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ARTICLE

Cyanidin-3-*O*-glucoside Protection against TNF-α-Induced Endothelial Dysfunction: Involvement of Nuclear Factor-κB Signaling

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Oxidative stress and inflammation are considered to play a pivotal role in vascular endothelial dysfunction by triggering activation of transcription factors, such as NF-κB, functionally dependent on cellular redox status. The anthocyanin cyanidin-3-*O*-glucoside (C3G), as well as other phytochemicals recognized as potent antioxidants and free radical scavengers, may act as modulators of gene regulation and signal transduction pathways. This study demonstrates that C3G is able to protect human endothelial cells against alterations induced by TNF-α, including the activation of NF-κB, increased gene expression of adhesion molecules, leukocyte adhesion to endothelium, and intracellular accumulation of H_2O_2 and lipid peroxidation byproducts. These observations contribute to provide a conceptual background for the understanding of the mechanisms underlying the role of C3G, as well as other dietary plant polyphenols, in the prevention of diseases associated with inflammation and oxidative stress, including atherosclerosis.

KEYWORDS: Anthocyanins; atherosclerosis; endothelial dysfunction; NF-κB; cyanidin-3-*O*-glusoside

INTRODUCTION

The vascular endothelial cell is a preferential target for therapy in various pathological conditions, including cardiovascular disease, neurodegenerative disease, and cancer, all of which are underlying alterations of this cell type. Vascular endothelial dysfunction may be induced by genetic and lifestyle-associated factors such as aging, smoking, inflammation, trauma, hyperlipidemia, or hyperglycemia, which are in fact among a myriad of risk factors that may contribute to the pathogenesis of many cardiovascular and degenerative diseases. Oxidative stress and inflammation are considered among the prominent pathways of vascular endothelial dysfunction in many pathophysiological conditions.

Many studies have demonstrated that the increased adhesion of circulating monocytes to the injured endothelial layer is a critical early event in the development of atherogenesis (*1*). In fact, endothelial cells recruit monocytes by selectively expressing various cell surface adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) (*2*). Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), can induce the expression and release of chemotactic factors and additional cytokines that can each further contribute to inflammation (*2*). Low-grade inflammation and increased circulating levels of proinflammatory cytokines are frequently observed in patients with atherosclerosis and hypertension (*3*).

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Increased oxidative stress is considered to be an important causative factor of vascular endothelial dysfunction. In recent years, it has been hypothesized that reactive oxygen species (ROS) may play a specific role as signaling molecules modulating a number of metabolic pathways and cellular responses (*4*). In addition, NF-κB, as well as other transcription factors, has been shown to be, at least in part, functionally dependent on cellular redox status, which is in turn controlled by antioxidant availability in the cellular milieu (*5*,*6*). Binding sites for redox-sensitive transcription factors are found in the promoter regions of many proinflammatory cytokines and immunoregulatory mediators important in the induction of acute inflammatory responses and associated with chronic and degenerative diseases. Therefore, dysregulated intracellular signaling may not only negatively affect immune responses but underlie a wide spectrum of chronic diseases.

Epidemiological and experimental data indicate that a dietary profile has an important effect on vasculature. The association between the consumption of specific food items, in particular, fruits and vegetables, and the risk of cardiovascular diseases has been attributed to their capacity to provide a high intake of components having a putative antioxidant capacity (*7*). An important part of this effect has been attributed to phenolic compounds present in foods of plant origin and more specifically to flavonoids. However, beyond the antioxidant activity, diverse mechanisms have been proposed to explain the biological activity of polyphenols, including regulation of signal transduction and modulation of redox-sensitive transcription factors, including NF-κB (*8*, *9*).

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Anthocyanins represent one of the most important and interesting classes of flavonoids. They are widespread in dark red-colored fruits and vegetables such as berries, red grapes, purple sweet potatoes, and red cabbages and seem to play a role in preventing human diseases related to oxidative stress (*10*, *11*). Cyanidin-3-*O*-glucoside (C3G), largely present in the human diet, is probably the best-known and investigated anthocyanin. Cyanidin-glycosides are reported as incorporated from the digestive tract into the bloodstream in their intact glycosylated forms (*12*). Furthermore, several in vitro and in vivo studies suggest that C3G and metabolites possess different biological properties and potentially beneficial effects in various human pathologies (*13*). In particular, anthocyanins have been proposed to exert significant cardiovascular health-promoting effects (*14*). However, the precise molecular mechanisms underlying these effects are still unknown.

In this study we investigated, at transcriptional level, the protective effect of C3G against endothelial dysfunction, by testing its capability to alter the response to TNF-α in primary human umbilical vein endothelial cells (HUVECs), which are a model system widely used to identify the effects of and targets for deleterious vascular risk factors (*15*). TNF-α was selected as a stressor because endothelial dysfunction induced by TNF-α is mediated through its ability to promote intracellular ROS formation and activation of the redox-sensitive transcription factor NF-κB.

MATERIALS AND METHODS

Chemicals and Reagents. C3G was supplied from Polyphenols AS, Sandnes, Norway, and was of HPLC grade. Methanol, ethanol, acetonitrile, chloroform, and isopropyl alcohol were obtained from Carlo Erba Reagent (Milan, Italy) in their highest commercially available purity grade. All other reagents, if not specified, were purchased from Sigma-Aldrich (Milan, Italy).

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 by Jaffe and co-workers (*16*) and were cultured in medium 199, supplemented with 20% fetal bovine serum (FBS), 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-pretreated flasks. Cells were maintained in a humidified atmosphere containing 5% CO_2 in an incubator at 37 °C. Cells used in this study were from the second to fourth passage.

For all experiments, C3G was always freshly dissolved in DMSO and used immediately. The final concentration of DMSO in the culture medium during different treatments was $< 0.1\%$ (v/v). The subconfluent cells were treated for 24 h in serum-free medium with various doses of C3G (20−40 μM), whereas control cells were treated with 0.1% DMSO only. After this incubation time, cells were washed with phosphate-buffered saline under sterile conditions and then incubated for 2 h with serum-free medium containing various doses of recombinant human TNF-α (range = 10−20 ng/mL) as previously reported (*17*). Control cells were not exposed to TNF-α. At the end of the exposure time, cells were immediately processed and/or preserved at −80 °C until analysis as expected for each test.

Cytotoxicity Assay. The cytotoxic effect of TNF-α on HUVECs pretreated or not with C3G was evaluated by means of two assay methods, lactate dehydrogenase (LDH) leakage assay (LDH assay kit, Sigma, Milan, Italy) and trypan blue exclusion assay. The LDH leakage assay evaluates cytotoxicity by measuring the activity of lactate dehydrogenase released from damaged cells. The LDH activity of both medium supernatant and cell homogenate was measured as the rate of consumption of NADH in the presence of pyruvate. Viability was expressed as the percent of LDH leakage, which corresponds to the ratio between the LDH activity in the medium and the total LDH activity.

Cell viability was also assessed using a dye exclusion test with Trypan blue. Briefly, 10 μL of cell suspension was mixed with 30 μL of Trypan blue isotonic solution (0.4% w/v) and loaded into a hemocytometer for both live and dead cell counting.

To avoid interferences due to antioxidant toxicity, we also used the same test to evaluate the cytotoxicity of C3G pretreatment in HUVECs.

Electrophoretic Mobility Shift Assay. Cells were lysed for 20 min in a hypotonic buffer in ice (10 mM Hepes, pH 7.8, and 1 mM $MgCl_2$, 10 mM KCl, 0.1 mM EDTA, 0.5 mM EGTA, and 5% glycerol), containing a cocktail of protease inhibitors. Nuclei were treated with 0.625% Nonidet 40 for 5 min and pelleted by centrifugation at 20000*g* for 30 s. Nuclear proteins were obtained by incubation with a hypertonic buffer (50 mM Hepes, pH 7.8, 400 mM NaCl, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM EGTA, and 10% glycerol) also containing a cocktail of protease inhibitors. After treatment, nuclei were centrifuged at 20000*g* for 5 min, and the supernatant was retained for use in the DNA binding assay. Annealed complementary oligonucleotides (10 pmol) were labeled in 1× kinase buffer with 5 U of T4 polynucleotide kinase (USB, Cleveland, OH) and 10 μCi of [γ^{32}P]-ATP (Perkin-Elmer, Boston, MA) at 37 °C for 30 min. After kinase inactivation at 65 °C for 5 min, probes were purified using Micro Biospin30 columns (Bio-Rad, Hercules, CA). Binding reactions were performed by incubating 5 μg of nuclear proteins for 20 min at room temperature with 2 μg of polydI-dC−polydI-dC and 50000 cpm (Cherenkov counting) of labeled oligonucleotides. DNA−protein complexes were resolved in 6% polyacrylamide (29:1 acrylamide/bisacrylamide) gels and then autoradiographed. The resulting films were subjected to densitometric scanning using the SCION IMAGE program and normalized against loading control. A 100-fold excess of unlabeled oligonucleotides was added as cold competitor as binding specificity control. The NF-κB oligonucleotide consensus sequence is 5′AGT TGA GGG GAC TTT CCC AGG C3′ (*10*).

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 DPBS, suspended in medium 199, and immediately used.

Two flasks with subconfluent HUVECs were treated with various doses of C3G (20−40 μM) and were incubated for 24 h at 37 °C in a humid 5% CO_2 atmosphere incubator. Two controls were treated with the medium containing only 0.1% DMSO. After this pretreatment, cells were washed with DPBS under sterile conditions and then cocultured with leukocytes (3×10^6 leukocytes/flask) and TNF-α 20 ng/mL for 2 h at 37 °C with gentle shaking. Cells not exposed to TNF-α were used as controls. After this incubation time, medium was removed and cells were washed with DPBS.

Cocultures were visualized under an inverted microscope and photographed using a digital camera. Four areas for each flask were selected and used to count the number of adherent leukocytes. Increase in leukocyte adhesion upon stimulation of HUVECs with TNF-α was calculated in relation to the basal adhesion of leukocytes to unstimulated HUVECs, which was set to 1 (*18*).

Quantitative RT-PCR. Total cellular RNA was isolated according to the TRIzol protocol. The quality of the RNA was tested in 1% formaldehyde−agarose gel stained with ethidium bromide (EtBr) and spectrophotometrically quantified. After reverse transcription (RT) with oligo (dT)15 primers, Polymerase Chain Reaction (PCR) was performed for identification of VCAM-1, ICAM-1, and E-Selectin mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene for normalization.

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 CAT CCC TGC-3′, reverse, 5′-GGG TGT CGC TGT TGA AGT CAG AGG-3′; ICAM-1, forward, 5′-GGG AGC TTC GTG TCC TGT ATG GCC-3′, reverse, 5′-AGT CTG TAT TTC TTG ATC TTC CGC TGG C-3′; VCAM-1, forward, 5′-GAA TGG GAG CTC TGT CAC TGT AAG C-3′, reverse, 5′-GAC CAA GAC GGT TGT ATC TCT GGG- 3′ (*19*); E-selectin, forward, 5′-CTG CCA AGT GGT AAA ATG TTC AAG-3′, reverse, 5′-TTG GAC TCA GTG GGA GCT TCA-3′ (*20*). Cycling conditions were 40 cycles of 94 °C denaturation (15 s), 60 °C annealing, and extension (1 min). A final dissociation stage was run to generate a melting curve for verification of amplification product specificity. Each sample was assayed at least three times from the same RNA. Data were collected and processed with SDS 1.3.1 software (Applied Biosystems, Monza, Italy) and given as threshold cycle (Ct). The fold increase compared with the control cells not treated and not exposed to TNF-α mRNA expression was determined using the

$2^{-\Delta\Delta Ct}$ method (*21*). Primer efficiencies for the test genes were comparable to those for GAPDH (reference gene).

Lipid Peroxidation Assay. Lipid peroxidation was quantified in samples by measuring the content of malondialdheyde (MDA) and 4-hydroxynonenal (HNE), degradation products of unsaturated fatty acids. After the appropriate treatment, cells were trypsinized, washed with DPBS, and lysed in ice-cold water followed by two freeze−thaw cycles. MDA and HNE were measured in the lysates by a colorimetric assay kit (Calbiochem, San Diego, CA). Briefly, a chromogenic reagent, *N*-methyl-2-phenylindole in acetonitrile, reacts with MDA and HNE at 45 °C. Condensation of one molecule of either MDA or HNE with two molecules of reagent yields a stable chromophore with maximal absorbance at 586 nm. Results are normalized with the protein content determined with the Bradford assay (*22*) and expressed as nanomoles per milligram of protein.

Intracellular Levels of Hydrogen Peroxide (H_2O_2). Intracellular levels of H_2O_2 were measured as previously described by Korolyuk and co-workers (*23*), measuring the absorbance at 405 nm of the yellow complex formed by the reaction between molybdate and hydrogen peroxide. Results are expressed as micromoles and normalized with the protein content determined with the Bradford assay.

Statistical Analysis. All of the experiments were performed in triplicate and repeated three times. Results are expressed as means ± SD from three experiments and statistically analyzed by a one-way ANOVA test, followed by Tukey's HSD, using the statistical software ezANOVA (http://www.sph.sc.edu/comd/rorden/ezanova/home.html). Differences in groups and treatments were considered to be significant for $P < 0.05$.

RESULTS

C3G Protects HUVECs from TNF-α-Induced Injury. To investigate the effect of C3G on TNF-α-activated endothelium, cells were pretreated with increasing concentrations of C3G (20−40 μM) and then exposed for 2 h to TNF-α (range = 10−20 ng/mL). The range of C3G concentrations used in the present study was consistent with that employed in many other studies concerning the protective effect of anthocyanins in cultured cells (*24*−*26*). Our results indicate a dose-dependent cytotoxicity of TNF-α; the highest dose tested is associated with 24.9 ± 2.4% cell death (**Figure 1A**). These results were also confirmed by the increase of LDH release in the culture medium resulting from membrane damage (**Figure 1B**). Cell pretreatment with C3G showed a dose-dependent protective effect against TNF-α-induced cell death. In both tests, the higher C3G dose (40 μM) was the more effective, with cell viability similar to untreated and unexposed control cells (**Figure 1**). Furthermore, C3G alone was unable to affect cell viability at the tested doses.

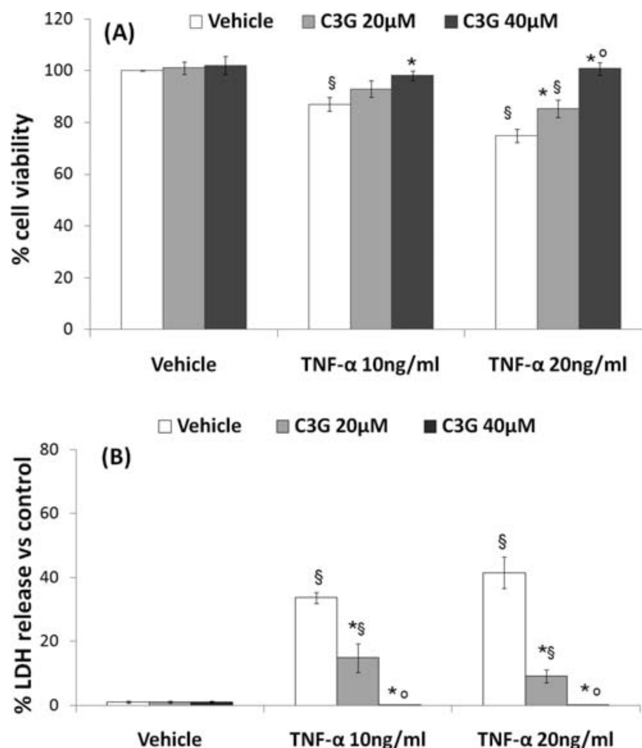

Figure 1. Cell viability after 24 h of cell pretreatment with C3G (20−40 μM) and 2 h of TNF-α exposure. Cultures treated with the vehicle alone (DMSO 0.1%) were used as controls. Each point represents the mean ± SD of three experiments. (**A**) Trypan blue assay. Data represent the percentage of viable cells (mean percentage) calculated from the number of viable cells in treated samples versus control untreated and unexposed. (**B**) LDH release assay. Viability is expressed as percent of LDH leakage, which corresponds to the ratio between LDH activity in the medium and total LDH activity. ∗, $P < 0.05$ versus respective control exposed to TNF-α; §, $P < 0.05$ versus respective control not exposed to TNF-α; °, $P < 0.05$ versus respective C3G 20 μM.

Effect of C3G on TNF-α-Dependent NF-κB Nuclear Translocation. Proinflammatory cytokines initiate adhesion of circulating monocytes by up-regulating the expression of adhesion molecules in the endothelium. The translocation of NF-κB in the nucleus is considered to be a key event triggering the up-regulation of the expression of cell adhesion molecules within an inflammatory response (*27*).

As shown in **Figure 2**, a significant NF-κB activation was observed in HUVECs exposed for 2 h to 20 ng/mL of TNF-α, according to the electromobility shift assay (EMSA).

The activation of NF-κB observed in TNF-α-exposed cells was remarkably inhibited by C3G pretreatment, at all tested doses. Furthermore, C3G alone is not able to affect NF-κB nuclear translocation.

Effect of C3G on TNF-α-Induced Endothelial Activation. To investigate the transactivating activity of NF-κB, real-time PCR was used to evaluate ICAM-1, VCAM-1, and E-selectin gene expression in endothelial cells as representative genes involved in cell adhesion. Gene expression was assessed in HUVECs after 24 h of incubation with C3G followed by 2 h of exposure to TNF-α 20 ng/mL. According to data reported in the literature, cell exposure to TNF-α significantly induced the surface expression of ICAM-1, VCAM-1, and E-selectin (**Figure 3**) (*28*). TNF-α-induced up-regulation of RNA expression of these adhesion molecules was significantly suppressed by pretreatment with C3G, and in some cases in a dose-dependent way (**Figure 3**). C3G per se, without any kind of stimulus, had no effects on the basal expression of these genes.

Effect of C3G on TNF-α-Induced Leukocyte Adhesion. NF-κB has been suggested to be the major transcriptional regulator of endothelial adhesion molecules as well as of chemokines. Adhesion molecules, in fact, are responsible for leukocyte adhesion to vascular endothelium, so promoting their migration into subendothelial space, an early event in atherogenesis. To confirm the inhibitory activity of C3G on endothelial activation induced by TNF-α, we investigated isolated leukocytes cocultured with HUVECs.

Figure 4 shows that the number of leukocytes adhered to the endothelium of cells exposed to TNF-α was higher than that observed in controls, but it appeared to be reduced by pretreatment with C3G.

These results confirm the inhibitory activity of C3G on TNF-α-induced endothelial activation by preventing the activation of the NF-κB pathway.

C3G Inhibits TNF-α-Induced Oxidative Damage. Many in vitro and in vivo studies have already demonstrated that ROS and other reactive free radicals are involved in the inflammatory response of endothelial vessel (*29*). In fact, antioxidant defenses have been found to be altered in endothelial cells after exposure to

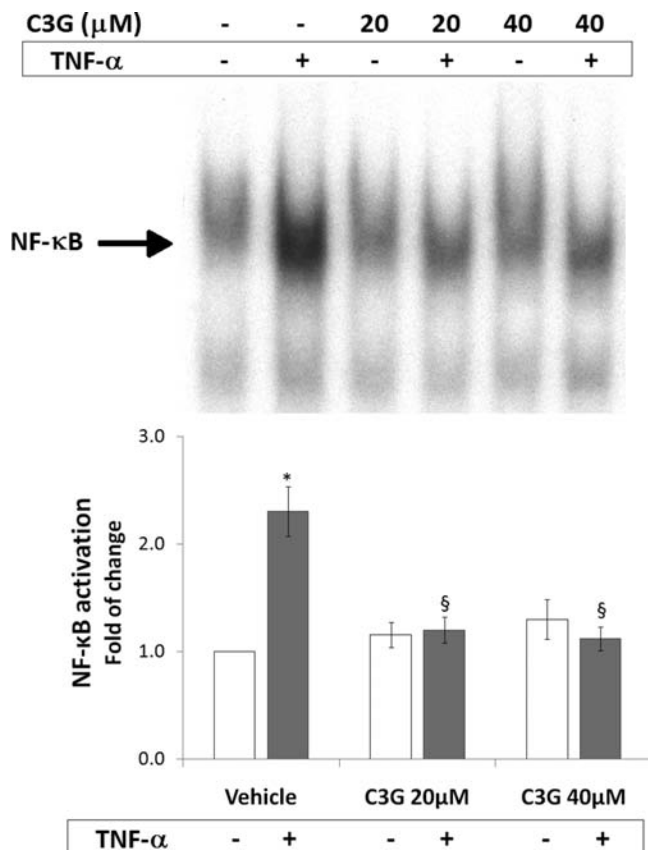

Figure 2. Modulation of NF-κB activation in HUVECs treated with various doses of C3G (20−40 μM) for 24 h and then exposed for 2 h to TNF-α 20 ng/mL. Cultures treated with the vehicle alone (DMSO 0.1%) were used as controls. The specificity of the DNA binding was confirmed by using binding reactions containing 100-fold excess of unlabeled oligonucleotides as cold competitors (not shown). Representative image from three independent experiments. Results by densitometry are reported as fold of change compared with control and expressed as mean ± SD of three experiments. $*$, $P < 0.05$ versus control not exposed to TNF-α; §, $P < 0.05$ versus control exposed to TNF-α 20 ng/mL.

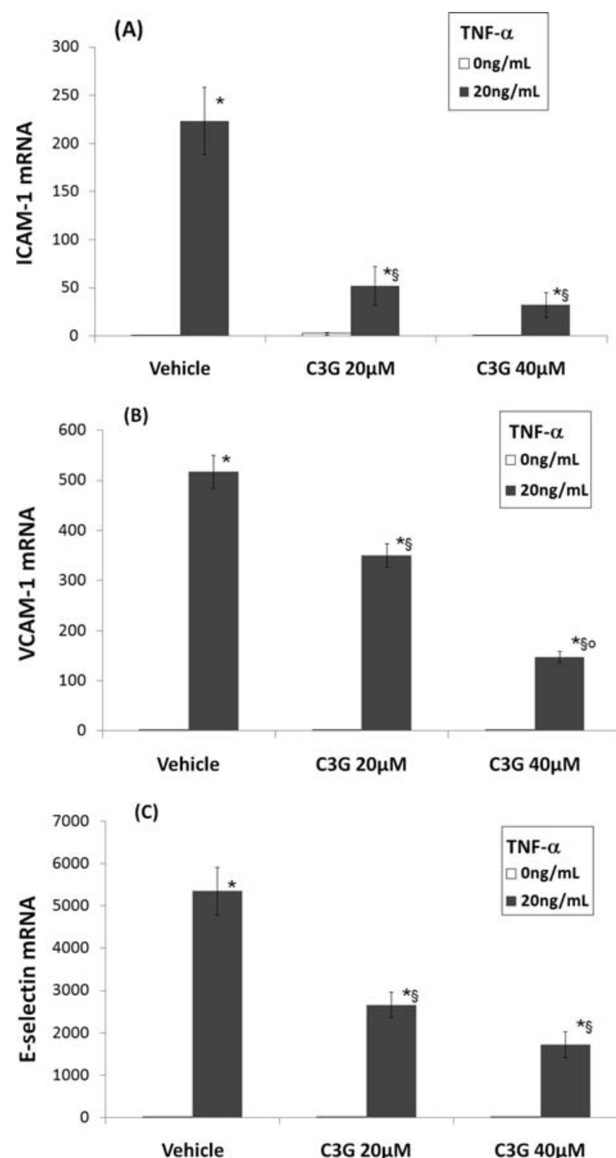

Figure 3. Effect of C3G on TNF-α-induced mRNA expression of adhesion molecules, ICAM-1 (**A**), VCAM-1 (**B**), and E-selectin (**C**), in HUVECs. Cells were treated with various doses of C3G (20−40 μM) for 24 h and then exposed for 2 h to TNF-α 20 ng/mL. Cultures treated with the vehicle alone (DMSO, 0.1%) were used as controls. Values are expressed as $2^{-\Delta\Delta Ct}$ normalized to control. $*$, $P < 0.05$ versus control not exposed to TNF-α; §, $P < 0.05$ versus control exposed to TNF-α 20 ng/mL; °, $P < 0.05$ versus C3G 20 μM exposed to TNF-α.

proinflammatory cytokines, suggesting that oxidative stress plays a major role in endothelial inflammation (*30*). On this basis, we studied the involvement of oxidative stress in endothelial alterations following TNF-α exposure and if C3G was able to modulate TNF-α-induced altered redox state in HUVECs. To evaluate the TNF-α-induced production of oxidative species and the resulting oxidative damage to cellular macromolecules, we measured the intracellular levels of H_2O_2 and of MDA and 4HNE, as byproducts of lipid peroxidation.

HUVECs exposure to TNF-α increased MDA and 4HNE levels when compared to unexposed control cells (**Table 1**). Pretreatment of HUVECs with C3G decreased TNF-α-induced oxidative damage, returning the levels of MDA/4HNE to those of unexposed control cells. Furthermore, TNF-α exposure caused a marked increase in intracellular levels of H_2O_2 (**Table 1**), a powerful cellular oxidant. C3G pretreatment suppressed intracellular H_2O_2 overproduction in a dose-dependent manner (**Table 1**).

DISCUSSION

Inflammation is considered to be a critical initiating step in the development of atherosclerosis. Several studies have demonstrated that atherosclerosis is associated with a proinflammatory shift in gene expression profile (*31*). Proinflammatory changes in endothelial phenotype, known as "endothelial activation", involve up-regulation of cellular adhesion molecules and an increase in endothelial−leukocyte interactions and permeability, as well as alterations in the secretion of autocrine/paracrine factors, which are pivotal to inflammatory responses (*32*). There is increasing evidence that NF-κB activation plays a key role in endothelial activation in atherosclerosis. The NF-κB activation pathway is triggered by a wide variety of stimuli including inflammatory cytokines, ROS, and mechanical forces acting on the vascular endothelial wall and leading to stimulation of transmembrane receptors. These activate intracellular signaling pathways leading to activation of a kinase (IκK) mediated phosphorylation/degradation of the inhibitor of NF-κB (IκB) and thus resulting in nuclear translocation of the NF-κB heterodimer (p65/p50 subunits and, perhaps, p65, RelB, c-Rel, p50, and p52), where it binds to promoters of gene targets. Some of these

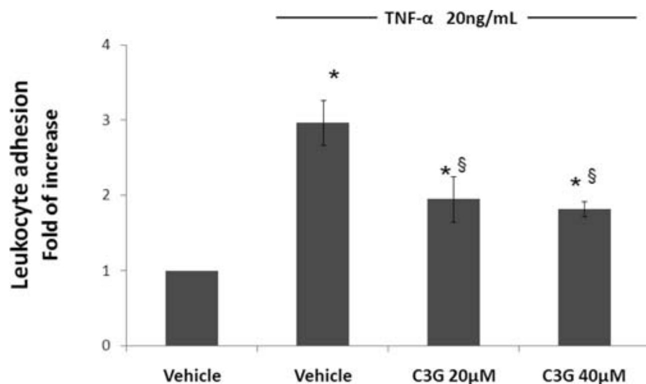

Figure 4. Leukocyte adhesion in HUVECs pretreated with various doses of C3G (20−40 μM) for 24 h and then exposed to TNF-α 20 ng/mL for 2 h with gentle shaking. A flask containing the coculture and not exposed to TNF-α was used as control. Increase in leukocyte adhesion upon stimulation of HUVECs with TNF-α was calculated in relation to the basal adhesion of leukocytes to nonstimulated HUVECs that was set to 1. $*$, $P < 0.05$ versus control not exposed to TNF-α; §, $P < 0.05$ versus control exposed to TNF-α 20 ng/mL.

Table 1. Levels of MDA and 4HNE and Intracellular Levels of Hydrogen Peroxide in HUVECs after 24 h of Pretreatment with C3G (20-40 μM) and 2 h of TNF-α Exposure[a]

	MDA-HNE (nmol/mg of protein)	H_2O_2 (μmol/mg of protein)
vehicle	20.9 ± 0.7	38.2 ± 2.5
C3G 20 μM	20.1 ± 0.3	38.1 ± 1.2
C3G 40 μM	20.4 ± 0.5	37.5 ± 1.5
vehicle + TNF-α 10 ng/mL	22.9 ± 0.3°	48.7 ± 2.2°
C3G 20 μM + TNF-α 10 ng/mL	20.0 ± 0.9*	47.3 ± 1.3°
C3G 40 μM + TNF-α 10 ng/mL	20.9 ± 0.4*	43.8 ± 1.4°*
vehicle + TNF-α 20 ng/mL	23.3 ± 0.6°	51.6 ± 1.5°
C3G 20 μM + TNF-α 20 ng/mL	20.4 ± 0.5§	45.1 ± 1.4§°
C3G 40 μM + TNF-α 20 ng/mL	19.4 ± 0.9§	40.8 ± 1.8§

[a] Cultures treated with the vehicle alone (DMSO 0.1%) were used as controls. Data are expressed as mean ± SD of the three independent experiments. °, $P < 0.05$ vs control not exposed to TNF-α; *, $P < 0.05$ vs control exposed to TNF-α 10 ng/mL; §, $P < 0.05$ vs control exposed to TNF-α 20 ng/mL.

potential gene targets predisposing the vasculature to endothelial dysfunction and to "proatherogenic" phenotype are pro-inflammatory molecules, receptors for advance glycation end products (RAGE) and adhesion molecules (*33*).

Within this picture, cellular and extracellular redox balance plays an important role. Accumulating evidence indicates that overproduction of ROS is a key event in the development of many cardiovascular diseases (*34*). ROS (including superoxide and hydrogen peroxide) are produced by endothelial cells and the adjacent smooth muscle cells, adventitial fibroblasts, and inflammatory cells. Whereas low levels of ROS contribute to normal vascular function, the uncontrolled higher concentrations of these chemical species mediate cell injury. TNF-α stimulates the production of ROS in a variety of cell types, and ROS can serve as signals facilitating NF-κB activation (*35*). The discovery of specific pathways affected by antioxidants, such as the redox-sensitive NF-κB regulated signaling, led to the hypothesis that phytochemicals endowed with antioxidant properties may act as modulators of gene regulation and signal transduction pathways (*36*). Our study demonstrates that C3G, an anthocyanin clearly recognized as a potent antioxidant and free radical scavenger (*37*, *38*), is able to protect endothelial cells from TNF-α-induced cell damage in a dose-dependent way.

In particular, C3G appears to be able to significantly inhibit the activation of NF-κB induced by TNF-α. As the concentrations of C3G used in this study do not affect cell viability, the decreased nuclear levels of NF-κB observed in TNF-α-challenged HUVECs pretreated with C3G are not due to cell death. In agreement with these findings, we have previously observed that C3G is able to prevent NF-κB activation induced by ultraviolet irradiation in human keratinocytes (*10*).

Previous studies have shown that NF-κB is involved in the cytokine-induced up-regulation of VCAM-1 and ICAM-1, early markers for atherosclerosis (*39*), in endothelial cells. It is known that promoter elements in the genes encoding for the adhesion molecule ICAM-1 and VCAM-1 contain from one to three NF-κB binding sites and that NF-κB provides an effective "transducer" for feed-forward activation of them (*40*). The data presented herein indicate that, as expected, TNF-α markedly induces the gene expression of VCAM-1, ICAM-1, and E-selectin in HUVECs (**Figure 3**) and that C3G pretreatment significantly inhibits the effects of TNF-α, in a dose-dependent way. The effect of C3G on the expression of adhesion molecules was confirmed at the functional level by leukocyte/HUVEC coculture experiments, which evidenced that C3G reduces leukocyte adhesion induced by TNF-α.

According to our observations, Xia and co-workers (*41*) have demonstrated that C3G is able to reduce CD40-induced adhesion molecules release in HUVECs. In their experimental model the protective effect of C3G has been found to be mediated by the inhibition of JNK and p38 activation.

Oxidative stress has recently been suggested to be an important regulator in TNF-α-induced NF-κB activation and endothelial dysfunction (*42*). Our results indicate that intracellular levels of H_2O_2 and lipid peroxidation byproducts (MDA/HNE) increase following treatment with TNF-α, corroborating the importance of a role for ROS in mediating NF-κB mobilization and the subsequent VCAM-1 and ICAM-1 induction. In our study, C3G has been demonstrated to restore the intracellular oxidative stress status and to reduce the oxidant species overproduction.

In conclusion, the results reported here clearly indicate that C3G can down-regulate the TNF-α-stimulated NF-κB signal transduction pathway, which responds to oxidative signals, and thus inhibit VCAM-1 and ICAM-1 induction in human endothelial cells. These findings are in agreement with previous papers showing that a wide variety of plant-derived agents can alter or correct undesired cellular functions caused by abnormal proinflammatory signal transmission mediated by inappropriately activated NF-κB (*43*). Our observations contribute to provide a conceptual background for the understanding of the mechanisms underlying the role of C3G, as well as other dietary plant polyphenols, in the prevention of diseases associated with inflammation and oxidative stress, including atherosclerosis.

ABBREVIATIONS USED

C3G, cyanidin-3-*O*-glucoside; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; E-selectin, endothelial adhesion molecule 1; HUVECs, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; LDH, lactate dehydrogenase; OPA, *o*-phthalaldehyde; SSA, sulfosalycilic acid; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1.

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