10832077]
30. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–3450. [PubMed: 3838314]
31. Martin S, Andriambeloson E, Takeda K, Andriantsitohaina R. Red wine polyphenols increase calcium in bovine aortic endothelial cells: a basis to elucidate signalling pathways leading to nitric oxide production. *Br J Pharmacol* 2002;135:1579–1587. [PubMed: 11906973]
32. Busse R, Mulsch A. Calcium-dependent nitric oxide synthesis in endothelial cytosol is mediated by calmodulin. *FEBS Lett* 1990;265:133–136. [PubMed: 1694782]

33. Muller JM, Davis MJ, Kuo L, Chilian WM. Changes in coronary endothelial cell Ca²⁺ concentration during shear stress- and agonist-induced vasodilation. *Am J Physiol Heart Circ Physiol* 1999;276:H1706–1714.
34. Long LH, Clement MV, Halliwell B. Artifacts in Cell Culture: Rapid Generation of Hydrogen Peroxide on Addition of (–)-Epigallocatechin, (–)-Epigallocatechin Gallate, (+)-Catechin, and Quercetin to Commonly Used Cell Culture Media. *Biochem Biophys Res Commun* 2000;273:50–53. [PubMed: 10873562]
35. Yang Z, Zhang A, Altura BT, Altura BM. Hydrogen peroxide-induced endothelium-dependent relaxation of rat aorta involvement of Ca²⁺ and other cellular metabolites. *Gen Pharmacol* 1999;33:325–336. [PubMed: 10523071]
36. Thomas SR, Chen K, Keaney JF Jr. Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J Biol Chem* 2002;277:6017–6024. [PubMed: 11744698]
37. Williams RJ, Spencer JP, Rice-Evans C. Flavonoids: antioxidants or signalling molecules? *Free Radic Biol Med* 2004;36:838–849. [PubMed: 15019969]
38. Choi EJ, Chee KM, Lee BH. Anti- and prooxidant effects of chronic quercetin administration in rats. *Eur J Pharmacol* 2003;482:281–285. [PubMed: 14660033]
39. Awad HM, Boersma MG, Vervoort J, Rietjens IM. Peroxidase-catalyzed formation of quercetin quinone methide-glutathione adducts. *Arch Biochem Biophys* 2000;378:224–233. [PubMed: 10860540]
40. Awad HM, Boersma MG, Boeren S, van Bladeren PJ, Vervoort J, Rietjens IM. Structure-activity study on the quinone/quinone methide chemistry of flavonoids. *Chem Res Toxicol* 2001;14:398–408. [PubMed: 11304128]
41. Boots AW, Kubben N, Haenen GR, Bast A. Oxidized quercetin reacts with thiols rather than with ascorbate: implication for quercetin supplementation. *Biochem Biophys Res Commun* 2003;308:560–565. [PubMed: 12914787]
42. Metodiewa D, Jaiswal AK, Cenas N, Dickancaite E, Segura-Aguilar J. Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. *Free Radic Biol Med* 1999;26:107–116. [PubMed: 9890646]
43. Canada AT, Giannella E, Nguyen TD, Mason RP. The production of reactive oxygen species by dietary flavonols. *Free Radic Biol Med* 1990;9:441–449. [PubMed: 1963417]
44. Cai H. Hydrogen peroxide regulation of endothelial function: Origins, mechanisms, and consequences. *Cardiovasc Res* 2005;68:26–36. [PubMed: 16009356]
45. Choi MH, Lee IK, Kim GW, Kim BU, Han Y-H, Yu D-Y, Park HS, Kim KY, Lee JS, Choi C, Bae YS, Lee BI, Rhee SG, Kang SW. Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* 2005;435:347–353. [PubMed: 15902258]
46. Panus PC, Radi R, Chumley PH, Lillard RH, Freeman BA. Detection of H₂O₂ release from vascular endothelial cells. *Free Radic Biol Med* 1993;14:217–223. [PubMed: 8425723]
47. Fitzpatrick DF, Hirschfield SL, Coffey RG. Endothelium-dependent vasorelaxing activity of wine and other grape products. *Am J Physiol* 1993;265:H774–H778. [PubMed: 8396352]
48. Ndiaye M, Chataigneau T, Lobysheva I, Chataigneau M, Schini-Kerth VB. Red wine polyphenols-induced, endothelium-dependent NO-mediated relaxation is due to the redox-sensitive PI3-kinase/Akt-dependent phosphorylation of endothelial NO-synthase in the isolated porcine coronary artery. *FASEB J*. 2004
49. Duarte J, Andriambeloson E, Diebolt M, Andriantsitohaina R. Wine polyphenols stimulate superoxide anion production to promote calcium signaling and endothelial-dependent vasodilatation. *Physiol Res* 2004;53:595–602. [PubMed: 15588126]
50. Fraile ML, Conde MV, Sanz L, Moreno MJ, Marco EJ, Lopez de Pablo AL. Different influence of superoxide anions and hydrogen peroxide on endothelial function of isolated cat cerebral and pulmonary arteries. *Gen Pharmacol* 1994;25:1197–1205. [PubMed: 7875545]
51. Doan TN, Gentry DL, Taylor AA, Elliott SJ. Hydrogen peroxide activates agonist-sensitive Ca(2+)-flux pathways in canine venous endothelial cells. *Biochem J* 1994;297(Pt 1):209–215. [PubMed: 8280101]

52. Klinge CM, Blankenship KA, Risinger KE, Bhatnagar S, Noisin EL, Sumanasekera WK, Zhao L, Brey DM, Keynton RS. Resveratrol and estradiol rapidly activate MAPK signaling through estrogen receptors alpha and beta in endothelial cells. *J Biol Chem* 2005;280:7460–7468. [PubMed: 15615701]
53. Li H, Förstermann U. Resveratrol: A Multifunctional Compound Improving Endothelial Function. *Cardiovascular Drugs and Therapy*. 2009
54. Wallerath T, Deckert G, Ternes T, Anderson H, Li H, Witte K, Forstermann U. Resveratrol, a polyphenolic phytoalexin present in red wine, enhances expression and activity of endothelial nitric oxide synthase. *Circulation* 2002;106:1652–1658. [PubMed: 12270858]
55. Wallerath T, Poleo D, Li H, Forstermann U. 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* 2003;41:471–478. [PubMed: 12575978]
56. Middleton E Jr, Kandaswami C, Theoharides TC. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 2000;52:673–751. [PubMed: 11121513]
57. Anter E, Thomas SR, Schulz E, Shapira OM, Vita JA, Keaney JF Jr. Activation of endothelial nitric-oxide synthase by the p38 MAPK in response to black tea polyphenols. *J Biol Chem* 2004;279:46637–46643. [PubMed: 15333638]
58. Frei B, Higdon JV. Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. *J Nutr* 2003;133:3275S–3284S. [PubMed: 14519826]
59. Cherubini A, Beal MF, Frei B. Black tea increases the resistance of human plasma to lipid peroxidation in vitro, but not ex vivo. *Free Radic Biol Med* 1999;27:381–387. [PubMed: 10468212]
60. Carbonneau MA, Leger CL, Monnier L, Bonnet C, Michel F, Fouret G, Dedieu F, Descomps B. Supplementation with wine phenolic compounds increases the antioxidant capacity of plasma and vitamin E of low-density lipoprotein without changing the lipoprotein Cu(2+)-oxidizability: possible explanation by phenolic location. *Eur J Clin Nutr* 1997;51:682–690. [PubMed: 9347289]
61. O'Donnell VB, Chumley PH, Hogg N, Bloodsworth A, Darley-Usmar VM, Freeman BA. Nitric oxide inhibition of lipid peroxidation: kinetics of reaction with lipid peroxy radicals and comparison with alpha-tocopherol. *Biochemistry* 1997;36:15216–15223. [PubMed: 9398249]
62. Kelley EE, Wagner BA, Buettner GR, Burns CP. Nitric oxide inhibits iron-induced lipid peroxidation in HL-60 cells. *Arch Biochem Biophys* 1999;370:97–104. [PubMed: 10496982]

Figure 1. Quercetin attenuates phenylephrine (PE)-induced contraction in rat aortic vessels
 Line graphs represent concentration-response curves of aortic ring segments to PE. **(A)** Contractile response of rat aortic ring segments incubated with vehicle control (DMSO), 5 and 10 μ M quercetin for 30 min. **(B)** Aortic rings were incubated with 100 μ M L-NAME for 15 minutes followed by DMSO or quercetin treatment for 30 minutes. Aortic ring segments were incubated with DMSO or 5 μ M quercetin for 30 min followed by an Ach dose response (3×10^{-9} – 3×10^{-5} M). Results are presented as mean $\pm$ SEM, n=8–14. #, $p < 0.05$, significant difference compared to control; *, $p < 0.05$, significant difference compared to all groups (control, L-NAME, and L-NAME and quercetin).

Figure 2. Quercetin stimulates eNOS phosphorylation at Ser¹¹⁷⁹, NO production, and cGMP
 Confluent BAECs were treated with 5 or 10 μ M quercetin for 30 and 60 min. (A) Top panel is a representative Western blot; bottom panel is the densitometric intensity determined digitally. (B) Quercetin treatment stimulated NO release from BAECs. NO generation was determined in quercetin-treated confluent BAECs for the indicated times using Sievers nitric oxide analyzer (NOA). The levels of nitrite have been normalized to protein concentration (basal NO₂⁻ = 1.04 $\pm$ 0.09 nmoles/mg protein) and are reported as a percent of the vehicle control (DMSO) at each individual time point (30, 60, and 120 min). A23187 was used as a positive control. (C) Rat aortic ring segments were incubated with or without 25 μ M BAPTA/AM for 30 min followed by the treatment of 5 μ M quercetin for 60 min. The aortic ring segments

were immediately snap frozen in liquid nitrogen and cGMP values were determined using the cGMP enzyme immunoassay assay from Cayman. Diethylenetriamine/NO (DETA/NO represented as D), a nitric oxide donor, was used as a positive control. Results were derived from at least four independent experiments and data were expressed as mean $\pm$ SEM. *, $p < 0.05$ vs control; &, $p < 0.05$ vs all groups.

Figure 3. Quercetin increases intracellular Ca^{2+} pools in BAECs while BAPTA/AM blocks quercetin-induced eNOS phosphorylation at Ser¹¹⁷⁹

(A) BAECs were loaded with the fluorescent probe fura2/AM (5 μM) for 15 minutes, rinsed with HBSS, placed in a recording chamber mounted on a fixed-stage upright microscope, equilibrated and then treated with 2.5 μM quercetin (left panel) or DMSO (right panel). A representative recording from a single coverslip showing Ca^{2+} traces from individual cells following the treatment of quercetin. Each run had 10–20 cells per experiment. The arrows indicate when quercetin and DMSO were added. Results were derived from at least seven independent experiments. (B) BAECs were pretreated with 25 μM BAPTA/AM for 30 min followed by a 5 μM quercetin treatment for 30 min. Top panel is a representative Western blot of eNOS phosphorylation at Ser¹¹⁷⁹ and eNOS was used as a loading control; bottom panel is the densitometric intensity determined digitally. Data is represented as the mean $\pm$ SEM, $n=4-6$. *, $p<0.05$ vs control

Figure 4. Overexpression of catalase inhibits quercetin-stimulated eNOS phosphorylation at Ser¹¹⁷⁹

(A) Catalase transduction using 12.5, 25, 50 MOI Ad.Cat significantly increased catalase activity in BAECs compared to Ad.Empty transduced controls. (B) Catalase transduced BAECs were treated with DMSO or quercetin for 30 min. Top panel is a representative Western blot of eNOS phosphorylation at Ser¹¹⁷⁹ and eNOS was used as a loading control; bottom panel is the densitometric intensity determined digitally. Results are expressed as mean $\pm$ SEM, n=6. Ø, no treatment; 25E, 25 MOI Ad.Empty; 50E, 50 MOI Ad.Empty. * P <0.05, significant difference compared to all groups (non-transduced controls, 25 and 50 Ad.Empty transduced BAECs); *, p <0.05 vs Ad.Empty transduced BAECs treated only with DMSO; %, p <0.05-

quercetin vs DMSO for respective adenovirus control (i.e.- BAECs transduced with 50 MOI Ad.*Empty* treated with quercetin compared to DMSO).

Figure 5. Quercetin treatment induces EDHF

Rat aortic ring segments were equilibrated (basal) and then contracted with 70 mM KCl. The rings were rinsed and allowed to re-equilibrate back to baseline and then treated with or without 50 nM charybdotoxin (ChTx) for 30 min. Immediately after, the rings were contracted with another dose of 70 mM KCl (maximal contraction) followed by 5 μ M quercetin treatment for 30 min in intact (A) and denuded vessels (B). At the end of the experiments, acetylcholine (Ach) dose responses were performed to ensure maximal relaxation. Results are presented as mean $\pm$ SEM, n=8–14. *, p<0.05, significant difference compared to all groups.