JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Polyphenolics from Açaí (*Euterpe oleracea* Mart.) and Red Muscadine Grape (*Vitis rotundifolia*) Protect Human Umbilical Vascular Endothelial Cells (HUVEC) from Glucose- and Lipopolysaccharide (LPS)-Induced Inflammation and Target MicroRNA-126

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**ABSTRACT:** Endothelial anti-inflammatory effects of açaí (Ac) and red muscadine grape (Gp) polyphenolics have not been extensively investigated. It was hypothesized that polyphenolics from Ac and Gp exert comparable protective effects in human vascular endothelial cells (HUVEC) upon inflammatory stress. Furthermore, this study investigated whether microRNAs relevant to endothelial function might be regulated by Ac and Gp. Results showed that Ac and Gp (5−20 mg gallic acid equivalent/L) protected HUVEC against glucose-induced oxidative stress and inflammation. Glucose-induced expression of interleukin-6 and -8 was down-regulated by Ac and Gp at mRNA and protein levels. Upon lipopolysaccharide (LPS; 1 $\mu$g/L)-induced inflammation, Ac and Gp inhibited gene expression of adhesion molecules and NF-$\kappa$B activation to similar extents, although Gp was more effective in decreasing PECAM-1 and ICAM-1 protein. Of the screened microRNAs, only microRNA-126 expression was found to be modulated by Ac and Gp as the underlying mechanism to inhibit gene and protein expression of VCAM-1.

**KEYWORDS:** açaí, muscadine grape, HUVEC, inflammation, hyperglycemia

## INTRODUCTION

Obesity is a major problem worldwide that increases the risk for a variety of chronic diseases, including type 2 diabetes. According to the World Health Organization, type 2 diabetes comprises 90% of people with diabetes around the world including >220 million people worldwide. Projections indicate that diabetes deaths will double between 2005 and 2030. Vascular complications resulting from deterioration of endothelial function in a high-glucose environment and the activation of genes relevant to inflammation, immunity, and atherosclerosis are linked to mortality in diabetes patients. The mechanisms by which hyperglycemia results in tissue damage involve oxidative stress, inflammation, and the induction of adhesion molecules in vascular endothelial cells during the early development of atherosclerosis.[1]

The endothelial-protective effects of grape (*Vitis vinifera*) extracts rich in flavan-3-ols, phenolic acids, anthocyanins, flavonols, and resveratrol were previously demonstrated.[2,3] Similar effects from red muscadine (*Vitis rotundifolia*) grape and açaí fruit (*Euterpe oleracea*) extracts are less extensively investigated. These fruits share many similarities in polyphenolic composition with noted exceptions including flavonoid *C*-glycosides in açaí fruit along with predominance of cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside.[4] The polyphenol profile of açaí has been previously studied and reported.[4−7] The main compounds present in açaí pulp are anthocyanins, including cyanidin-3-glucoside, cyanidin-3-rutinoside, cyanidin-3-arabinosylarabinoside, cyanidin-3-arabinoside, cyanidin-3-acetylhexose, peonidin-3-rutinoside, peonidin-3-glucoside, pelargonidin-3-glucoside; flavones, including homoorientin, orientin, taxifolin deoxyhexose, and isovitexin; various flavanol derivatives, including (+)-catechin, (−)-epicatechin, and procyanidin dimers and trimers; and phenolic acids, including protocatechuic, *p*-hydroxybenzoic, vanillic, syringic, and ferulic acids.[4]

Muscadine grapes are notably different due to their high concentration of free ellagic acid, ellagic acid glycosides, and ellagitannins. The predominant anthocyanins are 3,5-diglucosides of delphinidin, cyanidin, and petunidin;[8,9] the phenolic acids hydroxybenzoic and hydroxycinnamic; stilbenes; and flavonols, including quercetin, myricetin, and kaempferol.[10,11] Increasing evidence shows that polyphenolics present in these fruits might protect endothelial cells against inflammation, insulin resistance, and atherosclerotic risk factors through regulation of gene expression; moreover, these events might be exerted at physiologically low concentrations of polyphenolics.

This study aims to determine how Ac and Gp polyphenolics can protect HUVEC against induced oxidative stress and inflammation. Oxidative stress may arise from an imbalance between the production of reactive oxygen and nitrogen species (ROS/NOS) and the activity of protective endogenous antioxidant defense systems. The resulting inflammation involves cytokines such as interleukins IL-6 and IL-8, via nuclear factor-$\kappa$B (NF-$\kappa$B)

**Received:** March 15, 2011
**Revised:** June 3, 2011
**Accepted:** June 17, 2011
**Published:** June 17, 2011





dx.doi.org/10.1021/jf201056x | *J. Agric. Food Chem.* 2011, 59, 7999–8012

activation.[12] Moreover, the formation of ROS in the vascular system may play an important role in the crosstalk between vasoactive substances, that is, angiotensin II (AngII) and pro-inflammatory cytokines such as IL-6.[13] Additionally, angiotensin II induced by intracellular oxidative stress has been shown to stimulate endothelial vascular cell adhesion molecule-1 (VCAM-1) and insulin resistance.[14] In general, chronic inflammation produced by pro-inflammatory cytokines seems to be involved in most of the events causing both type 1 and type 2 diabetes and related complications due to its apparent relationship to glucose homeostasis,[13] the extent of leukocyte attraction,[12] and the adhesive properties of endothelial cells.[1] More recently, plant polyphenolics in general have been investigated for greater understanding of their properties that do not directly relate to antioxidant function. Plant polyphenolics have been shown to act at the molecular level to improve endothelial function and inhibit platelet aggregation.[15] Some flavonoid endothelial-protective mechanisms have been associated with the induction of antioxidant defense systems through modulation of expression and activity of mitochondrial antioxidant enzymes, inhibition of endothelial cell adhesion molecules mediated by interference with the NF-$\kappa$B-dependent transcription pathway, and regulation of blood pressure through inhibition of angiotensin-converting enzyme activity.[15,16] These mechanisms have been extensively studied, and recent findings demonstrate that microRNAs (miRs) can modulate them by inducing mRNA degradation or blocking translation. Likewise, recent studies demonstrate that plant polyphenolics can modulate the expression of miRs.[17,18] Therefore, this study aims to screen some miRs expressed in endothelial cells that play a role in endothelial function through post-transcriptional regulation of innate immune response, redox signaling, inducible adhesion molecules, and hypertension.[19]

miRs, as regulators of vascular biology, are approximately 22-ribonucleotide-long ncRNAs, with a potential to recognize multiple mRNA targets guided by sequence complementarity and RNA-binding proteins. They can regulate gene expression at the post-trancriptional level by binding to the 3′-UTR of mRNA (reviewed by Sen et al.[19]). miR-146 has been directly implicated in mechanisms by which innate immune response is regulated by a negative feedback loop that attenuates chronic inflammation, causes desensitization of immune cells, and reduces production of pro-inflammatory cytokines.[20] The target mRNAs of miR-146 were found to be the Toll-like and cytokine receptors' downstream components, IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6), which activate NF-$\kappa$B. NF-$\kappa$B, in turn, activates miR-146a, which inhibits the gene expression of IRAK1 and TRAF6, serving as a negative feedback loop.[20] Pregnane X receptor (PXR) is one of the determined target genes of miR-148a, which was found to be inversely correlated to the expression of PXR. This implies a role of miR-148a in cell antioxidant defenses and inflammation.[21] PXR is involved in the regulation of drug-metabolizing and antioxidant enzymes and transporters such as the cytochrome P450 (CYP) family, glutathione *S*-transferase (GST), and multidrug-resistant protein 1 (MDRP1).[22,23] Moreover, PXR and NF-$\kappa$B have been found to be mutually repressive through interactions at the transcription factor level.[24] miR-155 was found to be involved in macrophage inflammatory responses[25] and regulation of antimicrobial defense.[20] Additionally, miR-155 was identified as a negative regulator of blood pressure mediated by the post-trancriptional repression of the angiotensin II type 1 receptor (AGTR1).[26] miR-126 was found to be expressed in endothelial cells and to inhibit VCAM-1.[27] The role of VCAM-1, a member of the adhesion molecule superfamily to which monocytes and lymphocytes can bind, is being considered as a potential therapeutic target in the prevention of atherosclerosis.[28] A reduced expression of VCAM-1 was associated with impaired progression and scope of atherosclerotic lesions in an animal model study.[28] The goal of this study was to compare the endothelial-protective effects of açaí and red muscadine grape polyphenolics against oxidative stress and inflammation, as well as to investigate the potential involvement of relevant microRNAs in endothelial function.

## MATERIALS AND METHODS

**Plant Material.** Seedless pulp from red muscadine grapes (*V. rotundifolia*) (cv. Noble) was obtained from a local vineyard in central Florida. Muscadine grape pulp and skin were obtained after juice pressing and seed removal. Açaí (*E. oleracea* Mart.) frozen pasteurized pulp imported from Brazil was kindly donated by the Bossa Nova Beverage Group (Los Angeles, CA). Açaí (Ac) and muscadine grape (Gp) polyphenolics were extracted with 100% methanol (0.01% 12 N hydrochloric acid) (1:1 ratio). Details regarding the chemistry and polyphenolic profile of the extracts used in this study have previously been described for açaí[4−7] and muscadine grapes.[8−11] Preparation of the methanolic extracts was performed as described by Cardona et al.,[9] with some modifications. Briefly, methanolic extracts were filtered through Whatman no. 1 filter paper, solvent was removed under reduced pressure at 45 °C. The aqueous extract was loaded on the $C_{18}$ cartridge preconditioned with 50 mL of methanol and 50 mL of nanopure water. After washing with 50 mL of water to eliminate organic acids and sugars, polyphenols bound to the matrix were eluted with 50 mL of 100% methanol and evaporated using a Speedvac (Savant, Thermo Scientific Inc., Pittsburgh, PA). The dried polyphenolics were stored at −20 °C in nitrogen atmosphere. For cell culture assays, dried polyphenolics were redissolved in DMSO to a known concentration based on total phenolics content,[29] quantified against a gallic acid standard curve, and expressed as milligrams of gallic acid equivalent (GAE) per liter. All polyphenolic extracts redissolved in DMSO were normalized to a final concentration of 0.2% DMSO in culture medium when applied to the cells; a control with 0.2% DMSO was included in all assays.

**Chemicals, Antibodies, and Reagents.** The Folin−Ciocalteu reagent, dichlorofluorescein diacetate (DCFH-DA), and lipopolysaccharide (LPS) were purchased from Fisher Scientific (Pittsburgh, PA). Dimethyl sulfoxide (DMSO) and Triton X-100 were obtained from Sigma (St. Louis, MO). HPLC grade solvents and hydrogen peroxide (Acros) were purchased from VWR International (Bristol, CT). Bradford reagent was obtained from Bio-Rad (Hercules, CA), and antibodies against NF-$\kappa$Bp65 and phospho-NF-$\kappa$Bp65, ICAM-1, phospho-ERK1/2, and PECAM-1 were obtained from Cell Signaling Technology (Beverly, MA); $\beta$-actin was from Sigma-Aldrich (St. Louis, MO); VCAM-1 and ERK1 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and AGTR1 was obtained from Abcam (Cambridge, MA).

**Cell Line.** The human umbilical vascular endothelial cells (HUVEC) were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured using ECM medium supplemented with 5% of fetal bovine serum, 1% of endothelial growth supplement (ECGS), and 1% of penicillin/streptomycin solution (ScienCell). Cells were maintained at 37 °C with a humidified 5% $CO_2$ atmosphere.

**Cell Proliferation.** Cells ($1.5 \times 10^4$) were seeded onto a 24-well plate and incubated for 24 h to allow cell attachment before exposure to various concentrations of Ac and Gp polyphenolics. A pretreatment number of cells (0 time value) was quantified with an electronic cell

counter (Z1 Series, Beckman Coulter, Inc.), and medium was replaced containing the muscadine grape (Gp) or açaí (Ac) polyphenolics dissolved in DMSO. The difference in the number of cells between final incubation time (24 and 48 h) and 0 time represents net growth.

**Generation of ROS.** The DCFH-DA assay was used to determine the intracellular generation of ROS by high glucose as described by Wang et al.[30] HUVEC ($6 \times 10^4$/mL) were seeded in a 96-well plate and incubated for 24 h. After that, HUVEC were pretreated for 60 min with different concentrations (0−40 mg GAE/L) of Ac or Gp polyphenolics. Cells were then washed with phosphate buffer solution, pH 7.0 (PBS), and incubated with 5 $\mu$M DCFH-DA for 30 min at 37 °C. After incubation, cells were centrifuged at low speed (1000 rpm) for 3 min and then were gently washed with PBS. The cells were then stimulated with 25 mM glucose, and the fluorescence intensity was measured after 30 min using a fluorescent microplate reader (BMG Labtech Inc., Durham, NC) at 485 nm excitation and 538 nm emission; relative fluorescence units (RFU) were normalized to control cells not treated with Ac or Gp polyphenolics.

**mRNA and microRNA Analysis.** Cells ($3 \times 10^5$) were seeded onto a 12-well plate and incubated for 24 h to allow cell attachment, and then cells were pretreated with varying concentrations of Ac or Gp polyphenolics for 30 min and stimulated with glucose (25 mM) for 24 h or LPS (1 $\mu$g/mL) for 3 h before mRNA extraction and analysis. Total RNA was isolated according to the manufacturer's recommended protocol using the *mir*Vana extraction kit (Applied Biosystems, Foster City, CA), and samples were evaluated for nucleic acid quality and quantity using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Isolated RNA was used to synthesize cDNA using a Reverse Transcription Kit (Invitrogen Corp., Grand Island, NY) according to the manufacturer's protocol. qRT-PCR was carried out with the SYBR Green PCR Master Mix from Applied Biosystems on an ABI Prism 7900 sequence detection system (Applied Biosystems). Primers were designed using Primer Express software (Applied Biosystems). Each primer was homology-searched by an NCBI BLAST search to ensure that it was specific for the target mRNA transcript. The pairs of forward and reverse primers were purchased from Integrated DNA Technologies, Inc. (San Diego, CA). Product specificity was examined by dissociation curve analysis. The sequences of the primers used were as follows: TATA binding protein (TBP) (forward, 5′-TGC ACA GGA GCC AAG AGT GAA-3′; reverse, 5′-CAC ATC ACA GCT CCC CAC CA-3′); NF-$\kappa$B (forward, 5′-TGG GAA TGG TGA GGT CAC TCT-3′; reverse, 5′-TCC TGA ACT CCA GCA CTC TCT TC-3′); PXR (forward, 5′-TGA CAA AAG CAG CAC AAG GAA-3′; reverse, 5′-CAG TGC CCG CCA TCA CA-3′); ICAM-1 (forward, 5′-TGG CCC TCC ATA GAC ATG TGT-3′; reverse, 5′-TGG CAT CCG TCA GGA AGT G-3′); E-selectin (forward, 5′-CCC GTG TTT GGC ACT GTG T-3′; reverse, 5′-GCC ATT GAG CGT CCA TCC T-3′); VCAM-1 (forward, 5′-ACA GAA GAA GTG GCC CTC CAT-3′; reverse, 5′-TGG CAT CCG TCA GGA AGT G-3′); IL-6 (forward, 5′-AGG GCT CTT CGG CAA ATG TA-3′; reverse, 5′-GAA GGA ATG CCC ATT AAC AAC AA-3′); IL-8 (forward, 5′-CAC CGG AAG GAA CCA TCT CA-3′; reverse, 5′-AGA GCC ACG GCC AGC TT-3′); CYP1A1 (forward, 5′-TCC TGG AGA CCT TCC GAC ACT-3′; reverse, 5′-CTT TCA AAC TTG TGT CTC TTG TTG T-3′); MDRP1 (forward, 5′-CAGACAGGGAAGGGAAACATTTT-3′; reverse, 5′-CAT GAA ACC TGA CTT GCT TCT GAA-3′); catalase (forward, 5′-CTG GAG AAG TGC GGA GAT TCA-3′; reverse, 5′-AAT GCC CGC ACC TGA GTA AC-3′); AGTR1 (forward, 5′-TGC TTC AGC CAG CGT CAG T-3′; reverse, 5′-CAA TGC TGA GAC ACG TGA GTA GAA-3′).

Quantification of microRNAs miR-148a and miR-146a was performed after 24 h of glucose challenge; miR-155 and miR-126 were analyzed after 3 h of LPS challenge using the Taqman microRNA reverse transcription kit (Applied Biosystems) and qRT-PCR reaction using TaqMan2X Universal PCR Master Mix (No AmpEraseUNG) (Applied Biosystems) according to the manufacturer's specifications. TATA binding protein (TBP) and miR-NU6B small nuclear RNA were used as endogenous controls to determine relative mRNA and microRNA expression, respectively.

**Western Blot Analysis.** Cells ($1 \times 10^6$) were seeded onto a 10 cm culture plate in regular growing medium and incubated for 24 h to allow cell attachment. Cells were pretreated with Ac and Gp polyphenolics for 30 min before glucose (25 mM) or LPS (1 $\mu$g/mL) stimulation. After 24 h incubation, medium was discarded and cells were washed with PBS and removed by scraping using PBS. After centrifugation, cell pellets were lysed with nondenaturing buffer (10 mM Tris-HCl, 10 mM $NaH_2PO_4$, 130 mM NaCl, 1% (v/v) Triton X-100, 10 mM sodium pyrophosphate, pH 7.5) and 1% proteinase inhibitor cocktail (Sigma-Aldrich) for 30 min in ice. Solid cellular debris was removed by centrifugation at 10000 rpm for 10 min at 4 °C. The supernatant was collected and stored at −80 °C. Protein content was determined using the Bradford reagent (Bio-Rad, Hercules, CA) following the manufacturer's protocol. For each lane, 60 $\mu$g of protein was diluted with Laemmli's loading buffer, boiled for 5 min, loaded on an acrylamide gel (10%), and subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis at 100 V for 2 h. Proteins were transferred by wet blotting onto 0.2 $\mu$m PVDF membrane (Bio-Rad). Membranes were blocked using 5% milk in 0.1% PBS-Tween (PBS-T) for 30 min and incubated with primary antibodies (1:1000) in 3% bovine serum albumin in PBS-T overnight at 4 °C with gentle shaking, followed by incubation with the secondary antibody (1:2000) in 5% milk PBS-T for 2 h. Reactive bands were visualized with a luminal reagent (Santa Cruz Biotechnology) after 1 min of reaction.

**Multiplex Bead Assay.** Cells ($1 \times 10^5$) seeded onto 12-well plates were incubated for 24 h to allow cell attachment and subsequently pretreated with Ac and Gp polyphenolics for 30 min before glucose (25 mM) stimulation. The proteins released into culture media after 24 h were evaluated by multiplex bead-based immunoassays (Luminex) using the human cytokine/chemokine kit (Millipore, Billerica, MA) according to the manufacturer's protocol. The kit contained the 14 cytokine/chemokine panel: granulocyte-macrophage colony-stimulating factor (GM-CSF); interferon $\gamma$ (IFN$\gamma$); interleukins (IL-) IL-1$\beta$, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, and IL-13; monocyte chemotactic protein 1 (MCP-1); and tumor necrosis factor $\alpha$ (TNF$\alpha$). Data were analyzed using Luminex xPonent 3.0 software. Quantitative results (pg/mL) were normalized to the untreated control.

**Enzyme-Linked Immunosorbent Assay (ELISA).** Cells seeded ($1 \times 10^5$ cells/well) onto 12-well plates were incubated for 24 h to allow cell attachment and pretreated with Ac and Gp polyphenolics for 30 min before LPS (1 $\mu$g/mL) stimulation. Cell culture supernatants were evaluated by ELISA using an Invitrogen kit (Invitrogen Corp.) for ICAM-1 according to the manufacturer's protocol. The final ICAM-1 (ng/$\mu$g protein) was calculated after quantifying the protein concentration using Bradford reagent (Bio-Rad) following the manufacturer's protocol. Results were normalized to untreated control cells.

**Glutathione *S*-Tranferase (GST) Activity.** Cells ($1 \times 10^6$) were seeded onto 10 cm culture plates and incubated for 24 h to allow cell attachment. The effects of 24 h of incubation with Ac and Gp on GST activity were evaluated as were the effects of a pretreatment with Ac and Gp for 30 min followed by glucose (25 mM) stimulation for 24 h on GST activity. Cell lysates were obtained as described in the Western blot procedure, and GST activity was assessed following the procedure described by Habig et al.[31] with some modifications. Briefly, the reaction was carried out in a 96-well plate with 0.2 mL total reaction volume by mixing 5 $\mu$L of cell lysate, 10 $\mu$L of freshly prepared 100 mM glutathione reduced free acid (Calbiochem, EMD Gibbstown, NJ), pH 7.0, 180 $\mu$L of potassium phosphate buffer, pH 6.5, and 5 $\mu$L of a 40 mM ethanolic solution of 1-chloro-2,4-dinitrobenzene (CDNB) (Sigma-Aldrich).

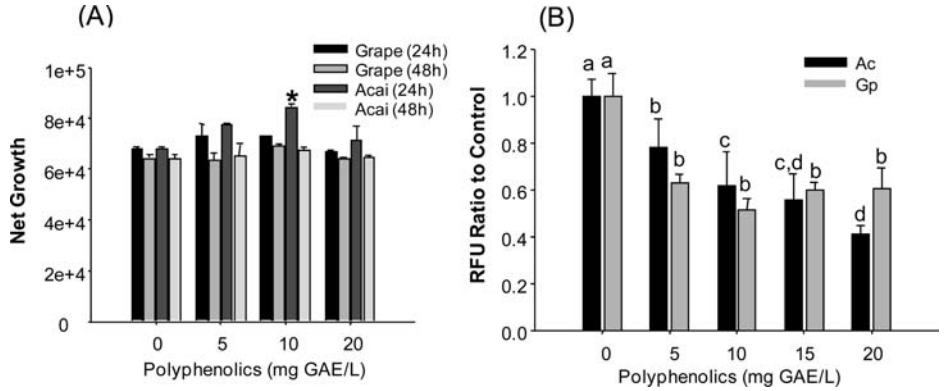


**Figure 1.** Concentration-dependent impact of Ac and Gp polyphenolics on the growth of HUVEC (A) and high glucose-induced ROS formation (B). Values are the mean of three replicates ± SD. * indicates significant difference from untreated control within plant material ($p \leq 0.05$). Different letters within plant material indicate a significant difference ($p \leq 0.05$).

The increase in absorbance was monitored at 340 nm using a 96-well plate reader (BMG Labtech Inc., Durham, NC), immediately after the addition of the CDNB, and correction was made for the increase of absorbance in the absence of cell lysate.

GST activity was calculated using the corrected CDNB extinction coefficient of 0.00503 $\mu M^{-1}$ adjusted for the path length of the solution in the well (0.6 cm) as follows:

$$\text{GST activity (nmol/}min\text{/mL)}$$

$$= (\Delta A_{340/min}/0.00503\ \mu M^{-1})(0.2\ \text{mL}/0.005)(\text{sample dilution})$$

The final GST activity (nmol/min/mg protein) was calculated after quantification of the protein concentration on cell lysates using Bradford reagent (Bio-Rad) following the manufacturer's protocol. Results were normalized to untreated control cells.

**Transfection Assays.** Transfections of 100 pmol of miRNA-126 inhibitor (Dharmacon, Inc., Lafayette, CO) into 10 $\mu$L of cell suspension containing $1 \times 10^5$ cells were performed using the Neon Transfection System according to the manufacturer's protocol (Invitrogen, Carlsbad, CA); the controls for the transfection used an equal amount of a nonspecific oligonucleotide. After transfection, cells were seeded onto 12-well plates and allowed to stabilize for 24 h. Total mRNA was extracted after treatment with the Ac and Gp for 24 h and after 30 min of pretreatment with Ac and Gp followed by LPS stimulation for 24 h. Analysis of miR-126 expression was performed as described under mRNA and microRNA analysis.

**Immunofluorescence Analysis.** Cells ($8 \times 10^4$) seeded onto four-chamber coverslip culture were incubated for 24 h to allow cell attachment followed by treatment with Ac (10 mg GAE/L) and Gp (10 mg GAE/L) for 24 h; controls received the same amount of DMSO. Monolayers were prepared for immunofluorescence analysis by undergoing two washes with PBS, followed by fixation with 3.8% paraformaldehyde and permeabilization in 0.5% Triton X-100. Blocking was performed for 2 h in PBS containing 5% BSA. Then, goat polyclonal IgG PXR primary antibody (Santa Cruz Biotechnology) (1:250 dilution) was applied for 2 h in the blocking solution; no primary antibody was used in the negative control. All subsequent steps were performed in the dark. The secondary antibody donkey anti-goat IgG-FITC (Santa Cruz Biotechnology) (1:200 dilution) was applied for 45 min in the same way as the primary antibody. After a rinse with PBS, mounting of the coverslip was performed with Prolong Gold Antifade-DAPI reagent (Invitrogen, Carlsbad, CA). All microscopic images were taken in 40×X amplification with a Zeiss Axioplan 2 microscope (Carl Zeiss, Thornwood, NY) fitted with an Axiocam high-resolution digital camera and Axiovision 4.1 software.

**Statistical Analysis.** Quantitative data represent mean values with the respective standard deviation (SD) or standard error of the mean (SE) corresponding to three or more replicates. Data were analyzed by one-way analysis of variance (ANOVA) using SPSS version 15.0 (SPSS Inc., Chicago, IL). Duncan pairwise comparisons were used for establishing statistically significant differences.

## RESULTS

**Cell Growth and Generation of ROS.** The net growth of HUVEC assessed by cell counting after 24 and 48 h of incubation was not inhibited by Ac or Gp polyphenolics within the tested concentration range (Figure 1A). Moreover, Ac and Gp protected cells against the production of ROS induced by hyperglycemic conditions (25 mM glucose) within a dose range of 5−40 mg GAE/L (Figure 1B). Ac protected HUVEC in a dose-dependent manner by decreasing the production of ROS to 0.4-fold, whereas Gp decreased the production of ROS to 0.6-fold of untreated controls, although values were not statistically different among concentrations (5−20 mg GAE/L).

**Effects of Ac and Gp Polyphenolics on NF-κBp65 and Pro-inflammatory Cytokines.** NF-κB is a central pro-inflammatory transcription factor that is involved in the induction of several pro-inflammatory cytokines among other activities and is activated through phosphorylation. Glucose increased the phosphorylation of NF-κBp65, whereas Gp and Ac polyphenolics reversed this effect. The constitutive NF-κBp65 remained constant; this indicates that decreased phospho-NF-κBp65 is due to the inhibitory effect of Ac and Gp polyphenolics on NF-κB activation (Figure 2A). In addition, mRNA expression of NF-κB increased by 1.1- fold after glucose challenge. This effect was reduced by Ac and Gp polyphenolics to 0.94- and 0.93-fold of untreated control cells, respectively (Figure 2B). Correspondingly, mRNA and protein expressions of pro-inflammatory cytokines/chemokines IL-6 and IL-8 were significantly up-regulated upon glucose stimulation. Glucose induced IL-6 mRNA by 1.24-fold, and Ac at 10 mg GAE/L reversed this effect to 0.37-fold. Likewise, Gp decreased IL-6 mRNA in a dose−response manner to 0.58-fold of untreated control cells (Figure 2C). For IL-8, mRNA was up-regulated by 1.15-fold by glucose, and this effect was reversed by Ac at 5 mg GAE/L to 0.64-fold and by Gp at 10 mg GAE/L to 0.77-fold of untreated controls (Figure 2D).

Secretion of IL-6, IL-8, and GM-CSF proteins was also increased by glucose stimulation. Among the 13 cytokine/chemokines

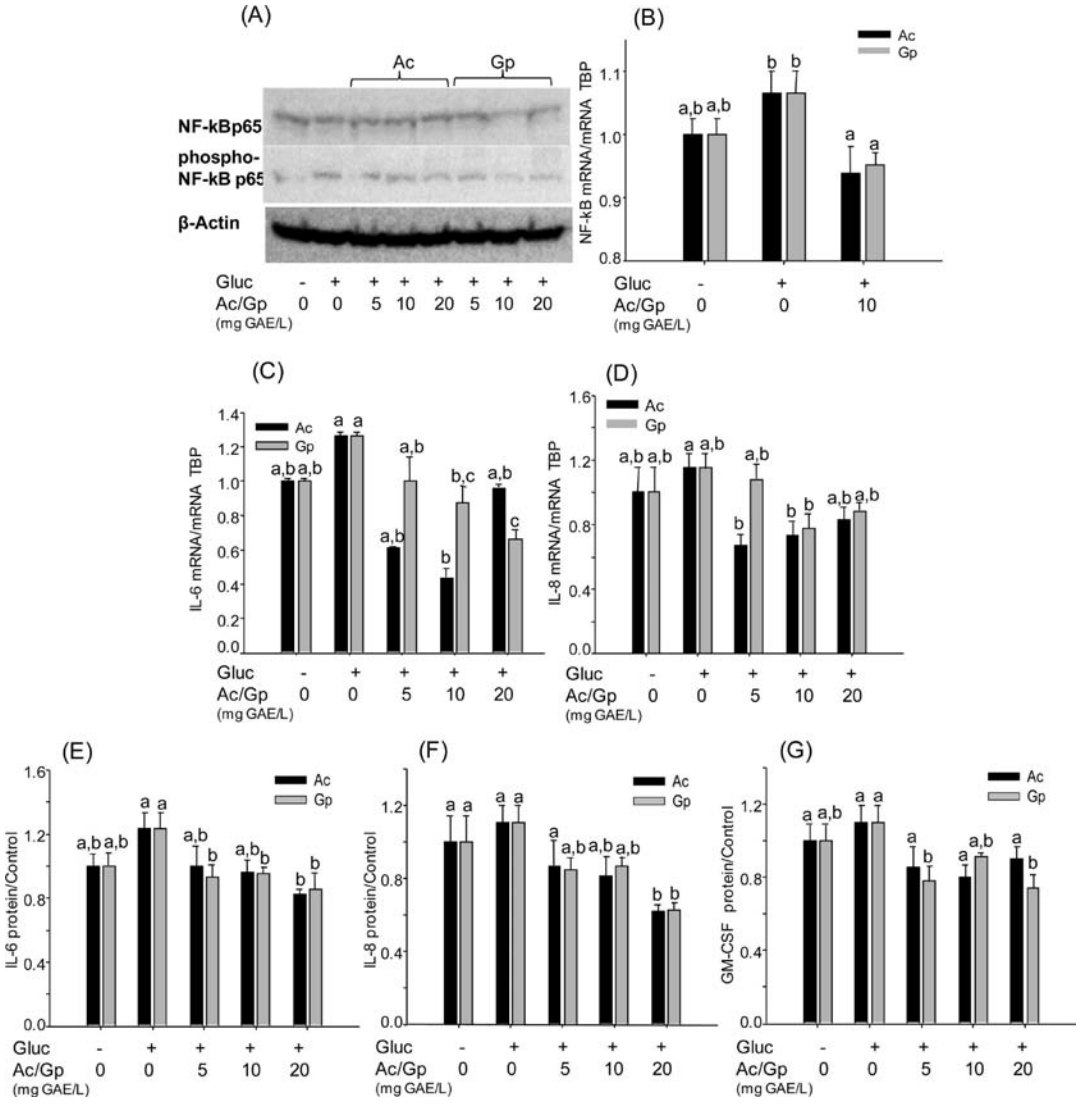


**Figure 2.** Effect of Ac and Gp polyphenolics in glucose-challenged endothelial cells. (A) Phosphorylation and activation of NF-κBp65. Cells were treated with solvent (DMSO) or different concentrations of Ac or Gp (5−20 mg GAE/L) for 30 min before glucose challenge (25 mM), and expression of proteins was determined after 24 h of incubation in whole-cell lysates by immunoblot analysis as described under Materials and Methods; total β-actin was detected to show the same amount of protein load. (B) Gene expression of NF-κB. Pro-inflammatory cytokines (C) IL-6 and (D) IL-8. Relative mRNA levels were determined after 24 h of glucose (25 mM) challenge by qRT-PCR as described under Materials and Methods. Protein levels of (E) IL-6, (F) IL-8, and (G) GM-CSF released into culture media. Protein expression was assessed after 24 h of glucose (25 mM) challenge in culture media by multiplex bead assay as explained under Materials and Methods. Quantitative data are the mean ± SE for gene expression and ± SD for protein levels ($n = 3$). Different letters within plant material indicate a significant difference ($p \leq 0.05$).

evaluated in the assay, only these three were found in quantities that fall within the standard curve values. Glucose challenge induced IL-6 secretion by 1.23-fold, and this increase was reversed by Ac and Gp to 0.66- and 0.69-fold of glucose-stimulated treatments, respectively (Figure 2E). Accordingly, the glucose-induced IL-8 secretion by 1.1-fold was down-regulated by Ac and Gp to 0.54- and 0.56-fold of glucose-stimulated treatments (Figure 2F). Additionally, the secretion of GM-CSF was increased by 1.1-fold by glucose and decreased by Ac (10 mg GAE/L) and Gp (20 mg GAE/L) to 0.72- and 0.67-fold of glucose-stimulated treatments, respectively (Figure 2G). Overall, these results indicate that Ac and Gp polyphenolics reversed glucose-induced intracellular and secretory inflammation biomarkers.

**Effect of Ac and Gp Polyphenolics on PXR.** PXR is a transcription factor and efficient regulator of genes involved in all phases of drug metabolism and excretion: the phase 1 drug metabolism genes including several CYP450s; the phase 2 drug metabolism genes encoding UDP-glucoronosyltransferases, glutathione-*S*-transferases; and the phase 3 drug efflux pumps.[22] Likewise, activation of PXR by dietary phytochemicals has been demonstrated.[23]

The immunofluorescence analysis (Figure 3A) showed that when cells were treated with Ac and Gp at 10 mg GAE/L, nuclei appeared more illuminated, indicating increased translocation and activation of PXR. Western blotting demonstrated that Ac at 5 mg GAE/L and Gp at 5 and 10 mg GAE/L reversed the PXR glucose repression effect (Figure 3B). Glucose decreased PXR mRNA to 0.37-fold. This effect was reversed by Ac at ≥10 mg GAE/L and Gp at 5−20 mg GAE/L, whereas PXR mRNA was induced to 2.9-fold by Ac and by Gp to 4.48-fold of glucose-challenged cells (Figure 3C).

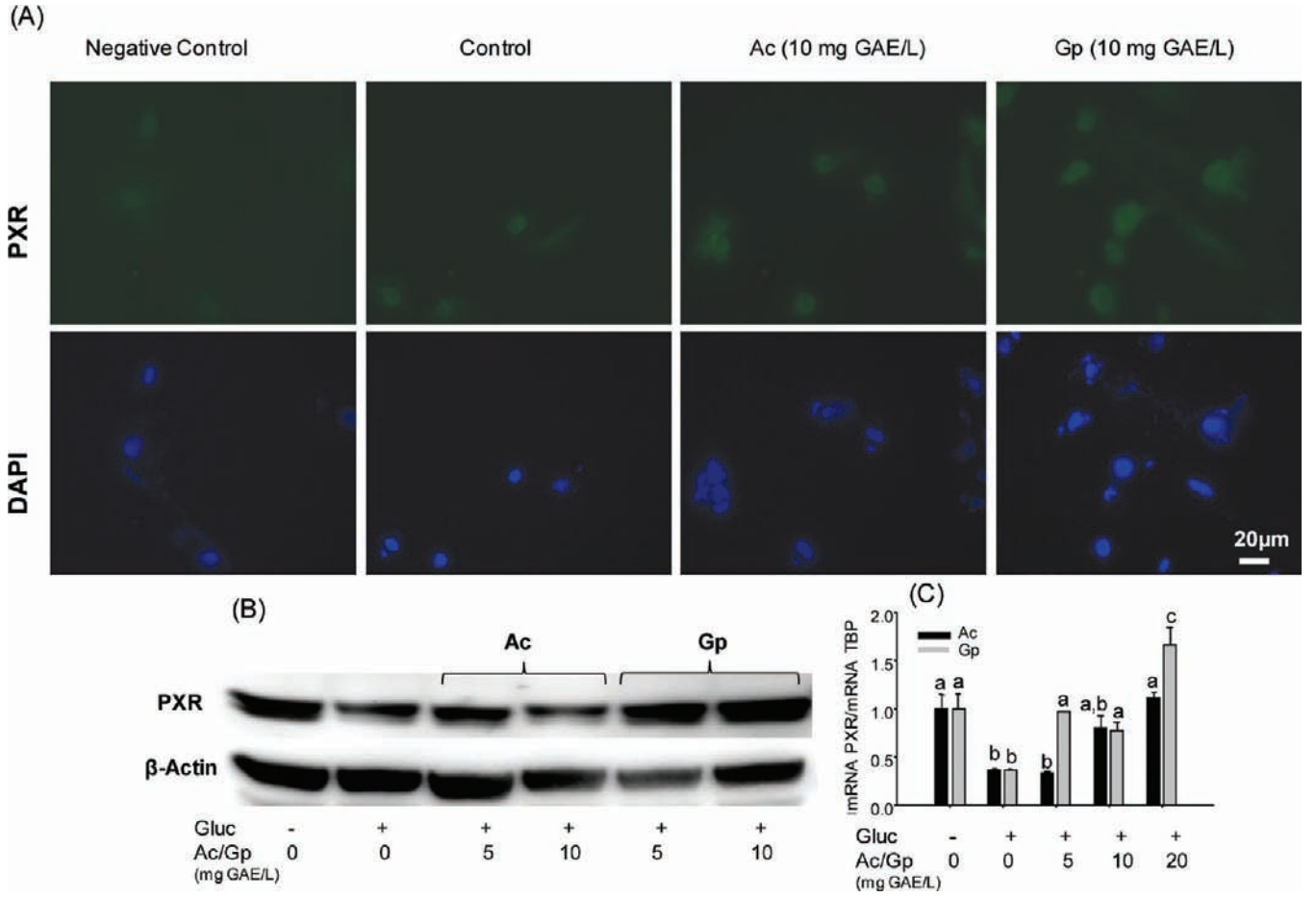


**Figure 3.** Effect of Ac and Gp polyphenolics on PXR expression and activation. (A) Immunofluorescence analysis. Cells were treated with solvent (DMSO) or Ac (10 mg GAE/L) or Gp (10 mg GAE/L) for 24 h and analyzed by immunofluorescence analysis assay as explained under Materials and Methods; width of each field on microscopy analysis is 20 $\mu$m. (B) Total PXR protein. Cells were treated with solvent (DMSO) or different concentrations of Ac or Gp (5−10 mg GAE/L) for 30 min before glucose challenge (25 mM), and expression of proteins was determined after 24 h of incubation in whole-cell lysates by immunoblot analysis as described under Materials and Methods; total $\beta$-actin was detected to show the same amount of protein load. (C) Gene expression of PXR. Cells were treated with solvent (DMSO) or different concentrations of Ac or Gp (5−20 mg GAE/L) for 30 min before glucose challenge (25 mM) for 24 h, and relative mRNA levels were determined by qRT-PCR as described under Materials and Methods. Quantitative data are the mean ± SE ($n$ = 3). Different letters within plant material indicate a significant difference ($p \leq 0.05$).

**Effect of Ac and Gp Polyphenolics on Metabolizing and Antioxidant Enzymes.** Previous studies have shown that an up-regulation of PXR may cause an up-regulation of antioxidant and metabolizing enzymes.[23] Results showed that glucose challenge decreased mRNA of MDRP1 and catalase by 0.50- and 0.49-fold, respectively, but not mRNA of CYP1A1. The glucose-induced CYP1A1 by 1.28-fold was enhanced by Ac (10 mg GAE/L) and Gp (20 mg GAE/L) to 1.49- and 1.86-fold, respectively, compared to untreated control cells (Figure 4A). Glucose challenge decreased mRNA of MDRP1 and catalase to 0.5-fold, and Ac at 5−10 mg GAE/L significantly increased the mRNA of MDRP1 to 1.52-fold, whereas Gp (5−20 mg GAE/L) restored basal levels found in untreated control cells (Figure 4B). Similar to the response on mRNA of MDRP1, Ac at 10 mg GAE/L induced catalase expression to 1.28-fold, and Gp restored the basal levels found on untreated controls (Figure 4C). Overall, Ac extract was shown to be more effective in inducing gene expression of the investigated enzymes when used at doses not higher than 10 mg GAE/L, whereas Gp polyphenolics showed a dose−response effect up to 20 mg GAE/L.

GST is also regulated by PXR activation;[22] it contributes to the detoxification process by conjugating electrophilic and/or lipophilic compounds and facilitates their dissolution and elimination from the body. However, neither Ac nor Gp polyphenolics induced GST at gene expression level (data not shown); instead, both of them induced GST activity. Ac (10−20 mg GAE/L) induced GST activity in a dose−response manner to 1.66-fold, whereas the Gp (10 mg GAE/L) induced GST to 1.92-fold (Figure 4D). When cells were challenged with glucose, Ac and Gp induced GST activity to similar extents (Figure 4E); maximum induction (1.54- and 1.43-fold, respectively) was exerted at 10 mg GAE/L.

**Effect of Ac and Gp Polyphenolics on miR-148a and miR-146a.** As Ac and Gp polyphenolics were shown to inhibit the mRNA and protein levels of PXR and NF-$\kappa$B regulated genes, the involvement of potentially associated miRs was investigated. miR-148a has been reported to recognize the 3′-untranslated region of human PXR mRNA and regulate its expression at post-transcriptional level,[21] whereas miRNA-146a was reported to negatively regulate NF-$\kappa$B activity and suppress expression of the NF-$\kappa$B target genes IL-8 and IL-6 within a negative feedback loop involving TRAF6 and IRAK1.[32] We therefore evaluated whether Ac and Gp polyphenolics might regulate these miRs on

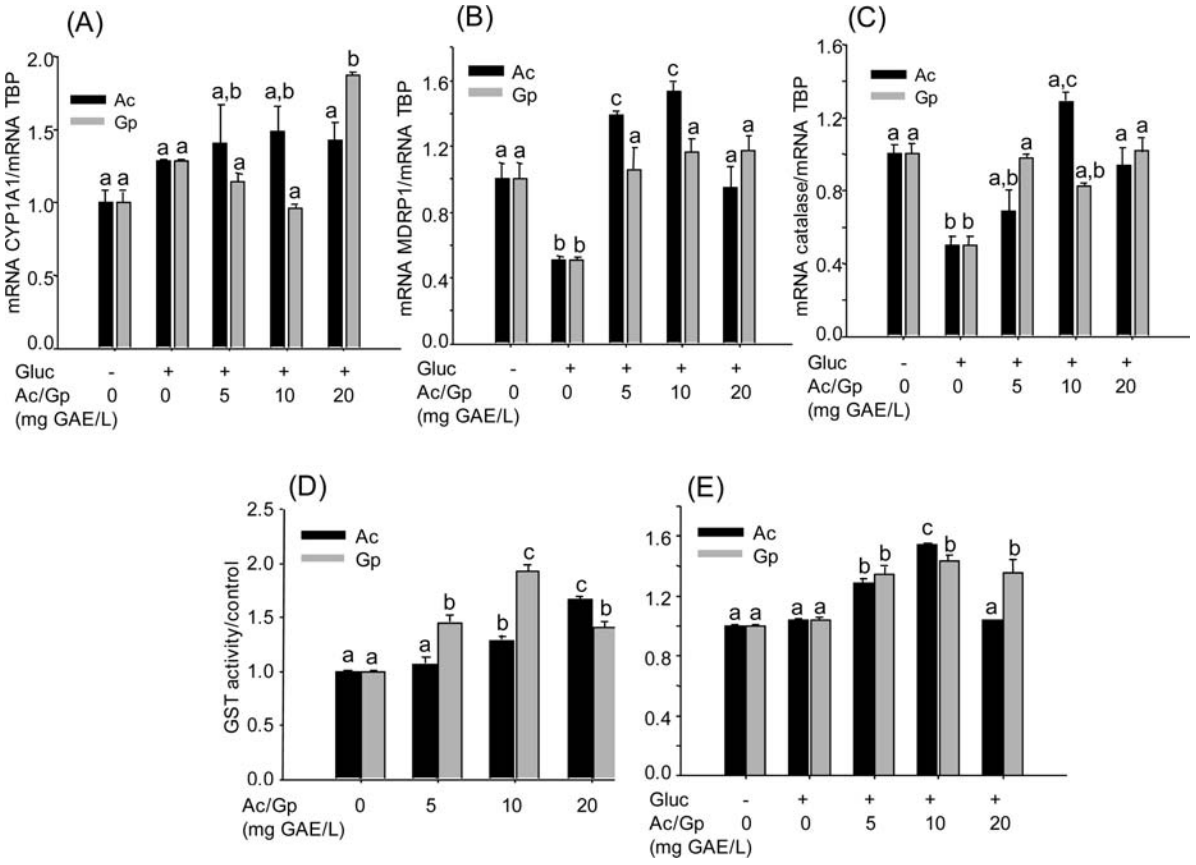


**Figure 4.** Ac and Gp polyphenolics induced metabolizing and antioxidant enzymes. mRNA levels of (A) CYPA1, (B) MDRP1, and (C) catalase. Cells were treated with solvent (DMSO) or different concentrations of Ac or Gp (5−20 mg GAE/L) for 30 min before glucose challenge (25 mM) for 24 h, and relative mRNA levels were determined by qRT-PCR as described under Materials and Methods. GST activity on endothelial cells under (D) physiological and (E) hyperglycemic conditions. Whole-cell lysates were analyzed by GST activity, and values were normalized to protein concentrations relative to untreated controls as described under Materials and Methods. Values are the mean ± SE ($n = 3$) for gene expression data and the mean ± SD ($n = 4$) for enzyme activity data. Different letters within plant material indicate a significant difference ($p \leq 0.05$).

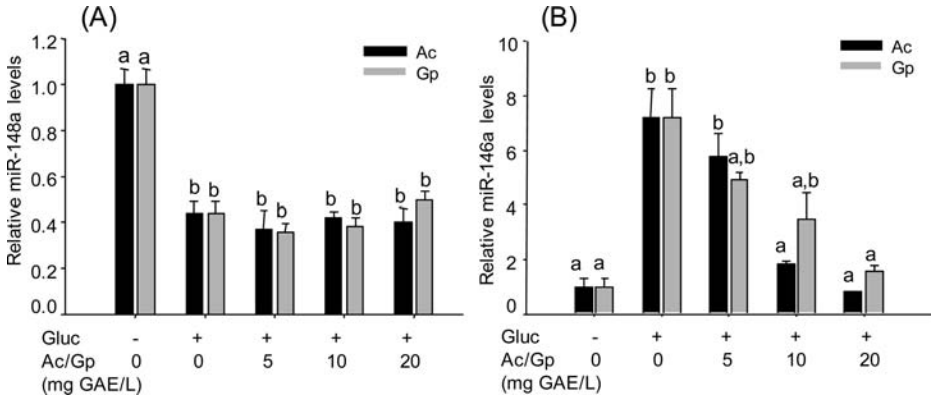


**Figure 5.** Modulation of miRs on glucose-challenged HUVEC. (A) miR-148a and (B) miR-146a. Cells were treated with solvent (DMSO) or different concentrations of Ac or Gp (5−20 mg GAE/L) for 30 min before glucose challenge (25 mM) for 24 h, and relative microRNA levels were determined by qRT-PCR as described under Materials and Methods. Values are the mean ± SE ($n = 3$). Different letters within plant material indicate a significant difference ($p \leq 0.05$).

HUVEC exposed to hyperglycemic conditions. Results showed that expression of miR-148a was down-regulated to 0.44-fold upon glucose challenge, and pretreatment with Ac or Gp polyphenolics did not induce any further significant change (Figure 5A). Potentially, the observed effects of these polyphenolics on PXR do not involve this miR under hyperglycemic conditions. Expression of miR-146a was up-regulated by glucose to 7.2-fold of untreated cells. Treatments with Ac and Gp at 5−20 mg GAE/L decreased the glucose-induced expression of miR-146a to 0.85- and 1.5-fold, respectively; these values were not statistically

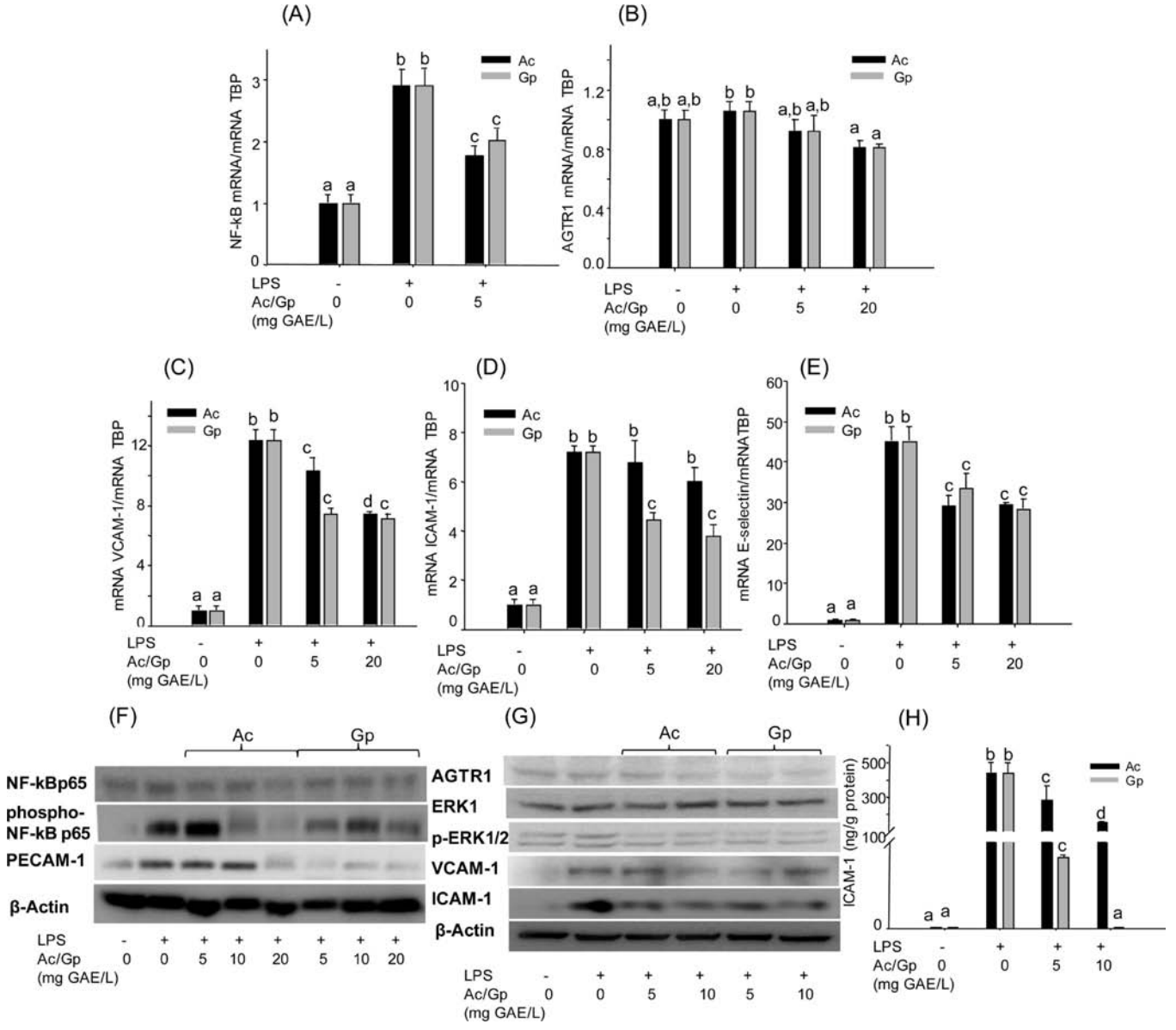


**Figure 6.** Modulation of inflammatory and cell adhesion markers on LPS-challenged HUVEC. Gene expression of the (A) NF-$\kappa$B, (B) AGTR1, and adhesion molecules (C) VCAM-1, (D) ICAM-1, and (E) E-selectin. Cells were treated with solvent (DMSO) or different concentrations of Ac or Gp (mg GAE/L) for 30 min before LPS challenge (1 $\mu$g/mL) for 3 h, and relative mRNA levels were determined by qRT-PCR as described under Materials and Methods. (F) Protein levels of phospho-NF-$\kappa$Bp65 and PECAM-1 modulated by Ac and Gp polyphenolics (5−20 mg GAE/L). (G) AGTR1, ERK1, phospho-ERK1/2, VCAM-1, and ICAM-1 modulated by Ac and Gp polyphenolics (5−10 mg GAE/L). Cells were treated with solvent (DMSO) or different concentrations of Ac or Gp for 30 min before LPS challenge (1 $\mu$g/mL), and expression of proteins was determined after 24 h of incubation in whole-cell lysates by immunoblot analysis as described under Materials and Methods. Total $\beta$-actin was detected to show the same amount of protein load. (H) Relative amount of ICAM-1 protein excreted to culture media. Cell culture supernatants from cells treated with solvent (DMSO) or different concentrations of Ac or Gp (5−10 mg GAE/L) for 30 min before LPS challenge (1 $\mu$g/mL) for 24 h were analyzed by ELISA. Values were normalized to protein concentrations relative to untreated controls as described under Materials and Methods. Quantitative data are the mean ± SE for gene expression and the mean ± SD for protein expression values ($n$ = 3). Different letters within plant material indicate a significant difference ($p \leq 0.05$).

different from the basal levels found in untreated control cells (Figure 5B).

**Effect of Ac and Gp Polyphenolics on LPS-Induced Inflammation and Adhesion.** We investigated the effect of Ac and Gp polyphenolics upon LPS stimulation, as an additional model to assess the extract's protective effects in vascular inflammation. Cell signals exerted by oxidized products of low-density lipoprotein (LDL) induce innate immune responses similar to those exerted by LPS due to common cell membrane ligands that initiate the NF-$\kappa$B cell signaling cascade and inflammatory events via the expression of intercellular adhesion molecules and interleukins that regulate leukocyte recruitment; therefore, LPS was also used in this study to induce vascular inflammation and cellular adhesion.

Results showed that LPS drastically induced NF-$\kappa$B gene expression after 3 h of stimulatio to 2.9-fold of untreated control cells. Ac and Gp at 5 mg GAE/L partially reversed this effect and decreased NF-$\kappa$B expression to 0.58- and 0.69-fold of

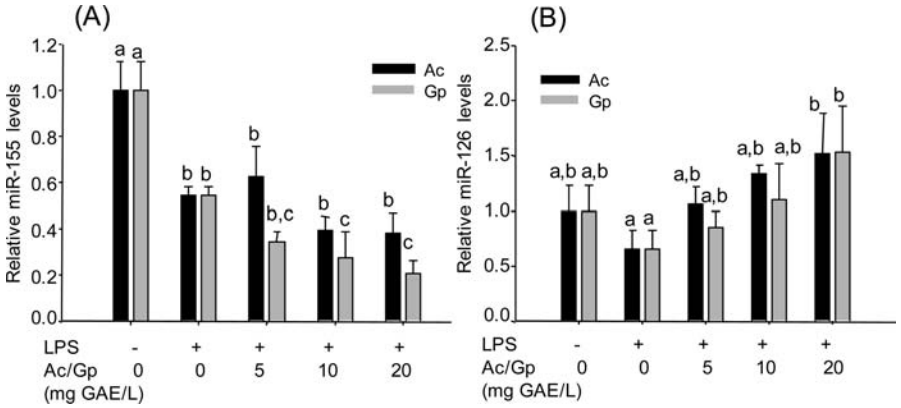


**Figure 7.** Modulation of miRs on LPS-challenged HUVEC. (A) miR-155 and (B) miR-126. Cells were treated with solvent (DMSO) or different concentrations of Ac or Gp (5−20 mg GAE/L) for 30 min before LPS challenge (1 $\mu$g/mL) for 24 h, and relative microRNA levels were determined by qRT-PCR as described under Materials and Methods. Values are the mean ± SE ($n$ = 3). Different letters within plant material indicate a significant difference ($p \leq 0.05$).

LPS-stimulated cells (Figure 6A). The AngII type 1 receptor (AGTR1) plays a key role in the development of hypertension and atherosclerosis.[33] LPS stimulation did not significantly induce mRNA expression of AGTR1; however, relative to LPS-induced cells, Ac and Gp at 20 mg GAE/L significantly down-regulated its expression to 0.77- and 0.76-fold, respectively (Figure 6B).

Cellular adhesion plays a key role in the process of leukocyte adhesion and migration and involves several adhesion molecules.[1] The mRNAs of cell adhesion molecules VCAM-1, ICAM-1, and E-selectin were up-regulated by LPS to 12.3-, 7.2-, and 45.1-fold of untreated control cells, respectively; Ac and Gp at 5−20 mg GAE/L reversed this effect and down-regulated the expression of VCAM-1 to 0.60- and 0.57-fold; of ICAM-1 to 0.83- and 0.51-fold; and of E-selectin to 0.64- and 0.62-fold, respectively, of LPS-challenged cells (Figure 6C−E). Overall, the respective effects of Ac and Gp polyphenolics on these NF-$\kappa$B target genes, VCAM-1, ICAM-1, and E-selectin were not significantly different between the selected doses of 5 and 20 mg GAE/L. At protein level, activation of NF-$\kappa$B by phosphorylation was increased by LPS, whereas Ac and Gp polyphenolics decreased this effect within 5−20 mg GAE/L (Figure 6F). However, polyphenolics from Ac at higher concentrations seemed to be more effective than those from Gp. Protein expression of constitutive NF-$\kappa$Bp65 remained constant, indicating that differences on its active phosphorylated form are due to the inhibitory effect of Ac and Gp polyphenolics. Platelet endothelial cell adhesion molecule-1 (PECAM-1) plays a critical role in the regulation of leukocyte transendothelial migration and causes inflammation.[34] Polyphenolics from Ac and Gp decreased PECAM-1 protein expression; Gp seemed to be more efficacious compared to Ac even at a low concentration of 5 mg GAE/L.

Likewise, Ac and Gp polyphenolics down-regulated protein expression of AGTR1 where the effects of polyphenolics were less evident compared to NF-$\kappa$B phosphorylation and PECAM-1. An important consequence of AGTR1 activation, particularly in the cardiovascular system, is the production and release of ROS,[33] which might be involved in insulin resistance,[14] activation of extracellular signal-regulated kinase (ERK1/2), increased contraction and hypertension,[35] and, ultimately, the accelerated progression of vascular lesions[33] due to enhanced expression of VCAM-1 via NF-$\kappa$B activation during the early stages of atherosclerosis.[36] In this study, the decrease of AGTR-1 was accompanied by a decrease of ERK1/2 phosphorylation (Figure 6G) for both extracts. Constitutive levels of ERK1 were not affected by treatments, which indicated that the effects on phospho-ERK1/2 were a response modulated by LPS and Ac and Gp polyphenolics.

Additionally, Ac and Gp polyphenolics were also effective in partially reversing LPS-induced up-regulation of the intercellular adhesion molecules ICAM-1 and VCAM-1 (Figure 6G): both of them down-regulated intracellular ICAM-1 within 5−10 mg GAE/L. The amount of ICAM-1 protein excreted to culture media after 24 h of LPS stimulation was increased to 440-fold compared to untreated control cells. Ac decreased this up-regulation to 0.34-fold of LPS-challenged cells, and Gp restored ICAM-1 basal protein levels excreted by the untreated control cells (Figure 6H).

**Effect of Ac and Gp Polyphenolics on miR-155 and miR-126.** The post-translational regulation of AGTR1 by miR-155 has been reported to be inversely correlated to blood pressure.[26] The study showed that miR-155 overexpression reduced the target AGTR1 protein expression, rather than mRNA levels.[26] Therefore, we investigated whether the down-regulation of AGTR1 protein exerted by Ac and Gp polyphenolics might be due to an induced up-regulation of miR-155. Conversely, neither Ac nor Gp polyphenolics induced expression of miR-155 (Figure 7A), implying that their role in the AGTR1 regulation is not mediated through miR-155.

miR-126 has been demonstrated to play an important role the regulation of VCAM-1 in vascular endothelial cells. The expression of miR-126 was found to be inversely correlated to leukocyte adherence, indicating its involvement in the control of vascular inflammation.[27] Results showed that LPS did not significantly decrease miR-126 expression; however, a decreasing trend was observed (Figure 7B), and this response was inversely correlated to the expression of adhesion molecules at both gene and protein expression levels (Figure 6). Ac and Gp polyphenolics (5−20 mg GAE/L) induced a dose−response increase of miR-126 expression to 2.32- and 2.33-fold of LPS-challenged cells, respectively (Figure 7B). To further confirm that Ac and Gp polyphenolics target the expression of miR-126 as one of the underlying mechanisms that protect endothelial cells from inflammation and atherogenesis, the specific miR-126 inhibitor was transfected

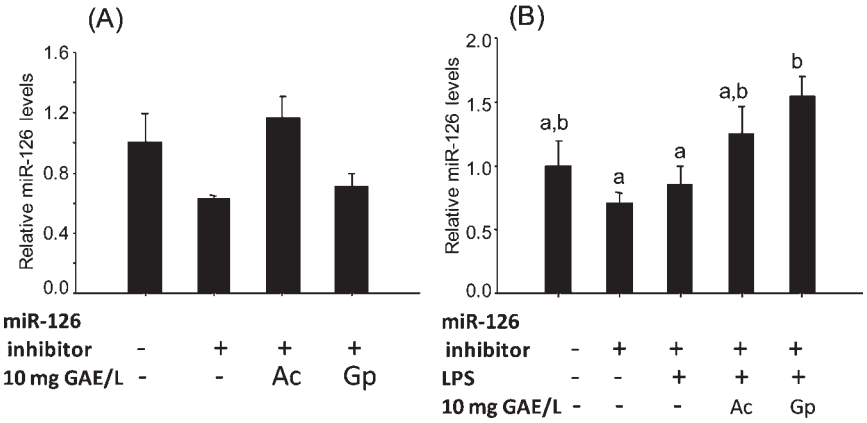


**Figure 8.** Ac and Gp polyphenolics reversed the effect of miR-126 inhibitor on transfected cells. miR-126 on (A) transfected and on (B) LPS-treated transfected cells. Transfected cells were treated with solvent (DMSO) or Ac or Gp (10 mg GAE/L) for 24 h or 30 min before LPS challenge (1 $\mu$g/mL) for 24 h. Relative miR-126 levels were determined by qRT-PCR as described under Materials and Methods. Values are the mean ± SE ($n = 3$). Different letters indicate significant difference ($p \leq 0.05$).

into HUVEC, and miR-126 gene expression was assessed. Transfection decreased miR-126 expression to 0.63-fold, and Ac but not Gp polyphenolics reversed this effect and induced its expression to 1.84-fold of transfected cells, yet this effect was not significant (Figure 8A). When transfected cells were exposed to LPS stimulation, Ac and Gp induced miR-126 expression to 1.47- and 1.81-fold of LPS-challenged cells, respectively, where the effect of Gp was statistically significant (Figure 8B).

## DISCUSSION

Plant polyphenolics have been reported to possess radical-scavenging and antioxidant properties, and it is known that a diet rich in fruits and vegetables might help to prevent cardiovascular complications related to metabolic syndrome and vascular inflammation.[15] The different mechanisms by which ROS production may induce endothelial dysfunction and inflammation are presented in Figure 9. The protective properties of grape (*V. vinifera*) extracts in endothelial cells were demonstrated in several studies;[37] however, muscadine grapes (*V. rotundifolia*) have not frequently been investigated, and at the time of submission of this study, no previous studies have reported the protection of vascular endothelial cells by polyphenolics from açaí fruit (*E. oleraceae* Mart). On the basis of the similarities of the polyphenolic profiles of red vinifera grapes with red muscadine grapes and açaí,[5,8,9] comparable results were expected in this study. Overall, Ac and Gp exerted a protective effect against glucose-induced ROS in HUVEC cells; however, a dose-dependent response was more pronounced for Ac (Figure 1B).

These findings are supported by a study using grape extracts enriched in different polyphenolic families, which were able to prevent ROS production in an in vivo model of metabolic syndrome using high-fructose-fed rats.[38] The protection of various tissues, such as the heart and aorta, against ROS was related to inhibition of the NADPH oxidase overexpression.[38] Grape seed proanthocyanidins have also been extensively investigated, and consistent data indicate their cardiovascular protection through interference with ROS generation and their anti-apoptotic effects.[39] Specifically, the antioxidant and cell protective effects of anthocyanins present in Ac and Gp have been documented. Cyanidin-3-*O*-glucoside is present in the mixture of anthocyanins on both Ac and Gp extracts and has been shown to protect against peroxynitrite-induced endothelial dysfunction and reduced mitochondrial respiration on HUVEC.[40] Likewise, cyanidin derivatives were shown to protect cells from DNA cleavage due to their free radical scavenging activity, suggesting their role in treatment of pathologies in which free radical production plays an important role.[40]

More relevant than the antioxidant properties of plant polyphenolics seems to be the regulation of mechanisms that decrease inflammation. Hyperglycemia is recognized as a risk factor for developing diabetes-associated atherosclerosis,[1] and plant polyphenolics have been shown to modify signal transduction pathways contributing to delaying or preventing inflammation.[3] The anti-inflammatory properties of açaí and muscadine grape polyphenolics have been reported for in vitro and in vivo models.[41,42] Likewise, individual phenolics present in açaí and grapes have been demonstrated to be anti-inflammatory via NF-$\kappa$B-dependent mechanisms in vitro and in vivo.[37] Significant anti-inflammatory effects of anthocyanins, flavanols, flavonols, and procyanidins (oligomeric flavonoids) have been demonstrated in mice, rats, and humans (reviewed by Xia et al.[37]). In this study, Ac and Gp polyphenolics were found to decrease NF-$\kappa$B phosphorylation as well as mRNA and mRNA and protein levels of glucose-stimulated inflammatory cytokines in HUVEC (Figure 2). Cytokines and chemokines such as IL-6 and IL-8 are involved in the initiation and amplification of inflammation.[12,13] The signaling events induced by IL-6 have been proven to contribute to atherosclerotic plaque development via a number of mechanisms including the release of other pro-inflammatory cytokines.[13] Likewise, GM-CSF secretion is induced by cytokine stimulation in endothelial cells and functions as a white blood cell growth factor. Moreover, IL-8 are chemokines mostly released by endothelial cells under oxidative stress, which attract leukocytes into the sites of inflammation and may be involved in the whole process of leukocyte transmigration into tissues characteristic of inflamed endothelium.[12] An overview of the mechanisms by which Ac and Gp decrease NF-$\kappa$B activation, and thereby the transcription of pro-inflammatory molecules, is presented in Figure 9.

This study also investigated the effects of Ac and Gp on PXR (Figure 3). Several studies have reported the role of plant phytochemicals in activating PXR, thereby regulating detoxification enzymes and transporters [23,43−45] as presented in Figure 9.

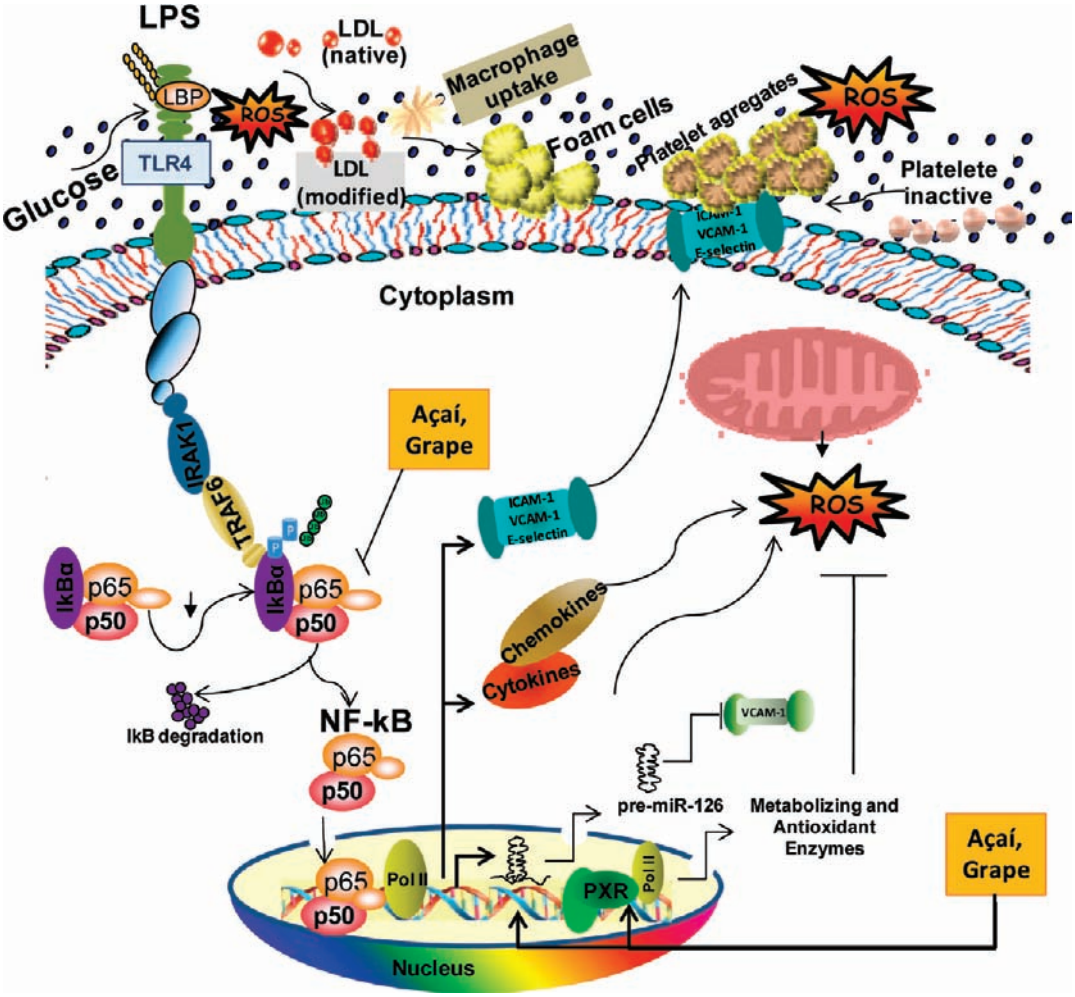


**Figure 9.** Molecular mechanisms for protection of HUVEC by Ac and Gp polyphenolics. Ac and Gp protect endothelial cells by decreasing ROS as a primary mechanism, which is directly mediated through their radical scavenging and antioxidant properties and indirectly mediated through inhibition of NF-κB activation, which result in decreased levels of cell adhesion molecules, inflammatory cytokines, and chemokines, and induction of metabolizing and antioxidant enzymes. The induction of miR-126 was a secondary mechanism by which Ac and Gp polyphenolics inhibited translation of VCAM-1 mRNA.

PXR plays a regulatory role in various physiological and pathophysiological processes such as lipid metabolism, glucose homeostasis, and inflammatory response. Moreover, PXR and NF-κB have been found to be mutually repressive, where negative regulation of NF-κB and its target genes such as intercellular adhesion molecules and several interleukins by PXR activation was reported.[24] Several flavonoids have shown their stimulatory effects in PXR activation; however, some controversy exists regarding the mechanisms of activation. Some flavonoids identified in açaí, such as luteolin and apigenin, were found to induce PXR through inactivation of cyclin-dependent kinases (Cdk5),[46] whereas PXR activation by plant extracts and individual phytochemicals through binding interactions and/or enhanced promoter activity was also reported.[46] In this study, activation of PXR as assessed by nuclear translocation and increased protein levels was induced by Gp polyphenolics (Figure 3A,B), but to a lesser extent by Ac at 5 mg GAE/L.

mRNAs of phase 1 CYP1A1, the phase 2 antioxidant catalase, and phase 3 drug efflux MDRP1 were induced by Ac and Gp polyphenolics (Figure 4). Catalase is an enzyme that scavenges superoxide and hydrogen peroxide; its depleted function is associated with an increased production of ROS. In general, the protective effects of Ac and Gp polyphenolics through increasing antioxidant and metabolizing enzymes might contribute to decreased ROS and harmful metabolites and thus delay vascular complications in diabetic patients. This might contribute to slow the development of vascular complications in diabetic patients; however, further research is needed on undesirable effects, such as pharmacokinetic interactions with coadministered drugs, which might also occur.

Induction of GST activity by Ac and Gp polyphenolics was another protective mechanism against the insult induced by high-glucose stress. This is relevant in complications produced by hyperglycemia due to the depletion of antioxidant defenses. The covalent binding of glucose to various physiological proteins alters their structure and their function, thus increasing the susceptibility of diabetic patients to vascular oxidative damage.[47] Similarly, apple extracts also rich in flavonoids and phenolic acids were shown to protect HUVEC from glycated protein toxicity through restoration of the levels of depleted antioxidant enzymes.[47]

Recent studies on regulation of metabolic disorders by miRs demonstrated their pivotal role in the onset and development of inflammatory-related diseases.[19] Research on miRs that might

play an important role in metabolic disorders and endothelial function led us to focus on the possible modulation of miR-148a, a post-transcriptional regulator of PXR,[21] and miR-146a, a negative regulator of the NF-κB inflammatory cascade.[32] The effects of Ac and Gp polyphenolics on miR-148a expression were not inversely correlated to the induction of PXR as we might have hypothesized on the basis of the study showing that a potential miR-148a recognition element was identified in the 3′-untranslated region of human PXR mRNA.[21] Our data, instead, indicate that the up-regulation of PXR and target genes is mediated by alternative mechanisms. Additionally, miR-146a, known to be induced by bacterial infection in NF-κB-dependent fashion, acts as negative regulator of pro-inflammatory cytokines/chemokines transcription and secretion by targeting the TRAF6−IRAK1 axis.[32] In concordance with the observed down-regulation of IRAK1 and TRAF6, miR-146a was expected to be up-regulated by treatment with Ac and Gp. In contrast, glucose induced the expression of miR-146a, whereas Ac and Gp decreased its expression, which indicates that the extract-induced decrease of TRAF6, IRAK1, and NF-κB does not involve miR-146a as a key regulator. In previous studies, miR-146a was involved in the down-regulation of TRAF6 and IRAK1 human lung alveolar epithelial cells.[32]

Intercellular adhesion molecules VCAM-1, ICAM-1, and E-selectin were not significantly up-regulated by high glucose (data not shown); however, the pro-inflammatory stimulus with LPS induced the expression of NF-κB transcription factor, as well as AGTR1, VCAM-1, ICAM-1, and E-selectin (Figure 6). Correspondingly, it has been reported that elevation of extracellular D-glucose levels is not sufficient to promote intercellular cell adhesion molecules or leukocyte adhesion to HUVEC in vitro, although glucose potentiated both under pro-inflammatory conditions.[1] This highlights the pro-atherosclerotic actions of hyperglycemia, especially under the inflammatory environment in diabetes.

Grape polyphenolics were previously demonstrated to decrease the expression of intercellular adhesion molecules in vitro and in vivo;[15,37,39] however, neither muscadine grape nor açaí has been extensively investigated for the potential to reduce atherosclerotic risk factors on endothelial cells. Grape polyphenolics attenuated atherosclerosis by reducing LDL oxidation; this is a key step in atherogenesis initiation. Oxidized LDL is taken up by macrophages in an unregulated manner to form foam cells and alter endothelial function, stimulating platelet activation and inflammation[15,37] (Figure 9). Grape seed proanthocyanidins have also been shown to exert an inhibitory effect on the expression of adhesion molecules of systemic sclerosis patients,[39] as well as decrease the generation of ROS and reduce DNA damage. In addition, resveratrol, a phyoalexin present in grapes, was demonstrated to reduce NF-κB activation and ICAM-1 expression, leading to suppression of tumor cell adhesion to endothelial cells.[37] Likewise, among other phenolics found in grape and açaí, catechin and (−)-epigallocatechin-3-*O*-gallate, which are also found in muscadine grape, were effective in reducing the progression of accelerated atherosclerotic plaque formation in an in vivo study using apolipoprotein E-deficient mice. One of the mechanisms targeted by epigallocatechin-3-*O*-gallate was the inhibition of the AngII-induced MAPK pathways leading to reduced adhesion molecule expression in HUVEC (reviewed by Naito and Yoshikawa[48]). A reduction of adhesion molecules was also observed in this study when LPS-challenged HUVEC were treated with Ac and Gp polyphenolics (Figure 6).

In general, Ac and Gp polyphenolics have been found to reduce gene expression of hypertensive and atherosclerotic risk factors AGTR1, VCAM-1, ICAM-1, and E-selectin to similar extents, although at protein expression level, Gp polyphenolics seemed more effective in the decrease of PECAM-1 and secretory ICAM-1 (Figure 6F,H). Overall, these results strongly suggest the down-regulatory effect of Ac and Gp polyphenolics on the AngII-ERK1/2-NF-κB cascade, which might contribute to down-regulate the expression of cell adhesion molecules and prevent the foam formation and development of atherosclerosis.[49]

Results also demonstrate that miR-155 is not likely to play a role in extract-induced down-regulation of AGTR1[26] or as part of the anti-inflammatory machinery modulated by Ac and Gp polyphenolics. miR-126, known to regulate endothelial expression of VCAM-1,[27] was induced by Ac and Gp polyphenolics (Figure 7B), and its increased expression was inversely correlated to the decreased VCAM-1 gene and protein expression (Figure 6C,E). In previous studies, it was demonstrated that miR-126 is complementary to the 3-UTR of the transcript for human VCAM-1 (between 604 and 625 nt)[27] with a proven role in decreasing cell adhesion and inflammation in endothelial cells.[19,27] Ac and Gp polyphenolics appeared to protect endothelial cells against atherosclerotic risk factors by decreasing the expression of VCAM-1 through induction of miR-126 as the underlying mechanism (Figure 9). This was further demonstrated when the effect of miR-126 inhibitor in transfected cells was reversed by these compounds (Figure 8), although this effect was significant only in cells exposed to LPS.

In conclusion, the present study has demonstrated that polyphenolics present in Ac and Gp have protective effects in vascular endothelial cells exposed to high glucose and LPS. The molecular pathways targeted by these compounds that contribute to maintaining cellular homeostasis and prevent endothelial dysfunction and cardiovascular complications include anti-inflammatory and antioxidant effects in addition to inducing PXR and PXR-dependent metabolizing enzymes. The anti-inflammatory protection and down-regulation of pro-atherogenic cell adhesion molecules induced by LPS were exerted by Ac and Gp polyphenolics to similar extents, although Gp was shown to be more effective in decreasing intracellular PECAM-1 and extracellular ICAM-1 protein levels. Finally, in the screening of potentially involved miR-126, -146a, -148a, and -155, only miR-126 was induced to similar extents by both Ac and Gp and seemed to be involved in the extract-derived reduction of VCAM-1 in LPS-stimulated HUVEC. Ac and Gp seem to be comparable in their anti-inflammatory effects (molecular pathways are summarized in Figure 9). Overall, despite the positive effects of Ac and Gp polyphenolics on endothelial function, vascular homeostasis, and reduction of atherogenesis, more in-depth mechanistic and translational studies in humans are needed to support these beneficial effects and to determine clinical relevance.

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## ■ ACKNOWLEDGMENT

We would like to thank Dr. Tian Yanan, Dept. of Veterinary Physiology & Pharmacology, Texas A&M University, College Station, TX for his technical assistance.

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