

Chlorogenic acid improves *ex vivo* vessel function and protects endothelial cells against HOCl-induced oxidative damage, via increased production of nitric oxide and induction of Hmox-1

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Abstract

Dietary polyphenols are potential contributors toward improved cardiovascular health. Coffee is one of the richest sources of dietary polyphenols in a coffee-drinking population, the most abundant form being chlorogenic acid (CGA). Endothelial dysfunction is an early and major risk factor for cardiovascular disease. Nitric oxide (NO) is a key factor in regulation of endothelial function. Heme oxygenase-1 (Hmox-1), an inducible isoform of heme oxygenase that is produced in response to stressors such as oxidative stress, may also play a role in vascular protection. The aim of this study was to investigate the effect of CGA on endothelial function with oxidant-induced damage in isolated aortic rings from C57BL mice. We further examine the mechanism by investigating cell viability, activation of eNOS and induction of Hmox-1 in human aortic endothelial cells (HAECs). We found that pretreatment of isolated aortic rings with 10- μ M CGA-protected vessels against HOCl-induced endothelial dysfunction ($P<0.05$). Pretreatment of cultured HAECs with 10- μ M CGA increased endothelial cell viability following exposure to HOCl ($P<0.05$). Moreover, CGA increased NO production in HAECs in a dose-dependent manner, peaking at 6 h ($P<0.05$). CGA at 5 μ M and 10 μ M increased eNOS dimerization at 6 h and induced Hmox-1 protein expression at 6 h and 24 h in HAECs. These results are consistent with the cardiovascular protective effects of coffee polyphenols and demonstrate that CGA can protect vessels and cultured endothelial cells against oxidant-induced damage. The mechanism behind the beneficial effect of CGA appears to be in part via increased production of NO and induction of Hmox-1.

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1. Introduction

Cardiovascular disease (CVD) is the leading cause of disability and death, representing 30% of global mortality, which contributes to the escalating costs of health care [1,2]. Lifestyle factors, such as unhealthy diet, physical inactivity and smoking can contribute toward cardiovascular risk [3].

Evidence from epidemiological research suggests that coffee consumption is associated with a lower risk of several chronic diseases including Type 2 diabetes, CVD and cancer, as well as neurodegenerative conditions such as Parkinson disease [4–6]. The majority of these studies are cross-sectional or longitudinal associations with self-reported coffee consumption, and there is limited evidence on the potential mechanisms that are involved [7].

There is considerable epidemiological evidence that diets rich in fruit and vegetables can reduce the incidence of noncommunicable diseases such as CVD, diabetes, cancer and stroke [8]. These protective effects are attributed, in part, to polyphenols, which are secondary metabolites in the plant [9]. Coffee is one of the richest sources of

polyphenols with the most abundant form being chlorogenic acid (CGA), which accounts for approximately 80% of total polyphenols [7]. In a coffee-drinking population, coffee is a major contributor to dietary polyphenol intake [10].

Endothelial dysfunction is considered as an important early biomarker for the development of CVD and events [11]. The endothelium synthesises and releases different molecules that orchestrate metabolic, vascular and cellular responses [12]. One of the most important compounds is nitric oxide (NO), which is produced by endothelial nitric oxide synthase (eNOS). NO is an endogenous vasodilatory gas that continually regulates the diameter of blood vessels and maintains an antiproliferative and antiinflammatory environment in the vessel wall [13–15]. Notably, reduced eNOS expression and/or NO bioavailability are associated with decreased endothelial cell survival and endothelial dysfunction [16]. Polyphenols from various fruits and vegetables, such as red wine, green tea and apple, have been shown to activate eNOS and induce endothelium-dependent vessel relaxation [17–19].

Oxidative stress has been suggested to play a critical role in the development of endothelial dysfunction, possibly via reduced bioavailability of NO [16]. Potent oxidants such as HOCl, produced by myeloperoxidase, can induce endothelial dysfunction which can lead

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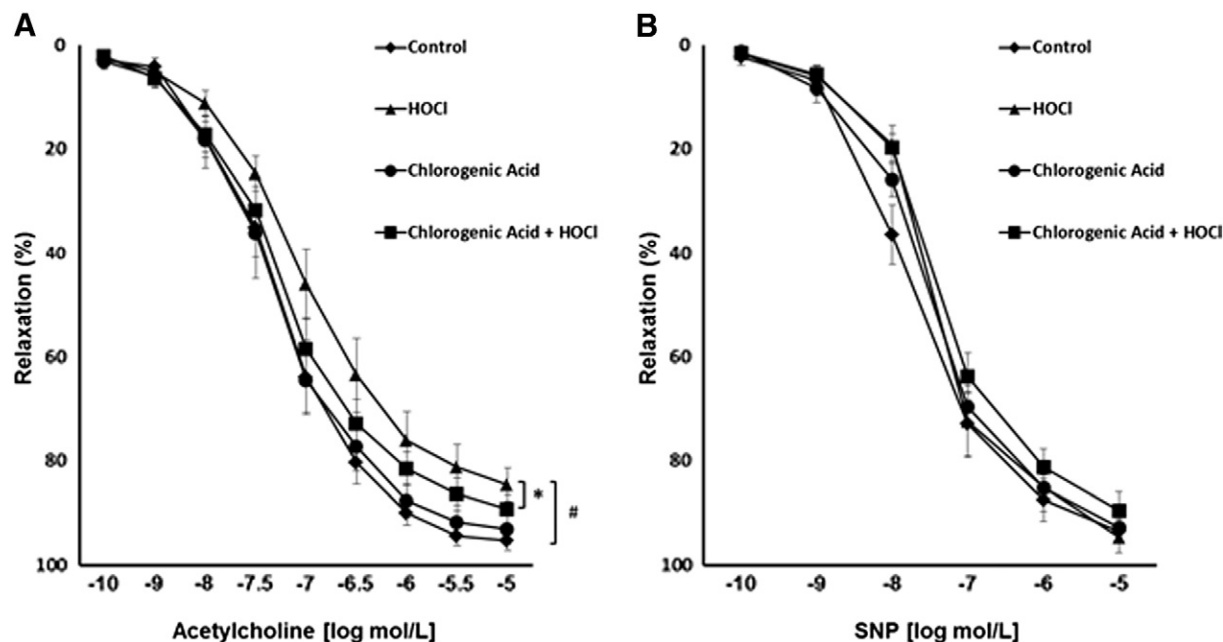


Fig. 1. CGA protects aortic rings against HOCl-induced endothelial dysfunction in *ex vivo* vessel function assessment. (A) Acetylcholine-mediated relaxations of mouse abdominal aortic rings with or without 10- μ M CGA pretreatment for 6 h and subsequent exposure to 100- μ M HOCl (60 min); (B) SNP-mediated relaxations of mouse abdominal aortic rings with or without 10- μ M CGA pretreatment for 6 h and exposure to 100- μ M HOCl (60 min). Data shown are mean $\pm$ S.E.M. * P <0.05; # P <0.01. n =6.

to eNOS dimer instability and subsequent increased superoxide formation [20]. Heme oxygenase-1 (Hmox-1) is an inducible isoform of heme oxygenase that is produced in response to stressors such as oxidative stress and may provide vascular protection [21]. Various polyphenols have been shown to provide antioxidant and anti-inflammatory protection via the induction of Hmox-1 [22,23]. The most recognized mechanism of Hmox-1 expression is through the Nrf2-ARE (antioxidant response elements) pathway [24]. CGA has been found to contribute to Nrf2 nuclear translocation and ARE-dependent gene expression [25].

In order to better understand the potential protective role of coffee polyphenols on the vasculature, we investigated the effects of CGA in mouse aortic rings and in human aortic endothelial cells (HAECs) exposed to oxidative damage by HOCl. We hypothesised that CGA would protect vessels and endothelial cells against oxidant-induced endothelial dysfunction by activating eNOS, increasing NO production and inducing Hmox-1 expression.

2. Materials and methods

2.1. Preparation of CGA and HOCl

CGA (Sigma-Aldrich, Australia) was dissolved in DMSO and diluted in ultrapure water to a final DMSO concentration of 0.1%. Notably, while Sigma lists this product as 3-O-CGA, it is actually 5-O-CGA according to correct IUPAC nomenclature. HOCl solution was freshly prepared by dilution of a concentrated stock solution of sodium hypochlorite (Sigma-Aldrich, Australia). To determine HOCl concentrations, the optical absorbance at 292 nm and pH 11 was measured using a molar extinction coefficient of 350 M⁻¹ cm⁻¹.

2.2. *Ex vivo* vessel function assessment

Aortic rings from 10-week-old C57BL/6J mice were isolated following anaesthesia by inhalation of methoxyflurane (Medical Developments International) and perfusion of the animal with 0.9% saline. Abdominal aortas were then incubated with 10- μ M CGA or vehicle for 6 h before the aortas were cut into equal segments, approximately 2-mm long. All rings were mounted onto a multimyography system (620 M, DMT, Denmark) and allowed to equilibrate for 30 min in Krebs-Henseleit modified buffer, aerated with 95% O₂/5% CO₂ at 37°C, under nonstretched conditions. The aortic rings were then primed with KPSS buffer (containing 123.70-mM KCl, 1.17-mM MgSO₄, 1.18-mM KH₂PO₄, 2.50-mM CaCl₂, 25.00-mM NaHCO₃, 0.03-mM EDTA and 5.50-mM glucose) and

mechanically stretched in a step-wise procedure to 9 mN, which is similar to physiological tension and considered to be the standardised baseline reading. The rings were then incubated for 1 h with HOCl (100 μ M) or vehicle [20]. Rings were then washed and subject to increasing doses of phenylephrine (Phe) (10⁻⁹–10⁻⁵ M; Sigma Aldrich, Australia) to achieve a stable precontraction. Following this, increasing doses of ACh (10⁻¹⁰–10⁻⁵ M; Sigma Aldrich, Australia) were added, and relaxation as a percentage of precontraction was determined. Precontraction with Phe was then repeated, and increasing doses of the NO-donor, sodium nitroprusside (SNP) (10⁻¹⁰–10⁻⁵ M; Sigma Aldrich, Australia) were added with relaxation recorded to determine smooth muscle viability. The data from each aortic ring were analysed using LabChart (AD Instrument, Australia). Results are presented as means $\pm$ S.E.Ms.

The use of animals was approved by the Royal Perth Hospital Animal Ethics Committee. All animal experiments were compliant with the National Health and Medical Research Council (NHMRC) guidelines for the Care and Use of Laboratory Animals in Australia.

2.3. Cell culture

HAECs (Lonza Pty, Australia) were maintained in commercially available EBM-2 media (Lonza Pty, Australia). Cells were grown in T-75 flasks and passaged every 3–4 days as necessary. All experiments were performed on confluent cells between passages 4 and 10 in 6-well culture plates. Prior to experiments, cells were serum-starved for 6 h prior to treatment with CGA or vehicle (0.1% DMSO) for 2, 6 or 24 h. Cell lysates and culture media were collected and stored at -80°C until analysis.

2.4. Measures of cell viability after CGA treatment and HOCl-induced oxidative damage (MTS assay)

Following pretreatment of cells with CGA as described above, media was then removed, and cells were washed twice with Krebs-Henseleit modified buffer. Cells were then exposed to 100- μ M HOCl for 30 min and allowed to be stabilised in fresh buffer for 1 h. The cells were washed twice, and then, 500 μ l of buffer was added to each well along with 100 μ l of CellTiter 96 Aqueous One Cell Solution Reagent (Promega, Australia). Plates were returned to the incubator for 30 min. Two 100- μ l aliquots from each well were transferred to a clear 96-well plate, and absorbance at 490 nm was read by a spectrophotometer. A higher reading indicates more formation of formazan and, therefore, increased cell viability.

2.5. Hmox-1 and eNOS expression and phosphorylation

To determine protein expression following treatment, cells were washed once with ice-cold PBS before being lysed and collected in 200- μ l lysis buffer, containing Laemmli's sample loading buffer (Bio-Rad, Australia), 5% β -mercaptoethanol and 10 $\times$ phosphatase inhibitor cocktail (PhosSTOP, Roche Diagnostics, Australia). Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 7.5% gels,

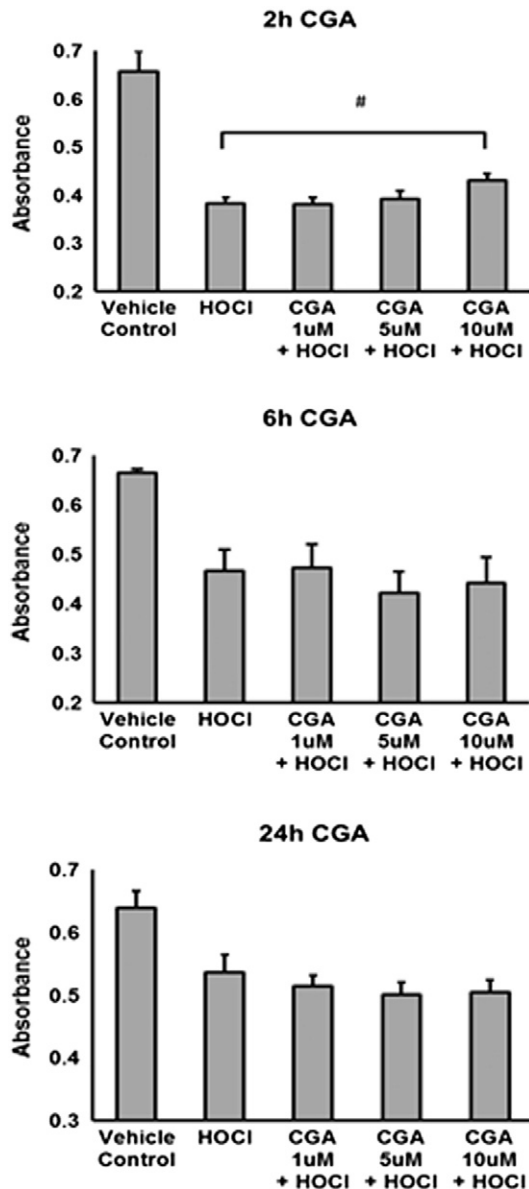


Fig. 2. Cell viability in HAECs with or without pretreatment of 1- μ M, 5- μ M or 10- μ M CGA for 2 h, 6 h and 24 h and exposure to 100- μ M HOCl (30 min). Data shown are mean $\pm$ S.E.M. $^{\#}P < 0.01$, $n = 6$.

transferred to a nitrocellulose membrane (GE Healthcare Life Sciences, USA) for 2 h and the membranes incubated with primary antibody overnight at 4°C. After washing and incubation with HRP-conjugated secondary antibody for 1 h at room temperature, protein bands were visualized using ECL reagents (GE Healthcare Life Sciences, USA) on a Flurochem FC2 (Alpha Innotech, USA). Protein bands were analysed by densitometry using the Flurochem software and normalized to Actin (loading control, Sigma–Aldrich, Australia). Antibodies against eNOS and p-eNOS (Ser1177) were purchased from Cell Signalling Technology, USA. Antibodies against Hmox-1 were purchased from Enzo Life Science, USA.

2.6. Assessment of eNOS monomer/dimer

eNOS dimerization was determined by low-temperature SDS-PAGE [26]. In brief, cells harvested from plates were lysed with ice-cold lysis buffer (100-mM Tris–HCl, pH 7.6, 0.5% NP-40, 1% Triton X-100, 0.5% sodium deoxycholate and 1 $\times$ phosphatase inhibitor cocktail). Lysates were centrifuged at 14,000 $\times$ g for 10 min at 4°C, and the supernatants were obtained for eNOS dimerization assay. Protein samples were mixed with an equal volume of ice-cold 2 $\times$ loading buffer. Samples were run on a 7.5% SDS-PAGE gel in prechilled 1 $\times$ SDS running buffer (Bio-Rad, Australia) and then transferred

in prechilled 1 $\times$ transfer buffer (Bio-Rad, Australia) at 4°C. The membranes were incubated with the eNOS antibody as described in Section 2.5.

2.7. NOx analysis

NOx (S-nitrosothiols, nitrite and nitroso species) in cell culture media were quantified by reductive denitrosation of samples using a mixture of iodine/iodide in glacial acetic acid and subsequent detection of the liberated NO by gas-phase chemiluminescence following reaction with ozone [27,28]. For calculation of the AUC of NO peaks, raw data from the chemiluminescence NO analyser (CLD 60, Eco Physics, Australia) was processed using PowerChrom software. Sodium nitrite external standards were used for quantification.

2.8. Metabolism of CGA by cultured endothelial cells

For the analysis of CGA metabolism, media samples were thawed, and the metabolites were extracted using a solid-phase extraction (SPE) method [29]. Bond Elut C18 500-mg cartridges were first conditioned with 2 ml of methanol followed by 2 ml of water. A 2 ml of media sample was spiked with 5 μ g of internal standard (1-hydroxy-2-naphthoic acid) used as a reference. The samples were acidified by adding 2 ml of 2% formic acid and then vortexed. After acidification, samples were applied to SPE cartridges and washed with water to remove any water-soluble interfering substances. Compounds were eluted with methanol and collected in a glass tube. The methanol was evaporated under a stream of nitrogen, and the residue was then reconstituted with 100- μ L methanol. The sample was then analysed by reverse-phase high-pressure liquid chromatography (HPLC) using a Agilent C18 250 $\times$ 4.5-mm column and a mobile phase as follows: A, 2% formic acid; B, acetonitrile at a flow rate of 1000 μ L/min, initiated at 10% of solvent B, then ramped to 100% from 5 to 15 min, then A (100%) from 16 to 25 min. Peaks were detected by UV detection at 325 nm.

Metabolites were further analysed using a Thermo Scientific TSQ QuantumUltra Triple Quadrupole LC-MS System (ThermoFisher Scientific) equipped with electrospray ionization source operated in negative-ion mode using a Zorbax eclipse XDB C18 2.1-mm $\times$ 100-mm/3.5- μ m column. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was performed as described previously [29]. CGAs and its metabolites were detected using the multiple reaction monitoring (MRM) mode based on retention time (Rt) and distinct MS² fragment patterns [MRM 353-191 collision energy (CE) 19eV, MRM 367-193 CE 15 eV, MRM 367-134 CE 15eV] [30–32]. The mobile phases were as follows: A, 2-mM ammonium acetate, pH 3.6; and B, methanol at a flow rate of 600 μ L/min. Chromatography was achieved using solvent A (100%) from 0 to 5 min, then ramped to B (100%) from 5 to 15 min and, then, A (100%) from 16 to 20 min. All solvents were of high-performance liquid chromatography (HPLC) grade. Water was obtained with Arium 611 VF water purification system (Startorius stedim biotech, Aubagne, France). CGA and metabolite extracted from culture media were also hydrolysed in the presence of ascorbic acid following the method of Nardini et al. [33] for subsequent HPLC analysis of phenolic acids.

2.9. Statistical analysis

Numerical data are presented as mean $\pm$ S.E.M. Comparisons between treatment groups were performed using one-way ANOVA or paired t tests as appropriate. Data from aortic ring studies were analyzed with mixed models in SAS using the PROC MIXED command. The overall effect of treatment was established using the P value for the effect of treatment. All analyses were carried out using the SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and SAS 9.2 (SAS institute Inc., Cary, NC, USA).

3. Results

3.1. CGA protects aortic rings against HOCl-induced endothelial dysfunction

Treatment with HOCl alone inhibited ACh-mediated relaxation, demonstrating oxidant-induced endothelial dysfunction. Incubation with 10- μ M CGA for 6 h significantly increased ACh-mediated endothelial-dependent relaxation following HOCl treatment (Fig. 1A). The presence of CGA had no effect on vessel relaxation in response to the NO-donor SNP (Fig. 1B).

3.2. CGA increases cell viability in HAECs following exposure to HOCl

Cell viability was significantly decreased following HOCl exposure. Pretreatment of cultured HAECs with 10- μ M CGA for 2 h increased endothelial cell viability following exposure to HOCl as compared to the HOCl exposure group ($P < 0.05$) (Fig. 2). The protective effect was not observed with 6 h and 24 h treatments.

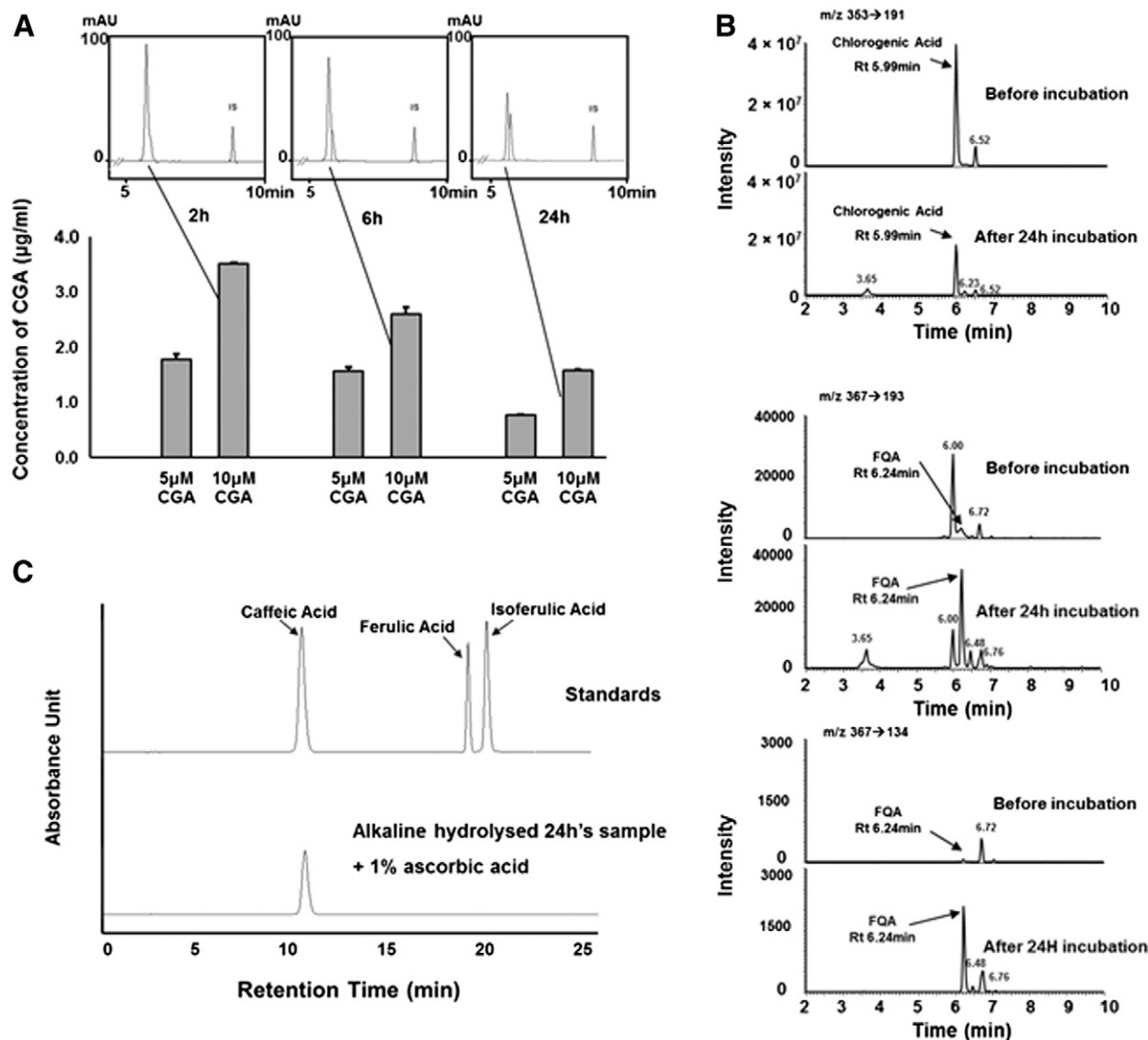


Fig. 3. Metabolism of CGA by cultured endothelial cells. (A) CGA concentration in cell culture media after 5- μ M or 10- μ M CGA treatment for 2 h, 6 h and 24 h using HPLC. Sample trace from the analysis of 10- μ M CGA treatment for 2 h, 6 h and 24 h was shown. (B) LC/MS/MS chromatogram trace of CGA and its metabolites FQAs identification by Rt and MRM transition of molecular ions: m/z 353 $\rightarrow$ 191 (CGA), and 367 $\rightarrow$ 193, 367 $\rightarrow$ 134 (FQA), at baseline and 24 h after incubation in cell culture media. Intensity of CGA peak (Rt, 5.99 min) decreased in a 24-h sample as compared to baseline. Intensity of FQA peak (Rt, 6.24 min) increased in a 24 h sample as compared to baseline. (C) HPLC analysis of hydrolysed CGA with or without protection by 1% ascorbic acid to generate the phenolic acid. Caffeic acid could be recovered with protection by ascorbic acid, whereas no ferulic or isoferulic acid could be identified as compared to standards.

3.3. Metabolism of CGA by cultured endothelial cells

To help explain the loss of protection at longer incubation times, we examined the breakdown or metabolism of CGA by HAECs. There was an increase in the breakdown of CGA over time with subsequent formation of a metabolite as determined by HPLC analysis (Fig. 3A). Mass spec analysis suggested that this metabolite might be feruloyl quinic acids (FQA) or isoferuloyl quinic acids [30,31] (Fig. 3B). To confirm the identity, we hydrolysed the products to generate the phenolic acid which were then analysed by HPLC. However, no ferulic or isoferulic acid could be identified (Fig. 3C), suggesting that the concentration of the metabolite may be too low to generate the phenolic acid after hydrolysis or it may have been methylated on the quinic acid residue rather than the phenolic residue.

3.4. CGA increases NOx level in HAECs culture media

Total NOx, including S-nitrosothiols, nitrite and nitroso species, reflect NO production and can also act as a physiological pool of NO

[34]. Sample trace record from the chemiluminescence NO analyser was shown (Fig. 4A). Following serum starvation, HAECs were treated for 2 h, 6 h or 24 h with CGA, and the cell media were collected and analysed for total NOx (Fig. 4B). Total NOx level significantly increased following treatment with 10- μ M CGA at 2 h. At 6 h, both 5- μ M and 10- μ M CGA significantly increased NOx production, while no increase was observed at 24 h. These results suggest that CGA can increase NO production in endothelial cells in a time-dependent and dose-dependent manner, peaking at 6 h. The 24 h data tie in with the previous data showing that CGA breaks down over 24 h.

3.5. Increase of NOx level in HAECs is via increased formation of eNOS dimer, but not via phosphorylation of eNOS

Treatment of HAECs with CGA at 5 μ M and 10 μ M for 2 h, 6 h or 24 h had no significant effect on eNOS phosphorylation at Ser1177 site compared to serum-starved cells alone (Fig. 5). However, eNOS dimer formation/stability was positively influenced by CGA treatment after 6-h incubation in a dose-dependent manner (Fig. 6).

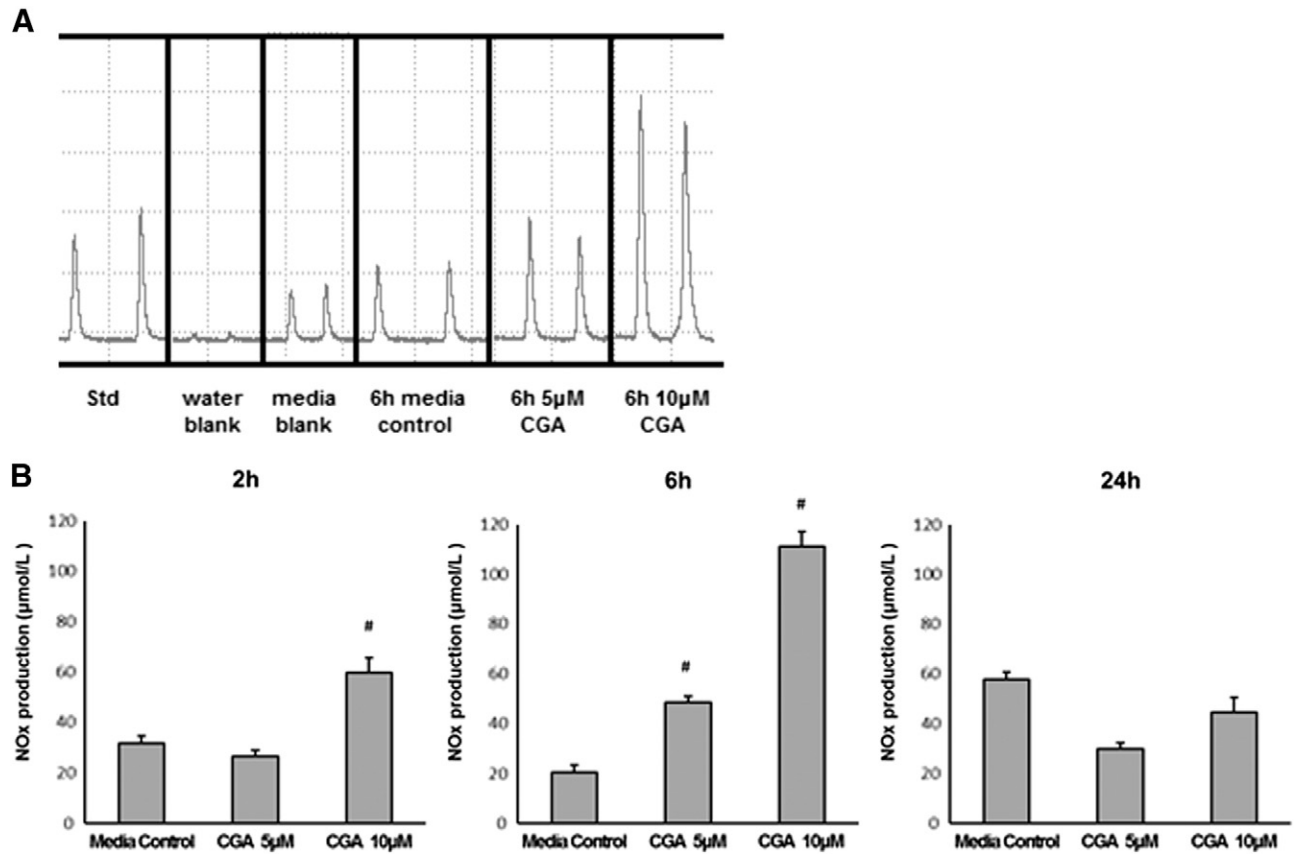


Fig. 4. NOx level in the culture media of serum-starved HAECs following treatment with 5-µM or 10-µM CGA for 2 h, 6 h and 24 h. (A) sample trace record from the chemiluminescence NO analyser; (B) 2 h, 6 h and 24 h quantified NOx production by CGA. All sample analysis was done in duplicates. Data shown are mean $\pm$ S.E.M. [#] $p < 0.01$ versus control; $n = 3$.

3.6. CGA induced Hmox-1 protein expression in HAECs

There was no significant increase in Hmox-1 expression at 2 h. At 6 h and 24 h, treatment of HAECs with CGA at 5 µM and 10 µM caused a significant increase in Hmox-1 protein expression, compared to serum-starved cells alone, in a dose-dependent and time-dependent manner (Fig. 7).

4. Discussion

The present study has shown that CGA can protect vessels against HOCl-induced endothelial dysfunction, a model of physiologically relevant oxidative damage [20,35]. CGA was also able to protect cultured endothelial cells against oxidant-induced damage. The protection was associated with increases in eNOS stability and NO production. CGAs are a family of nonflavonoid polyphenolic compounds and are particularly rich in coffee, with a single shot of espresso from commercial outlets providing between 24 and 422 mg [36,37]. The work presented here suggests a molecular mechanism of action on how CGA may be acting on endothelial cells and improving vascular function. The associated changes in eNOS stability and NO production suggest that the beneficial effects of CGA on the endothelium are mediated in part by an increase in NO bioavailability and/or bioactivity. The increase in Hmox-1 expression in the presence of CGA suggests that the protective effects may also be mediated in part via oxidative stress/Hmox-1 pathway.

The results of epidemiological and intervention studies on the effects of coffee on CVD risk varies with a mix of beneficial and harmful effects being reported. Nevertheless, long-term studies suggest that harmful effects are unlikely for moderate coffee consumption and it is

possible that polyphenolic compounds in coffee counteract the negative effects of caffeine and diterpenes [7]. In healthy subjects, decaffeinated coffee consumption is associated with an improved flow-mediated dilation response [38], suggesting that polyphenolic components in coffee may play an important role in improvement of endothelial function.

Endothelial dysfunction is considered as an important early biomarker for the development of CVD and cardiovascular events [11]. Endothelium-derived NO is a critical regulator of cardiovascular homeostasis that continually regulates the diameter of blood vessels and maintains an antiproliferative and antiinflammatory environment in the vessel wall [13–15]. Red wine polyphenols have been shown to increase eNOS expression in human umbilical vein endothelial cells [17]. Green tea polyphenols have also been shown to induce eNOS-mediated endothelium-dependent relaxation in rat aortic rings [18]. We have previously found that quercetin, a dietary flavonoid, can improve vessel function by inducing eNOS activity via phosphorylation of AMP-dependent kinase (AMPK) [19]. CGA was also reported to improved glucose and lipid metabolism in mice through the activation of AMPK [39]. Moreover, intake of CGA improved vascular function in spontaneously hypertensive rats which was associated with increased urinary excretion of NO metabolites [40]. Another polyphenolic compound syringaresinol has been shown to increase eNOS dimerization by activating AMPK [41].

The above findings are in agreement with our *ex vivo* vessel function study where significant improvements in endothelial-dependent relaxation were observed following treatment with CGA. This polyphenolic compound was able to reduce endothelial dysfunction induced by HOCl, a myeloperoxidase-derived oxidant [20,42], at the plausible pathophysiological concentration of 100 µM [43,44]. The

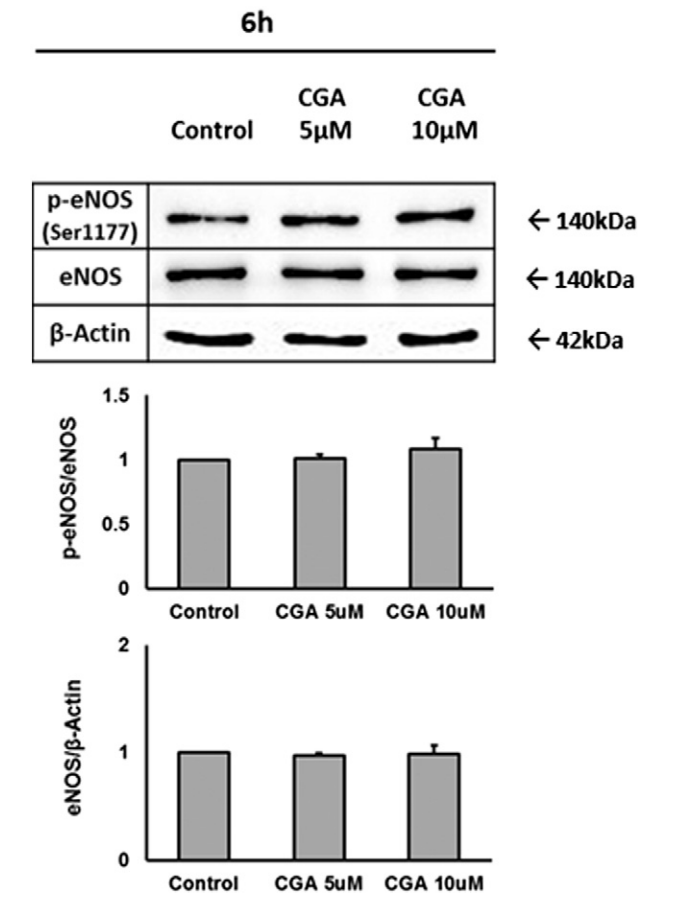


Fig. 5. eNOS phosphorylation at Ser1177 site as a ratio to total eNOS expression after 5-μM or 10-μM CGA treatment for 6 h. Data shown are mean±S.E.M. n=4. (Two hour's and 24 h's data are not shown.)

presence of HOCl-modified proteins in human atherosclerotic lesions implicates this oxidant in LDL modification *in vivo* [35]. Stocker *et al.* [20] has previously demonstrated that HOCl impairs endothelial

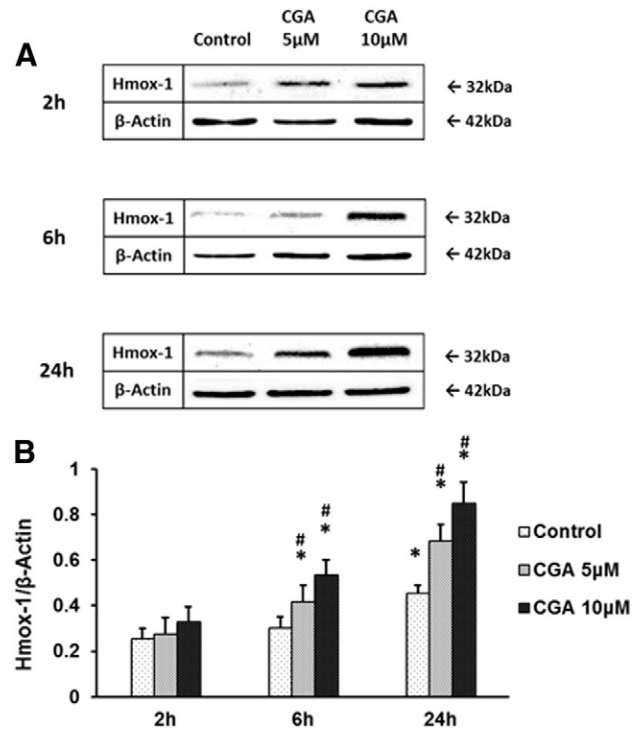


Fig. 7. Cellular Hmox-1 expression after 5-μM or 10-μM CGA treatment for 2 h, 6 h and 24 h. Data shown are mean±S.E.M. *P<0.05 versus 2 h control; #P<0.05 versus control at same time point. n=4–5.

function and is likely due to a superoxide-mediated decrease in NO and subsequent eNOS dimer instability. The HOCl-induced endothelial dysfunction was consistent with this previous study. However, we observed significant decrease of cell viability following exposure to 100-μM HOCl for 1 h, indicating that loss of endothelial cell viability is another mechanism by which HOCl impairs vessel function. In the present study, we have shown that CGA can enhance endothelial cell viability following HOCl-induced oxidative damage. This was

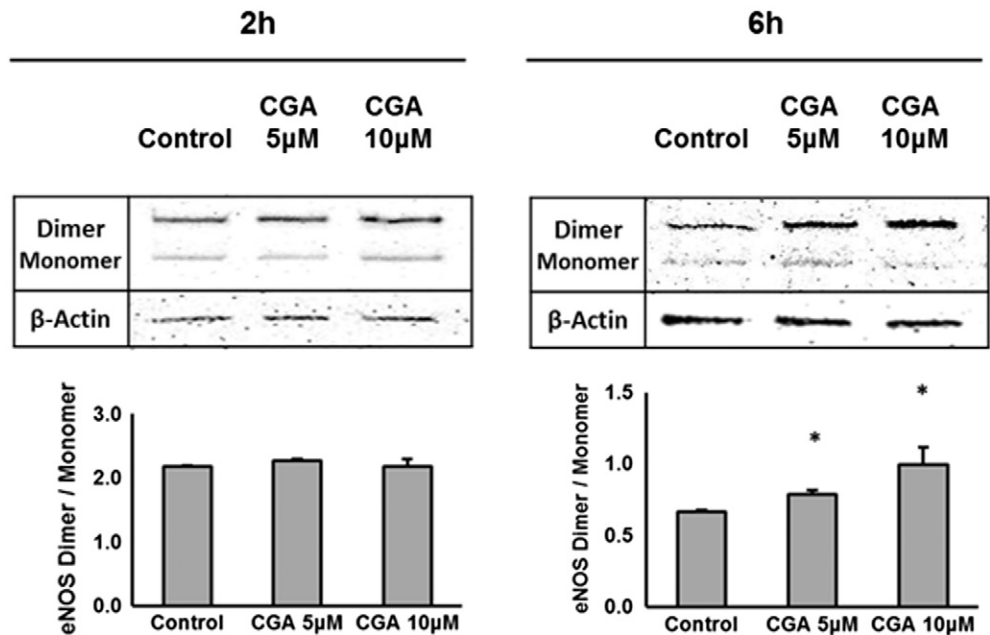


Fig. 6. eNOS dimer and monomer expression after 5-μM or 10-μM CGA treatment for 2 h and 6 h. Data shown are mean±S.E.M. *P<0.05 versus control. n=4.

associated with significant increases in NO release into culture media, possibly via activation of eNOS. This activation was indicated by increased eNOS dimerization, although there were no effects on phosphorylation of eNOS at Ser1177 site. It is interesting to note that we saw increases in eNOS activity without increased phosphorylation at Ser1177; a recent study suggests that eNOS dimerization is regulated by heat shock protein 90 rather than by phosphorylation [45]. Therefore, it is likely that CGA in part exerts its benefits through an eNOS/NO pathway.

We also tried to explain the loss of protection at longer incubation times in cell viability test by analyzing CGA and its metabolites after incubation with endothelial cells over different time periods. We observed a significant decrease in CGA concentration in a time-dependent manner by HPLC analysis. The subsequent LC/MS² results indicated the metabolite to be FQA; however, we were unable to confirm this with subsequent hydrolysis and HPLC analysis of phenolic acids (Fig. 3). A previous study did not observe endothelial protection by green coffee bean extract which was rich in CGA in mice fed with a high-fat diet (HFD) [46], suggesting that the metabolism, the bioavailability and the structure of CGA may play critical roles in its function *in vivo*, and further studies addressing the mechanism are required.

Alleviation of oxidative stress has been shown to improve endothelial dysfunction [47]. CGA has been found to act as an antioxidant *in vitro*; by directly scavenging a number of reactive oxygen species (ROS) [48], although this process is unlikely to occur *in vivo* [23]. The dose of CGA we used in this study is at low micromolar level (i.e., 5 μ M, 10 μ M) which is estimated to be supraphysiological [36,37], and this is a limitation to our study. Hmox-1 exerts an antiinflammatory and antioxidant action within the vasculature [49]. The beneficial effects of Hmox-1 on endothelial function and vascular repair are mediated by its three by-products, carbon monoxide, ferric ion and biliverdin, via multiple mechanisms [21,50–53]. Its induction can protect against ROS-induced oxidative damage and may modulate endogenous cellular ROS generation, further protecting endothelial cells against oxidative damage [54]. Pharmacological inducers of Hmox-1, such as probucol, have been shown to protect against vascular disease in various animal models of atherosclerosis [55]. Several polyphenols have been shown to provide vascular protection via the induction of Hmox-1 [22,44]. It is more likely that dietary polyphenolic compounds act *in vivo* by boosting antioxidant enzymes for damage removal and repair systems [23]. One such protective enzyme induced through Nrf2-ARE pathway is Hmox-1 [23,24]. Activation of AMPK has been associated with increased Hmox-1 expression via Nrf2-ARE pathway [24,56]. Arterial induction of Hmox-1 can protect eNOS from oxidative damage [55], and in turn, increased NO can induce Hmox-1 [57]. It has recently been shown that specific overexpression of Hmox-1 in adipocytes can attenuate vascular dysfunction in mice fed with an HFD [58]. CGA has recently been reported to contribute to Nrf2 nuclear translocation and ARE-dependent gene expression [25]. In our study, we have shown that CGA can increase the expression of Hmox-1 in HAECs. Together with the vessel function results, our data suggest that CGA is able to attenuate endothelial dysfunction, and these actions are likely to be mediated in part via oxidative stress/Hmox-1 pathway.

In conclusion, the present study has demonstrated that CGA was able to protect against HOCl-induced endothelial dysfunction in isolated mouse aortic rings and enhance endothelial cell survival following HOCl-induced oxidative damage. We also found that CGA increased production of NO, activated eNOS by increasing eNOS dimer formation/stability and induced Hmox-1 expression in HAECs. This suggests that the beneficial effects of CGA on vascular function may in part be mediated via eNOS/NO pathway and oxidative stress/Hmox-1 pathway. These results provide further evidence for the cardio-protective effects of this dietary polyphenol.

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