Research Communication
Low nanomolar caffeic acid attenuates high glucose-induced endothelial dysfunction in primary human umbilical-vein endothelial cells by affecting NF-κB and Nrf2 pathways

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Abstract

Hyperglycemia contributes to dysregulate endothelial function associated with diabetes, leading to initiation and propagation of vascular complications and dysfunction. Caffeic acid (CA), a dietary hydroxycinnamic acid abundant in coffee, has been reported to exert antidiabetic effects in rat models. Herein, we investigated the molecular effects of physiological concentrations of CA (10 nM) against endothelial dysfunction induced by high glucose (HG) in human endothelial cells (HUVECs). HUVECs were exposed to HG 25 mM, to mimic diabetic condition, in presence of CA. Intracellular redox status (reduced glutathione, superoxide dismutase (SOD) and total antioxidant activity levels), and NF-κB pathway were examined. We also evaluated the involvement of NF-E2-related factor 2 (Nrf2)/electrophile responsive element (EpRE) pathway. Our data show that CA inhibits HG-induced nuclear translocation of NF-κB and the downstream expression of endothelial adhesion molecule 1 and restores antioxidant levels by upregulating Nrf2/EpRE pathway. Our data suggest that CA can suppress several aspects of HG-induced endothelial dysfunction through the modulation of intracellular redox status controlled by the transcription factor Nrf2. These findings highlight that low physiological concentration of CA achievable specifically upon food consumption are able to prevent endothelial dysfunction associated with inflammation and oxidative stress induced by high concentration of glucose. © 2016 BioFactors, 43(1):54–62, 2017

Keywords: caffeic acid; cellular adaptive response; endothelial dysfunction; high glucose; Nrf2; oxidative stress

Abbreviations: CA, caffeic acid; EpRE, electrophile responsive element; E-selectin, endothelial adhesion molecule 1; GSH, reduced glutathione; HO-1, heme oxygenase-1; HUVECs, human umbilical vein endothelial cells; HG, high glucose; NF-κB (p65), nuclear factor kappa-light-chain-enhancer of activated B cells; Nrf2, NF-E2-related factor 2; ROS, reactive oxygen species; SOD, superoxide dismutase; TAA, total antioxidant activity

1. Introduction

Diabetes mellitus (type 2 diabetes, T2D) is a complex metabolic disorder characterized by chronic dysregulation of glucose metabolism and therefore by high levels of blood glucose (high glucose, HG). T2D is often associated with severe long term pathological consequences, such as, structural and functional abnormalities of heart, brain, and kidney, and to a higher risk for the development of cardiovascular disease [1], the main cause of morbidity and mortality in developed countries [2]. HG plays a critical role in the development of diabetic vascular complications, such as atherosclerosis and blindness. In fact, HG causes damage at the level of vascular endothelial cells, which are the first barrier of vessels, and dysregulates endothelial function leading to the initiation and propagation of further vascular complications [3]. Since damage of the
vasculature is one of the most evident hallmarks of diabetic complications, several studies have investigated the adverse effects of HG on vascular function using cellular models. Although, the underlying mechanisms in diabetes-related endothelial dysfunction are not fully understood, there is evidence that HG-induced endothelial dysfunction is often resulting from overproduction of reactive oxygen species (ROS) like hydrogen peroxide (H2O2) and superoxide radical (O2-) [4]. At molecular level, HG has been reported to impair cellular responses associated to an increased permeability, [5] and to a generalized condition of vascular inflammation, eventually leading to cell death [6]. Epidemiological studies suggested associations between the consumption of polyphenol-rich food and beverages and the prevention of many human diseases, including cardiovascular diseases, cancer, and chronic inflammation [7]. Hydroxycinnamic acids are among the main phenolic compounds derived from fruits, vegetables, and grains. Commonly found in the esterified form with quinic acid, they have attracted considerable interest thanks to their various biological activities, including putative antioxidant and anti-inflammatory capacities. Caffeic acid (3,4-dihydroxycinnamic acid, CA) is one of the most representative hydroxycinnamic acids in the diet, being present in many foods and beverages, including coffee. More recently, many studies have demonstrated the potential health benefits of daily coffee consumption, and several epidemiological studies reported that a lifelong regular though moderate consumption of coffee lowers the risk of diabetes and cardiovascular diseases [8]. In food, CA is mainly found as chlorogenic acid, the quinic acid conjugated form. In western countries, a typical consumption of coffee results in the ingestion of 0.5–1 g of chlorogenic acid and 250–500 mg of CA per day [9]. However, about 95% of CA is absorbed at intestinal level, about one-third of chlorogenic acid is absorbed in the gut, with the remaining being metabolized to CA in the colon [10].

These bioavailability data suggest that the biological effects of chlorogenic acid in the body are due to its metabolite CA. Several studies indicated that CA is able to protect endothelial cells against different types of insults and under different experimental conditions [11,12]. Available data clearly indicate that plasma concentration of a specific polyphenol has been reported rarely to exceed 1 μM after the consumption of 10–100 mg of a single compound [13]. In particular, plasma conjugated and aglycone CA levels have been reported in the range of 300–500 nM and 10–116 nM, respectively [14]. Irrespective of this evidence, the majority of the studies addressing the effects of phenolic acids on vascular cells have been carried out considering much higher concentrations (most frequently in the micromolar to millimolar range); therefore, introducing a strong and evident bias. Recently [15], we showed that CA, at a concentration of 10 nM, significantly reduced glucose uptake in endothelial Ea.hy926 cells counter-acting the HG-dependent increase of permeability and cell death. In particular, we have suggested that the reduced glucose entrance, exerted by CA, allows the cells to activate survival mechanisms that induce NF-κB pathway modulation and Bcl-2 activation. The aim of this study was to understand deeply the mechanism of survival activated by low doses of CA. Primary human umbilical vein endothelial cells (HUVECs) were utilized to demonstrate that CA significantly counters several dysfunctional effects of HG by modulating NF-E2-related factor 2 (Nrf2) nuclear translocation and electrophile responsive element (EpRE)-regulated gene expression.

2. Materials and methods

2.1. Cell culture and treatments

HUVECs were isolated from freshly obtained human umbilical cords by collagenase digestion of the interior of the umbilical vein as described elsewhere [16]. Then a pool of HUVECs from different healthy donors were cultured in medium 199, supplemented with 20% fetal bovine serum, 1% L-glutamine, 20 mM Hepes, 100 units/mL penicillin/streptomycin, 50 mg/mL endothelial cell growth factor, and 10 μg/mL heparin, in gelatin-prefretreated flasks. Cells used in this study were from the second to fourth passage. HG treatment was performed using 199 medium containing 25 mM d-glucose anhydrous. >99.5%, HPLC grade. Subconfluent cells were treated for 24 h in medium 199 containing HG in presence or not of CA.

Cells treated with CA vehicle (DMSO 0.1%) in medium 199 containing normal glucose concentrations (5.5 mM d-glucose) were used as controls. At the end of the exposure time, cells have been immediately processed and/or preserved at −80 °C until analysis as expected for each test.

2.2. Cytotoxicity assay

The cytotoxic effect of HG on HUVECs was evaluated by Trypan blue dye-exclusion test. Briefly, 20 μL of cell suspension were mixed with 20 μL of Trypan blue isotonic solution (0.4% w/v) and loaded into a hemocytometer for cell counting. Interferences were avoided by testing cytotoxicity of CA treatment in HUVECs.

2.3. Leukocyte adhesion

Mononuclear cells have been isolated from human whole blood with Histopaque-1077, following the procedure recommended by the manufacturer. Briefly, heparinized venous blood from healthy donors was centrifuged over Histopaque-1077; the mononuclear cell layer was collected, washed twice with dulbecco’s phosphate-buffered saline (DPBS), suspended in medium 199, and immediately used. Leukocyte adhesion assay was performed as described elsewhere [17]. Briefly, after the treatments described earlier, HUVECs were washed with DPBS and then cocultured with human leukocytes (3 × 10^6 leukocytes/flask) for 2 h at 37 °C with gentle shaking. Cocultures were visualized under an inverted microscope and photographed using a digital camera. Increase in leukocytes adhesion was calculated in relation to the basal adhesion of leukocytes to control cells that was set to 1 [17].

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2.4. Quantitative real-time RT-PCR

Total cellular RNA was isolated with E.Z.N.A® Total RNA Kit according to manufacturer’s instruction (OMEGA Bio-tek). After reverse transcription (RT) with oligo (dT)\textsubscript{24} primers, PCR was performed for identification of heme oxygenase-1 (HO-1) (HO-1) and endothelial adhesion molecule 1 (E-selectin) mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as housekeeping gene, since it showed low variability in expression levels between the different treatments. Gene expression was assessed by real-time PCR (Applied Biosystem 7300 Real-Time PCR System, Monza, Italy) coupled with the Sybr green JumpStart™ Taq Ready Mix kit. The specific primers set for the target genes were as follows: GAPDH, forward, 5’-GGC TCT CCA GAA CAT CATCCC TGC-3’, reverse, 5’-GGG TGT CGC TGT TGA AGT CAG AGG-3’; HO-1, forward, 5’-CAACATCCAGCTTTGAGG-3’, reverse, 5’-AGAAAGCTGAG TGTAGGAC-3’ [17]; E-selectin, forward, 5’-CTGCCAAGTGGT AAAATGTTCAAG-3’, reverse, 5’-TGGACTCAGTGGAGCT TCA-3 [18]. Cycling conditions were 40 cycles 94°C (reference gene).

2.5. SOD assay

SOD activity in cell lysate was assayed by a spectrophotometric test using a method based on the reduction of nitro-blue tetrazolium (maximum absorbance at 560 nm) by the superoxide radical generated by a β-nicotinamide adenine dinucleotide/phenazine metasulfate system, as previously reported [20]. Results are expressed as percentage of SOD activity versus control. Each analysis was carried out in at least in triplicate.

2.6. Intracellular-reduced glutathione (GSH) assay

Intracellular reduced glutathione (GSH) was determined using Ellman’s reagent (5-5’-dithiobis-2-nitrobenzoic acid, DTNB) as a modified method described by Pastore et al. [21]. Briefly, cell lysates were deproteinized with 10% sulfosalicylic acid and centrifuged at 17,000 g for 5 min at 4°C. The supernatant was collected and then diluted with 0.2 M sodium phosphate buffer (pH 8.0). Later, equal volume of 0.6 mM DTNB was added and, after 10 min, the optical density of the yellow-colored complex was measured. A standard curve was obtained with pure GSH. The intracellular levels of GSH were normalized with the protein content determined with the Bradford assay [22], and expressed as nmol/mg protein. Each analysis was carried out in triplicate.

2.7. Total antioxidant activity

Following appropriate treatments, cells were lysed with 0.05% Triton X-100. Cytosolic and membrane enriched fractions were subsequently separated by centrifugation at 14,000 rpm for 15 min at 4°C. Total antioxidant activity (TAA) in cell fractions was determined by decoloration of the radical cation ABTS\textsuperscript{**}, in terms of absorbance quenching at 740 nm. Briefly, this method determines the capacity of intracellular antioxidants to quench the ABTS\textsuperscript{**} radical. The antioxidants inhibit the reaction leading to an absorbance decrease and the extent of inhibition is proportional to the antioxidant concentration in the sample. Values obtained for each sample were compared with the concentration–response curve of a standard Trolox solution, and expressed as Trolox Equivalents (nmol/mg of protein) versus control [23]. Each analysis was carried out in triplicate.

2.8. ROS measurement by dichlorodihydro-fluorescein diacetate assay

Generation of ROS was measured by the oxidation-sensitive fluorescent probe, dichloro-dihydro-fluorescein diacetate (DCFH-DA), as a modified method described earlier [17]. Briefly, confluent HUVECs were treated in six-well plates with HG (25 mM) in presence or not of CA (10 nM) for 6 h. Cells exposed to 50 μM H\textsubscript{2}O\textsubscript{2} for 1 h were used as positive internal controls. Then, cells were washed three times with DPBS (without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}), and treated with 50 μmol/L DCFH-DA at 37°C for 30 min in the dark. DCFH-DA was then removed, and cells were washed three times with DPBS (pH 7.4) to remove excess probe. Finally, cells were trypsinized, washed, and resuspended in DPBS; after centrifugation, the supernatant was transferred to measure fluorescence intensity and analyzed by flow cytometry, at excitation and emission wavelengths of 485 nm and 530 nm, respectively. ROS levels were expressed as DCFH-DA relative fluorescence intensity and reported as percentage of control. Each analysis was carried out in triplicate.

2.9. Cellular and nuclear lysate preparation

Following appropriate treatment, nuclear and cytosolic lysates were prepared as follows. Briefly, cells were lysed for 10 min at 2°C in a hypotonic buffer (10 mM Heps, 1.5 mM MgCl\textsubscript{2}, 10 mM KCl, and 5% glycerol, pH 7.8), containing a cocktail of protease inhibitors (2 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mM benzamidine) and 1 mM 1,4-dithiothreitol (DTT), and treated with 0.65% Igepal (Sigma) for 5 min. Nuclei were recovered by centrifugation at 20,000 g for 1 min at 4°C, and the supernatant was kept as the cytoplasmic extract. Nuclear proteins were obtained by incubating with a hypertonic buffer (20 mM Heps, 400 mM NaCl, 1 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 1 mM EGTA, 10% glycerol, pH 7.8) also containing a cocktail of protease inhibitors (2 μg/mL aprotinin, 1 μg/mL leupeptine, and 1 mM benzamidine), and 1 mM DTT. All the protein fractions were stored at −70°C until use. Protein concentration in lysates was determined using Bradford reagent.
2.10. Western blotting analysis

For immunoblot analyses, 40 µg of protein lysates per sample were denatured in 4× SDS-PAGE reducing sample buffer and subjected to SDS-PAGE on 10% acrylamide/bisacrylamide gels. Separated proteins were transferred to nitrocellulose membranes (Hybond-P PVDF, Amersham Bioscience). Residual binding sites on the membranes were blocked by incubation in TBST (10 mM Tris, 100 mM NaCl, 0.1% Tween 20) with 5% (w/v) nonfat milk powder overnight at 4 °C. Membranes were then probed with specific primary antibodies: rabbit anti-Nrf2 polyclonal antibody raised against a peptide mapping at the C-terminus (C-20) (Santa Cruz Biotechnology) (1:400); rabbit anti-NF-κB (p65) polyclonal antibody (Santa Cruz Biotechnology) (1:400); mouse monoclonal β-actin (Santa Cruz Biotechnology) (1:600); mouse anti-Lamin B monoclonal antibody (Santa Cruz Biotechnology) (1:200); followed by peroxidase-conjugated secondary antibody HRP labeled goat anti-rabbit IgG (BD Pharmigen) (1:5000), goat anti-mouse IgM secondary antibody HRP conjugate (Thermo Scientific) (1:10000), and visualized with an ECL western blotting system (Amersham Biosciences). The equivalent loading of proteins in each well was confirmed by Ponceau staining and β-actin or Lamin B control.

2.11. Statistical analysis

All the experiments were performed in triplicate and repeated three separate times. Results are expressed as mean ± SD from three experiments and statistically analyzed by a two-way ANOVA test, followed by Turkey’s HSD, using the statistical software ezANOVA (http://www.cabiatl.com/mricro/ezanova/). Differences in groups and treatments were considered significant for $P < 0.05$.

3. Results

3.1. CA protects HUVECs from HG-induced cytotoxicity

To investigate the protective effect of CA on HG-induced endothelial damage, cells were treated for 24 h with HG medium in the presence of different concentrations of CA (1–10–100 nM). CA alone did not affect cell viability at the tested concentrations. Exposure of HUVECs to elevated concentrations of glucose (25 mM) induced a cell damage leading to a significant decrease of cell viability (Fig. 1). Results show that when cells were exposed to HG in presence CA > 10 nM, HG-cytotoxic effect was significantly reduced. Based on these results, 10 nM corresponds to the lowest concentration of CA associated to cell protection against HG-induced cell mortality. Furthermore, the highest concentration tested (100 nM) was unable to induce a protective effect higher, in a statistically significant way, than that induced by CA 10 nM. For these reasons, CA 10 nM was used for the following experiments.

3.2. CA and HG modulate cell endogenous antioxidant defense system

It has been frequently reported that HG induces an imbalance between the generation of reactive nitrogen and oxygen.
species and the cellular antioxidant ability. We therefore assessed if this event also occurred under our experimental condition. The significant decrease of SOD activity, GSH level, and TTA (see Table 1), confirmed that HG exposure induces a significant depletion of intracellular antioxidant defense in HUVECs, and that redox imbalance is involved in endothelial dysfunction associated to hyperglycemic conditions. The presence of 10 nM CA countered HG-induced redox imbalance and partially restored GSH level, TAA and SOD activity to values close to those observed in normo-glycemic controls. Interestingly, under normo-glycemic conditions (cells exposed to 5.5 mM glucose), CA treatment alone did not affect TAA, but induced a significant increase of cellular SOD activity (Table 1). These data let us hypothesize that in normoglycemic condition CA is associated to the induction of an adaptive response. On the other side, the coincubation of HG with CA allows the activation of the cellular protective mechanism.

3.3. CA reduced HG-induced ROS formation and oxidative stress in HUVECs

The production of reactive oxygen and nitrogen species has been mechanistically associated to endothelial cell dysfunction [24] and to the activation of NF-κB in the pathogenesis of atherosclerosis. A recent study indicates that hyperglycemia is sufficient to induce the generation of free radicals and oxidative stress in endothelial cells [25]. Accordingly, the role of ROS and NF-κB in the induction of apoptosis by HG has been demonstrated in primary cultured human endothelial cells [26].

In agreement with previous reports, in our experimental conditions, we observed higher levels of DCFH-DA fluorescence induced by HG, suggesting a significant increase of intracellular oxidants. This increase was significantly countered by CA treatment indicating a “protective” effect at the level of reactive species generation (see Fig. 2).

3.4. CA prevents NF-κB p65 nuclear accumulation induced by HG

Several transcription factors, including NF-κB, have been demonstrated to be activated by redox-sensitive mechanisms in

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**FIG 2**

Intracellular oxidants production in HUVECs after 6 h of cell exposure to HG (25 mM d-glucose) and effect of 10 nM CA. Cultures treated with d-glucose 5.5 mM and exposed to the vehicle only (DMSO, 0.1%) were used as controls. Cells exposed to 50 μM H2O2 for 1 h were used as positive control of increased intracellular ROS level. Results are expressed as % DCFH-DA fluorescence intensity relative to control. Bars indicate the mean (± SD) of three separate experiments. *P < 0.05 versus control; #P < 0.05 versus cells exposed to HG alone.

**FIG 3**

Modulation of NF-κB p65 translocation from cytosol to nucleus in HUVECs exposed to HG (25 mM d-glucose) and effect of 10 nM CA. Cells treated with d-glucose 5.5 mM and exposed only to the vehicle (DMSO, 0.1%) were used as controls. (A) One representative blot, out of three independent experiments. (B, C) densitometry plots reporting the fold change versus control. Bars indicate the mean (± SD) of three separate experiments, for cytoplasmic and nuclear levels, respectively. P65 intensity values were normalized to the corresponding Lamin B value. *P < 0.05 versus control; #P < 0.05 versus cells exposed to HG alone; §P < 0.05 versus cells treated only with CA.
cultured endothelial cells [27]. In this study, we sought to investigate if HG-induced redox imbalance could trigger NF-κB activation. Western blotting analysis revealed an increased NF-κB p65 nuclear translocation in HG-treated HUVECs. The presence of CA in the culture medium was associated to reduced NF-κB nuclear levels in comparison to control cells and cells exposed to HG (Fig. 3).

3.5. Effect of CA on HG-induced E-selectin expression and leukocytes adhesion

To investigate whether HG induces a pro-inflammatory condition and phenotypic changes in HUVECs, we assessed adhesion molecules expression and leukocytes adhesion. Cells were cultured with normal glucose (5.5 mM) or HG (25 mM) concentrations for 24 h. In particular, we focussed on E-selectin mRNA levels, a representative adhesion molecule gene which expression is related to an increase of the adhesion of leukocytes to the endothelium [17]. As shown in Fig. 4A, the presence of 25 mM glucose in the culture medium was associated with a significant increase of E-selectin expression. HG-induced mRNA expression of this adhesion molecule was significantly suppressed by cotreatment with CA.

Since the expression of adhesion molecules is responsible for leukocytes adhesion to vascular endothelium and their migration into subendothelial space, to confirm the inhibitory activity of CA on endothelial activation induced by HG, we cocultured HUVECs with isolated human leukocytes as described in Section 2.3. Figure 4B shows that the number of leukocytes adhered to the endothelial cells exposed to HG was higher than that observed in controls, but it appeared to be reduced by cotreatment with CA.

Furthermore, as observed for p65 nuclear levels, the presence of 10 nM CA was associated to a decrease of E-selectin gene expression, and then of leukocytes adhesion, irrespective to the presence of HG. It is possible to speculate that this effect is due to the modulation of redox status exerted by natural polyphenols as reported in previous studies [12,17,28].
This study aimed to identify and describe the molecular mechanisms involved in protective effects of CA at nanomolar concentrations against endothelial dysfunction induced by HG. Our results showed that the incubation of HUVECs with a HG concentration (25 mM) for 24 h determined a consistent reduction of cell viability, and that the presence of 10 nM CA significantly improved HUVECs viability. HG causes intracellular ROS overproduction and impairs constitutive antioxidant defenses leading to the deregulation of redox sensitive signaling pathways and then to defective endothelial and vascular functions. It has been reported that reducing oxidative stress by lowering ROS production is pivotal in the management of diabetes complications [32]. Our data indicate that HG leads to reduced activity of the endogenous antioxidant enzyme SOD, depleted GSH levels, and decreased cellular TAA, an index of total antioxidant cellular capacity. The presence of CA in the culture media was associated to increased SOD activity, intracellular GSH levels, and TAA. Interestingly, as concern GSH and SOD levels, this effect was evident independently of HG exposure. Furthermore, HG increased cellular ROS in HUVECs while the presence of CA significantly inhibited their production. Taken together, our data suggest that CA at a low concentration, normally achievable in plasma and possibly intracellularly under normal dietary western habits is potentially able to affect intracellular redox status and then protect against HG-induced vascular damage via antioxidant mechanism. ROS, and in particular hydrogen peroxide and lipoperoxides, act as signaling molecules and are known to activate NF-κB proinflammatory pathway. NF-κB is a ubiquitous transcription factor controlling the expression of genes encoding for cell adhesion molecules and cytokines, and it has been linked to inflammatory events associated with endothelial dysfunction [33].

Our data indicate that CA is able to reduce NF-κB nuclear translocation triggered by HG and has an inhibitory effect on NF-κB-dependent expression of genes involved in cell-to-cell adhesion, including E-selectin. The observed increased gene expression of E-selectin and leukocyte adhesion suggests the existence of HG-induced activation of pathogenic events leading to endothelial dysfunction and atherogenesis. Interestingly, CA was able to reduce adhesion molecule gene expression and leukocytes adhesion and therefore to potentially revert, at least in part, the pro-atherogenic phenotype induced by HG in cultured human endothelial cells. To understand the molecular basis involved in protective effects of CA at nanomolar concentrations against endothelial dysfunction induced by HG. Our results showed that the incubation of HUVECs with a HG concentration (25 mM) for 24 h determined a consistent reduction of cell viability, and that the presence of 10 nM CA significantly improved HUVECs viability. HG causes intracellular ROS overproduction and impairs constitutive antioxidant defenses leading to the deregulation of redox sensitive signaling pathways and then to defective endothelial and vascular functions. It has been reported that reducing oxidative stress by lowering ROS production is pivotal in the management of diabetes complications [32]. Our data indicate that HG leads to reduced activity of the endogenous antioxidant enzyme SOD, depleted GSH levels, and decreased cellular TAA, an index of total antioxidant cellular capacity. The presence of CA in the culture media was associated to increased SOD activity, intracellular GSH levels, and TAA. Interestingly, as concern GSH and SOD levels, this effect was evident independently of HG exposure. Furthermore, HG increased cellular ROS in HUVECs while the presence of CA significantly inhibited their production. Taken together, our data suggest that CA at a low concentration, normally achievable in plasma and possibly intracellularly under normal dietary western habits is potentially able to affect intracellular redox status and then protect against HG-induced vascular damage via antioxidant mechanism. ROS, and in particular hydrogen peroxide and lipoperoxides, act as signaling molecules and are known to activate NF-κB proinflammatory pathway. NF-κB is a ubiquitous transcription factor controlling the expression of genes encoding for cell adhesion molecules and cytokines, and it has been linked to inflammatory events associated with endothelial dysfunction [33].

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adaptive, pharmacologically or nutritionally induced expression of cytoprotective proteins regulated by Nrf2/EpRE, can significantly contribute to the atheroprotective and anti-inflammatory phenotype in endothelial cells [35,36]. In agreement with this, we have also reported a potential cross-talk between Nrf2 and NF-κB transcription factors. In fact, we demonstrated that the inhibition of Nrf2 nuclear accumulation by the inhibitor PD98059 was associated to an increase of NF-κB p65 nuclear translocation in HUVECs [36], resulting in a reduced protective effect of different molecules of nutritional interest. However, this mechanism needs to be confirmed by RNAi experiments. Furthermore, we have demonstrated that in HUVECs, Nrf2 activation induced by glutathione depletion (after the treatment with the inhibitor of glutathione synthesis, buthionine sulfoximine), reduced the expression of adhesion molecules following TNF-α treatment [36]. These observations confirmed that Nrf2 significantly interacts with NF-κB signaling. Taken together, our data further reinforce the hypothesis of a crosstalk between Nrf2 and NF-κB and, more specifically, the inhibitory effect of Nrf2/EpRE pathway on NF-κB transcription [17].

In conclusion, this study demonstrates that CA inhibits HG-induced phenotypic modulation of endothelium to a dysfunctional state through the inhibition of the NF-κB pathway and improving cellular redox status. Furthermore, the beneficial effects of CA appear to be due to its ability to activate cellular protective responses, involving a hormetic response associated with the activation of the transcription factor Nrf2. Based on these observations, our study suggests that the coordinate induction of endogenous cytoprotective proteins through activation of the Nrf2/EpRE pathway might act as mechanism to prevent or limit redox unbalance induced by HG levels.

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