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Cyanidin-3-glucoside attenuates angiotensin II-induced oxidative stress and inflammation in vascular endothelial cells

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ABSTRACT

Angiotensin II (Ang II) causes oxidative stress and vascular inflammation, leading to vascular endothelial cell dysfunction, and is associated with the development of inflammatory cardiovascular diseases such as atherosclerosis. Therefore, interventions of oxidative stress and inflammation may contribute to the reduction of cardiovascular diseases. Cyanidin-3-glucoside (C3G) plays a role in the prevention of oxidative damage in several diseases. Here, we investigated the effect of C3G on Ang II-induced oxidative stress and vascular inflammation in human endothelial cells (EA.hy926). C3G dose-dependently suppressed the free radicals and inhibited the nuclear factor-kappa B (NF-κB) signaling pathway by protecting the degradation of inhibitor of kappa B-alpha (IκB-α), inhibiting the expression and translocation of NF-κB into the nucleus through the down-regulation of NF-κB p65 and reducing the expression of inducible nitric oxide synthase (iNOS). Pretreatment with C3G not only prohibited the NF-κB signaling pathway but also promoted the activity of the nuclear erythroid-related factor 2 (Nrf2) signaling pathway through the upregulation of endogenous antioxidant enzymes. Particularly, we observed that C3G significantly enhanced the production of superoxide dismutase (SOD) and induced the expression of heme oxygenase (HO-1). Our findings confirm that C3G can protect against vascular endothelial cell inflammation induced by AngII. C3G may represent a promising dietary supplement for the prevention of inflammation, thereby decreasing the risk for the development of atherosclerosis.

1. Introduction

Atherosclerosis, the main risk factor for cardiovascular diseases (CVDs), induces a number of deaths worldwide [1]. Endothelial cells play a key role in the vascular homeostasis, which maintains the balance of blood pressure, and these cells have been implicated in several aspects of vascular biology, including the permeability barrier function, coagulation systems, blood clotting control, inflammation and angiogenesis [2]. Endothelial dysfunction is presented in the initial stage of atherosclerosis associated with inflammation and oxidative stress and the subsequent progression into vascular smooth muscle cells [1,3–8]. The inflammation of endothelial cells activates the nuclear factor-kappa B (NF-κB) signaling pathway, which induces the expression of adhesion molecules, cytokines and chemokines responsible for the recruitment of monocytes to the inflamed area [9,10]. Previous studies have reported that several extracellular stimulators such as lipopolysaccharide (LPS) [11], tumor necrosis factor-alpha (TNF-α) [12] and angiotensin II (Ang II) [13] induce inflammatory stimulation in endothelial cells. Among these factors, Ang II is a major mediator of the renin-angiotensin system (RAS), associated with cardiovascular diseases [14]. The pro-atherosclerotic actions of Ang II include binding to the angiotensin type 1-receptor (AT1R), thereby activating membrane-bound NAD(P)H oxidase for the formation of reactive oxygen species (ROS), particularly superoxide anion [15–18]. The ROS production, induced through Ang II, activates the NF-κB signaling pathway, which induces the expression of pro-inflammatory mediators such as inducible nitric oxide synthase (iNOS), intercellular adhesion molecule (ICAM), tumor necrosis factor-alpha (TNF-α) and other chemokines that affect many cells in the vessel wall [13,19,20]. Thus, ROS is an important cellular

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signaling mechanism that plays a major role in the induction of vascular wall injury [21], leading to structural changes in the vessel wall that facilitate the abnormal vascular growth observed in atherosclerosis leading to eventual organ damage. Thus, the inhibition of Ang II-induced oxidative damage or inflammation may provide benefits to reduce atherosclerosis.

Cyanidin-3-glucoside (C3G) is a major component of anthocyanins in the flavonoid family, which is typically present in dark-colored (violet, blue and red) vegetables and fruits such as black currant, red cabbages, red raspberry, blueberry, blackberry and purple rice bran [22–24]. C3G is primarily present in the human diet and shows potential beneficial effects in different human pathologies [23–26]. Several studies have reported the beneficial effects of C3G, such as activity against hypoxic conditions in endothelial cells [27], anti-tumor effects [28], obesity suppression [29] and activity against endothelial dysfunction and vascular failure [30]. Several studies have demonstrated that C3G induces the nuclear erythroid-related factor 2 (Nrf2) pathway and up-regulates heme oxygenase 1 (HO-1) expression, which inhibits ROS production, contributing to the prevention of endothelial injury [26]. Thus, C3G exerts advantageous effects through anti-oxidation and anti-inflammation for atherosclerosis prevention. Therefore, the aim of the present study was to investigate the protective effect and molecular mechanism of C3G against Ang II-induced inflammation in human EA.hy926 endothelial cells. Specifically, we focused on the effects of C3G on the production of ROS and nitric oxide (NO), as oxidative stress markers, and inflammatory cytokines through the NF-κB pathway and characterized the importance of Nrf2 pathway activation in protecting cells from Ang II-induced inflammation.

2. Materials and methods

2.1. Chemicals and materials

Cyanidin-3-glucoside (C3G), angiotensin II (Ang II), JSH-23, and apocynin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against IκB, nuclear factor κB p65 (NF-κB p65) and iNOS were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against Nrf2, HO1, lamin B1 and actin were purchased from Abcam (Cambridge, UK). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, and trypsin were purchased from Gibco-BRL (Gaithersburg, MD).

2.2. Cell culture

The human umbilical vein cell line (EA.hy926) was purchased from American Type Culture Collection (ATCC®, USA) and maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37 °C, 5% CO_2 and 95% humidity. These cells were used between passages 4 and 8 for all experiments.

2.3. Cell viability assay

EA.hy926 cells at a density of 5×10^4 cells/well in 96-well plates were allowed to adhere overnight. Subsequently, the cells were incubated with C3G (5 μM, 10 μM, or 20 μM) for 2 h, followed by the addition of 10^{-6} M Ang II for 24 h at 37 °C, 5% CO_2 and 95% humidity. The cell viability was assessed using an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay to measure mitochondrial function. Yellow tetrazolium salt, which is reduced to purple formazan in living cells, was solubilized using dimethyl sulfoxide (DMSO). Subsequently, the absorbance was measured at a wavelength of 540 nm using a microplate reader (Anthos, Italy).

2.4. Determination of intracellular ROS

To evaluate the production of intracellular ROS, EA.hy926 cells were seeded onto 96-well plates at a density of 5×10^4 cells/well. After 24 h, the cells were pretreated with C3G (5 μM, 10 μM or 20 μM) for 2 h, followed by the addition of 10^{-6} M Ang II for 24 h. In the NOX1 inhibition group, apocynin (100 μM) was added at 1 h prior to the addition of C3G. Subsequently, the cells were washed with PBS and incubated with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (20 μM) in fresh medium at 37 °C for 30 min. The fluorescence was measured at an excitation of 485 nm and an emission of 535 nm using a fluorescence microplate reader (DTX800, Beckman Coulter, Austria).

2.5. Determination of nitric oxide (NO)

The EA.hy926 cells were seeded onto 96-well plates at a density of 5×10^4 cells/well and pretreated with C3G (5 μM, 10 μM or 20 μM) for 2 h, followed by the addition of 10^{-6} M Ang II for 24 h. In the NF-κB inhibition group, JSH-23 (20 μM) was added at 1 h prior to the addition of C3G. After treatment, 100 μl of the culture media from cells was collected and transferred to a new 96-well plate. Subsequently, the culture media was incubated with 50 μl of 1% sulfanilamide in 5% phosphoric acid for 5 min, followed by the addition of 50 μl of 0.1% *N*-(1-naphthyl) ethylenediamine hydrochloride for 5 min. The absorbance was measured at 540 nm using a microplate reader. The NO_2^- concentrations from each sample were determined using sodium nitrite ($NaNO_2$) as a standard.

2.6. Determination of superoxide dismutase (SOD) activity

EA.hy926 cells were seeded at a density of 5×10^6 cells/plate. After treatment, the cells were washed with ice-cold PBS and lysed in lysis buffer. The cell lysate was collected and centrifuged at 13,000 × g for 5 min, and the supernatant was collected to examine the total SOD activity using a superoxide dismutase assay kit (Cayman Chemical, Ann Arbor, MI, USA). A volume of 10 μl of the supernatant was mixed with 200 μl of radical detector solution and xanthine oxidase enzyme. The absorbance was immediately measured at a wave length of 450 nm using a microplate reader after the incubating for 30 min at room temperature. The SOD activity in each sample was calculated by comparing its optical density with the known activity of the SOD enzyme standard.

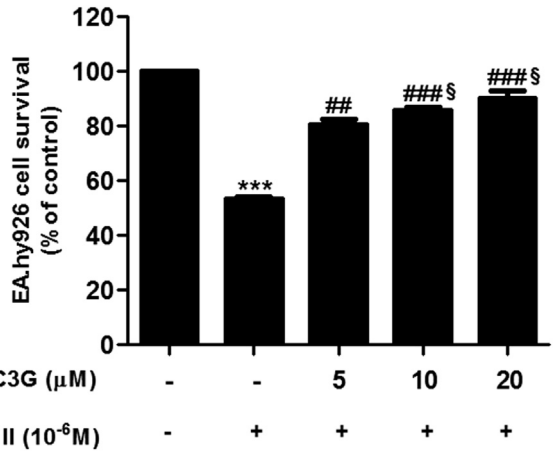

Fig. 1. Effect of C3G against Ang II-induced EA.hy926 cell death. Cells were pretreated with C3G for 2 h, followed by Ang II treatment. The cell viability was measured using the MTT assay. Values represent the means ± S.E.M. (n = 3 in each group). $^{***}p < 0.001$ vs. control group; $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. group treated with Ang II alone. $^{\S}p < 0.05$ vs. group treated with 5 μMC3G.