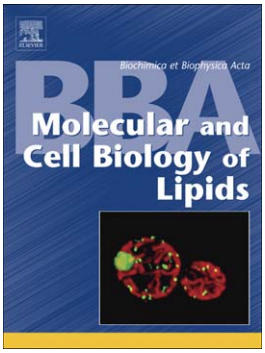

Cyanidin-3-O-glucoside ameliorates palmitate-induced insulin resistance by modulating IRS-1 phosphorylation and release of endothelial derived vasoactive factors

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Cyanidin-3-O-glucoside ameliorates palmitate-induced insulin resistance by modulating IRS-1 phosphorylation and release of endothelial derived vasoactive factors

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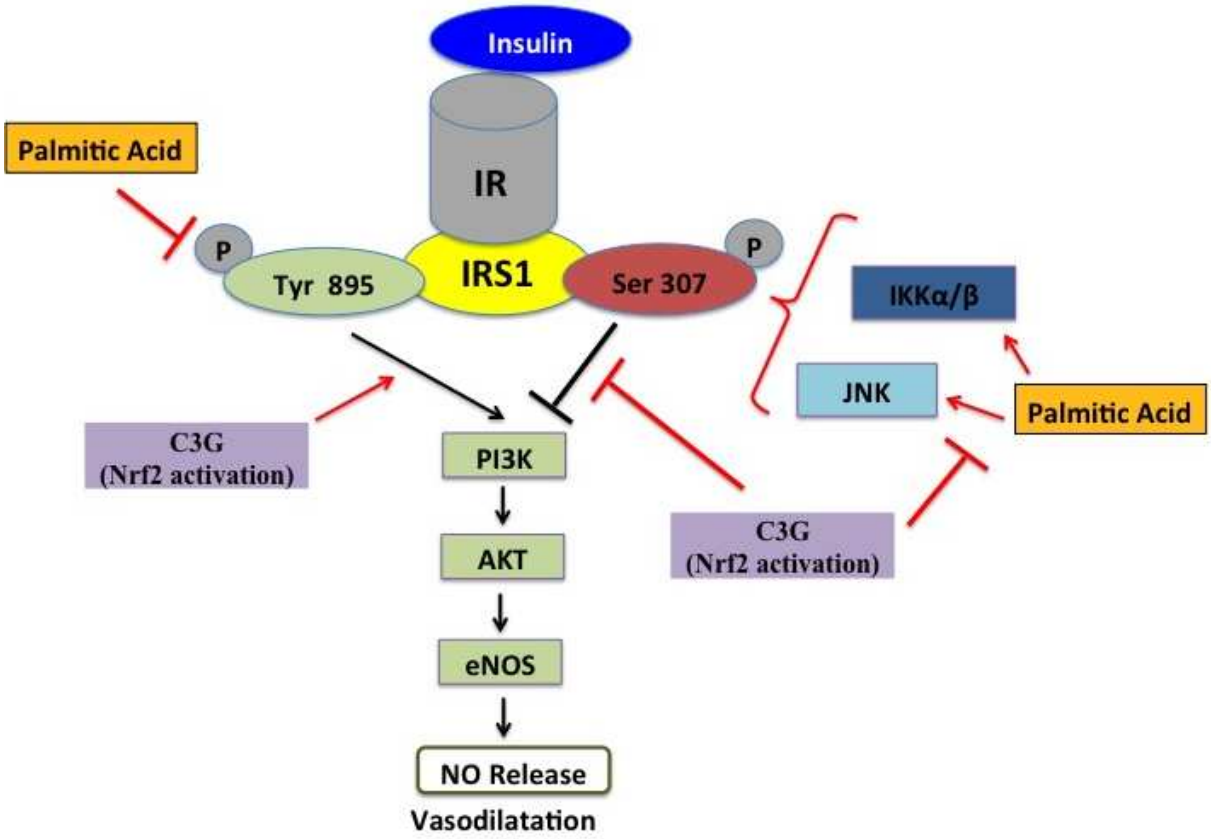
Graphical Abstract



Abstract

Increased plasma levels of free fatty acids, including palmitic acid (PA), cause insulin resistance in endothelium characterized by a decreased synthesis of insulin-mediated vasodilator nitric oxide (NO), and by an increased production of the vasoconstrictor protein, endothelin-1. Several in vitro and in vivo studies suggest that anthocyanins, natural phenols commonly present in food and vegetables from Mediterranean Diet, exert significant cardiovascular health-promoting activities. These effects are possibly mediated by a positive regulation of the transcription factor Nrf2 and activation of cellular antioxidant and cytoprotective genes.

The present study examined, at a molecular level, the effects of cyanidin-3-O-glucoside (C3G), a widely distributed anthocyanin, on PA-induced endothelial dysfunction and insulin resistance in human umbilical vein endothelial cells (HUVECs). Our results indicate that C3G pretreatment effectively reverses the effects of PA on PI3K/Akt axis, and restores eNOS expression and NO release~~,~~ altered by PA. We observed that these effects were exerted by changes on the phosphorylation of IRS-1 on specific serine and tyrosine residues modulated by PA through the modulation of JNK and IKK activity. Furthermore, silencing Nrf2 transcripts demonstrated that the protective effects of C3G are directly related to the activation of Nrf2.

Keywords

Cyanidin-3-*O*-glucoside; Insulin resistance; endothelial dysfunction; nitric oxide; palmitic acid.

List of abbreviations

C3G, cyanidin-3-O-glucoside; HUVECs, human umbilical vein endothelial cells; PA, palmitic acid; TNF-α, Tumor necrosis factor-alpha; SRB, Sulforhodamine B; ET-1, endothelin-1; PAI-1, plasminogen activator inhibitor-1; NO, nitric oxide.

1. Introduction

Elevated plasma levels of free fatty acids (FFAs) are frequently associated as a causative factor of endothelial insulin resistance in obese patients [1]. According to this mechanism, FFAs impair insulin pathway with a subsequent decrease in NO production, eventually leading to an overt endothelial dysfunction.

Consumption of fruits and vegetables has been frequently found to be inversely associated with risk of CVD most likely due to the abundance and variety of bioactive compounds present. Among these, anthocyanins are natural pigments providing the blue to red colors of many fruits and vegetables. The mean dietary AC intake has been estimated to be between 3 and 215 mg/day, depending on the specific dietary profile characteristic of the population under study. These levels are significantly higher than those reported for other dietary flavonoids such as genistein and quercetin [2, 3]. An inverse relationship between anthocyanins and anthocyanin-rich foods and CVD outcomes (e.g., mortality) and metabolic disorders has been observed in several epidemiological studies. Jennings and coll. [4, 5], in a cross-sectional study on 1,997 women, observed that higher anthocyanin and flavone intake was associated with significantly lower peripheral insulin resistance resulting from decreased insulin blood levels. The efficacy of anthocyanins in preventing insulin resistance has been yet demonstrated in different animal models. Dietary C3G supplementation improved hyperglycemia and insulin resistance in high fat diet fed mice and in genetically diabetic mice [6, 7]. Furthermore, in an in vitro model of HepG2 cells with high glucose/PA-induced insulin resistance, Yan and coll. [8] observed an evident increase on glucose consumption, glucose uptake, and glycogen content, following treatment with a mulberry anthocyanin extract. Zhao and coll. [9] used an in vitro model of HUVECs with endoplasmic reticulum (ER) stress, playing important roles in the development of obesity-induced insulin resistance, diabetes [10], and atherosclerosis [11]. Many reports suggest that C3G enhances glucose uptake through up-regulation of GLUT4, PPARγ activation, and activation of AMP-activated protein kinase (AMPK) [12-15]. However, the most promising targets have not been yet identified.

Insulin resistance is a major hallmark of several metabolic disorders that include glucotoxicity, lipotoxicity, and inflammation, all conditions leading also to endothelial dysfunction [16, 17]. Several studies suggest an association between the suppression of proinflammatory chemokines, growth factors, and adhesion molecules and the inhibition of NF-κB activation. Interestingly, this

effect seems to be modulated by a positive regulation of the transcription factor Nrf2 and the consequent activation of cellular antioxidant and cytoprotective genes [18].

On the basis of this background, we conducted a study aimed to explore, describe, and characterize the protective effects of cyanidin-3-O-glucoside (C3G), one of the most frequently consumed anthocyanins, on endothelial dysfunction induced by high FFAs on cultured primary Human Umbilical Vein Endothelial cells, focusing on the activation of Nrf2 as indirect mechanism involved in the effects observed.

2. Materials and Methods

2.1 Reagents

Cyanidin-3-*O*-glucoside (C3G) was supplied from Polyphenols AS, Sandnes, Norway, and was of HPLC grade (>97% purity). Methanol and ethanol were obtained from Carlo Erba Reagent (Milan, Italy) in their highest commercially available purity grade.

Palmitic acid (PA), Bovine serum albumin fatty acid free (FFA-free BSA), Insulin and all other reagents, if not specified, were purchased from Sigma Aldrich (Milan, Italy).

2.2 Cell culture and treatment

Human umbilical vein endothelial cells (HUVECs) were isolated from freshly obtained human umbilical cords by collagenase digestion of the interior of the umbilical vein as described elsewhere [19], and were cultured in medium 199, supplemented with 20% fetal bovine serum (FBS), 1% L-glutamine, 20 mM HEPES buffer, 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 an incubator with humidified atmosphere containing 5% CO_2 at 37°C. Cells used in this study were from the second to fourth passage.

For all the experiments, C3G was always freshly dissolved in DMSO and immediately used. The final concentration of DMSO in the culture medium during the different treatments was <0.1% v/v. C3G concentration was chosen on the basis of previous experiments demonstrating that 20 µM (out of two different concentrations, namely 20 and 40 µM) was the lowest one associated to cell protection against PA-induced cell death [20]. Furthermore, this concentration is within a range physiologically reachable with a dietary supplementation [2, 21]. The subconfluent cells were treated for 24 h with C3G 20µM, whereas control cells were treated with 0.1% DMSO only. After this incubation time, cells were washed with Dulbecco's phosphate buffered solution (DPBS) and then exposed for 3 h with 10 % FFA-free BSA medium containing PA 100 µM prepared as previously described [20]. After this exposure time, we incubated HUVECs with insulin 100nM or not for 15 min. Cells treated with C3G vehicle (DMSO 0.1%) and then exposed only to 10 % FFA-free BSA medium (containing ethanol 0.05% v/v) and insulin vehicle (water sterile-filtered) were used as control.

2.3 Preparation of Palmitic acid–albumin complexes

Lipid-containing media was prepared by conjugation of PA to BSA using a method described

elsewhere [20]. After dissolution pH was adjusted to 7.4 with 1N NaOH, and finally the solution was filter-sterilized. Final ethanol concentration was 0.05% w/w.

2.4 Nitric oxide measurement (Griess reaction assay)

NO concentration into cell medium was measured by the Griess reaction assay [22].

Concentration of nitrite in the sample was calculated using a standard curve and expressed in µM.

2.5 Immunoblotting

For immunoblot analyses, 40 µg of protein lysates per sample were denatured in 4× SDS-PAGE reducing sample buffer and subjected to SDS-PAGE on 10% acrylamide/bis-acrylamide gels. Separated proteins were transferred to nitrocellulose membrane (Hybond-P PVDF, Amersham Bioscience – Milan, Italy). Residual binding sites on the membrane were blocked with 5% (w/v in TBST) nonfat milk overnight at 4°C. Membranes were then probed with specific primary antibodies: mouse anti-cytoskeletal actin (Santa Cruz Biotechnology – Milan, Italy) (1:600), p-IRS-1 (Ser 307) polyclonal antibody (1:200) (Santa Cruz Biotechnology – Milan, Italy), p-eNOS polyclonal antibody, p-AkT monoclonal antibody, p-SAPK/JNK monoclonal antibody, p-IRS-1 (Tyr 985), all purchased from Cell Signaling® (Leiden -The Netherlands); followed by peroxidase-conjugated secondary antibody HRP labeled goat anti-rabbit Ig (BD Pharmigen – Milan, Italy) (1:5000), Goat anti-Mouse IgM Secondary Antibody, HRP conjugate (Thermo scientific – Milan, Italy) (1:10000); and visualized with an ECL plus detection system (Amersham Biosciences – Milan, Italy). The equivalent loading of proteins in each well was confirmed by Ponceau staining and actin control.

2.6 Quantitative RT-PCR

Total cellular RNA was isolated with E.Z.N.A ®Total RNA kit according to manufacturer's instruction (OMEGA bio-tek - Norcross, Georgia, USA). After reverse transcription (RT) with oligo $(dT)_{15}$ primers, PCR was performed for identification of Plasminogen activator inhibitor-1 (PAI-1) (FW 5'-ACCGCAACGTGGTTTTCTCA-3'), (RV 5'- TTGAATCCCATAGCTGCTTGAAT-3') (Primers Bank ID 383286746c1) and Endothelin-1 (ET-1) (FW 5'-AGAGTGTCTACTTCTGCCA-3'), (RV 5'-CTTCCAAGTCCATACGGAACAA-3') (Wang and Seed, 2003) mRNA levels. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as housekeeping gene since it showed low variability in expression levels between the different treatments. Gene expression was assessed by real-time PCR (Applied Biosystem 7300 Real-Time PCR System, Monza, Italy) coupled with the Sybr

green JumpStart™ Taq ReadyMix kit (see supporting information for more details about primer pairs and cycling conditions). Data were collected and processed with SDS 1.3.1 software (Applied Biosystems, Monza, Italy) and given as threshold cycle (C_t). The fold increase in comparison with the control cells not treated and not exposed to PA mRNA expression was determined using the $2^{-\Delta\Delta Ct}$ method [23].

2.7 Nrf2 gene silencing

HUVECs were plated in gelatin pretreated flasks and, after culturing overnight until reaching 30–50% confluency, medium was replaced with OPTI-MEM (Gibco – Milan, Italy).

HUVECs were transfected with 10 nM siRNA against Nrf2 (Silencer select pre-designed siRNA; Ambion® – Milan, Italy) or with non-targeting negative control siRNA (Silencer select Negative control #1 siRNA; Ambion® – Milan, Italy), in OPTI-MEM containing 10% FBS without antibiotics according to the manufacturer's instructions using lipofectamine RNAiMAX Transfection Reagent (Invitrogen – Milan, Italy), and incubated for 24 h. At the end of the incubation time, cells were pretreated for 24h with C3G (20μM), whereas control cells were treated with 0.1% DMSO only. Then medium was replaced with 10% FFA-free BSA medium containing PA (100 μM), prepared as described above, for 3 h, followed by a 15 min exposure to insulin (100 nM) or not. Nrf2 silencing reduced Nrf2 protein expression to the levels of the negative controls (data not shown). Consequently, the expression of NAD(P)H: quinine oxidoreductase 1 and heme oxygenase-1, genes regulated by Nrf2, were also maintained at the level of negative controls (data not shown).

2.8 Statistical analysis

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

3. Results

3.1 C3G restores insulin-mediated NO production and inhibits ET-1 and PAI-1 production altered by PA

Endothelial cells play a critical role in vascular function regulation by producing and secreting

important relaxing factors such as NO and prostacyclin (PGI2), and contracting factors such as ET-1, PAI-1, prostaglandins, and angiotensin II.

In our experimental conditions, NO production in response to insulin was reduced by PA exposure (Fig. 1A), indicating an impairment of endothelium-dependent vasodilation. C3G pretreatment effectively countered PA effects and restored insulin-mediated NO production to control level. Furthermore, according to Wang and coll. [24], when endothelial cells were exposed to insulin we observed slightly, but significantly, increased mRNA levels of ET-1 and PAI-1. When endothelial cells were exposed to PA, ET-1 and PAI-1 genes expression strongly increased (Fig. 1B-C) indicating an endothelial dysfunction in vascular tone control. As shown in Fig. 1 B-C, C3G pretreatment effectively countered ET-1 and PAI-1 overexpression induced by PA.

3.2 C3G modulates insulin receptor substrate-1 (IRS-1) serine/tyrosine phosphorylation in presence of PA

One of the most evident effects of insulin resistance is the impairment of the activation of PI-3K/Akt/eNOS signaling pathways. This impairment leads to a decreased NO production and increased ET-1 secretion. In the current study, we investigated the effects of PA-induced insulin resistance by focusing on the Ser to Tyr phosphorylation ratio in IRS-1 protein as a key regulator step in insulin signal transduction.

Figure 2B shows that the exposure of endothelial cells to PA is associated to an increased serine (S307) phosphorylation of IRS-1. This observation indicates the presence of a negative feedback control of insulin signaling, providing a potential mechanism for insulin resistance. Furthermore, insulin-induced phosphorylation of IRS-1 tyrosine residue was negatively affected by the presence of PA (Fig. 2A). The regulation of serine/threonine versus tyrosine phosphorylation is likely to be a pivotal mechanism of IRS-1 action, affecting insulin sensitivity.

C3G pretreatment was associated to a reduced PA-induced serine (S307) phosphorylation of IRS-1, and to an increased tyrosine phosphorylation. Interestingly, both the effect of PA on serine (S307) phosphorylation, and C3G inhibitory effect were present also in absence of insulin (Fig 2C).

3.3 C3G restores insulin-mediated Akt/eNOS axis altered by PA via Nrf2

Beside to its crucial metabolic role, insulin exerts effects on the maintenance of physiological endothelial function thanks to its ability to stimulate NO release [25]. This vaso-dynamic activity is

mainly exerted through IRS1/PI3K/Akt axis that controls downstream eNOS activation [25].

In our experimental conditions, PA reduced insulin-mediated Akt and eNOS phosphorylation (Fig. 3A,B), indicating the presence of an impaired PI3K/Akt signaling following insulin stimulation. This observation is congruent with IRS1 serine phosphorylation and lower insulin-induced NO levels in HUVECs exposed to PA reported above in this paper (Fig. 1A). Interestingly, C3G pretreatment effectively counters the inhibitory effect of PA on insulin-mediated Akt and eNOS activation (Fig. 3A,B) respectively. This mitigating effect results in the maintenance of baseline NO levels (Fig 1A). Furthermore, the presence of C3G alone was associated to an increase of Akt and eNOS phosphorylation.

We have previously reported that the protective effect of C3G on PA-induced endothelial dysfunction was due to its ability to activate the Nrf2/EpRE pathway triggering the expression of a spectrum of detoxifying and antioxidant enzymes [20]. In fact, in that study we demonstrated that C3G significantly inhibited the activation of NF-κB proinflammatory pathway and gene expression of adhesion molecules induced by PA in HUVECs. However, a direct demonstration of the involvement of the Nrf2 system in the protective effects of C3G in PA-induced endothelial insulin resistance has not been provided. At this aim, in the present study, we pointed to confirm the role of Nrf2 by transient silencing HUVECs with Nrf2 siRNA. Our data indicate the existence of a relationship between impaired insulin signaling by PA and Nrf2 activation. In fact, C3G-induced Akt and eNOS phosphorylation was quenched by the transfection with Nrf2 siRNA (Fig. 3C), suggesting that C3G protective effect on insulin pathway requires the activation of Nrf2.

3.4 C3G inhibits PA-induced IKK and JNK activation through Nrf2 modulation

IRS-1 activity on PI3-kinase activation and NO release is known to be modulated by the phosphorylating activity of specific ser/thr kinases JNK and IKK-β [26].

Accordingly, JNK and IKK-β activity have been found abnormally elevated in obesity and JNK deletion is associated to decreased adiposity, improved insulin sensitivity, and enhanced insulin receptor signaling capacity in animal models of obesity [27, 28]. Fig. 4A and 4B show that PA induces IKK-β and JNK phosphorylation, while C3G pretreatment attenuates this effect.

In addition, we aimed to correlate the role of Nrf2 in C3G protective effects. Interestingly, when the cells were transfected with Nrf2 siRNA, the reduction of JNK and IKK-β kinases exerted by C3G (Fig. 4A,B) was inhibited and an increase of phosphorylated kinases was observed (Fig. 4C).

4. Discussion

Insulin resistance is a major hallmark of several metabolic disorders including glucotoxicity, lipotoxicity, and inflammation, all conditions leading also to endothelial dysfunction [16]. Endothelial insulin resistance is typically accompanied by the loss of insulin-induced NO production and altered hemodynamic control [25]. Pathway-specific impairment in PI3K-dependent insulin signaling contributes to the relationships between insulin resistance and endothelial dysfunction that in turn promote the clustering of metabolic and cardiovascular diseases in insulin-resistant state.

It is well known that epidemiologic observations suggest that higher polyphenol intake from fruits and vegetables is associated with decreased risk for cardiovascular disease. Many studies have indicated that anthocyanins enhance the production of vasodilating factors such as NO and contribute to the endothelial-dependent vasodilation [2] although it is not clear the mechanism by which anthocyanins exert this effect. In fact, in a previous paper we had shown that C3G is able to improve the redox status altered by PA reducing, but not restoring, the levels of ROS [20], so these results were unable to support a ROS involvement in C3G protective effects. In addition, we demonstrated that C3G was able to elicit cell adaptive responses modulating the transcription factor Nrf2.

In the present study, we evaluated the effects of C3G on NO production and insulin PI3K signaling in PA-induced insulin resistance in HUVECs. Furthermore, we studied the molecular mechanisms involved in endothelial protection by C3G focusing on the activation of Nrf2 as indirect mechanism involved in the effects observed.

Our data indicate that cells exposed to high PA and stimulated with insulin display a reduced NO release and an increased mRNA levels of ET-1 and PAI-1 (Fig. 1), whose protein expression is known to be mainly modulated at transcriptional level in HUVECs [29-33]. These data suggest that PA generates an insulin resistance condition possibly contributing to a significant shift toward the development of prothrombotic state, predisposing the onset and progression of atherosclerosis [34]. C3G pretreatment countered the decrease of NO levels and restored a control of the expression of the atherothrombotic factors ET-1 and PAI-1.

The binding of insulin to its receptor results in phosphorylation of a specific tyrosine site of IRS-1 which then binds and activates PI3K signaling. PI3K stimulates phosphorylation and activation of Akt that in turn directly phosphorylates eNOS at Ser1177, resulting in increased eNOS activity and subsequent NO synthesis [35]. Emerging evidences indicate that specific serine or tyrosine

residues are key site of IRS-1 determining either negative or positive effects on insulin signaling in endothelial cells, upon various stimulations such as FFA, TNF-α, angiotensin II, and insulin [36]. In particular, serine phosphorylation induces IRS-1 conformational change that, in turn, renders it a poor substrate for insulin receptor [37]. On the contrary, tyrosine phosphorylation leads to activation of multiple signaling pathways required for insulin pleiotropic action. Given this background, we investigated the link between insulin resistance and C3G protective effects by focusing on the phosphorylation of Ser307/Tyr895 of IRS-1 and the downstream signaling. We observed that PA significantly induces IRS-1 serine phosphorylation, and attenuates tyrosine phosphorylation in response to insulin. The pretreatment with C3G effectively counters these alterations (Fig. 2).

It is known that insulin increases endothelial NO bioavailability via PI3K/Akt/eNOS axis through IRS-1 modulation [37]. In our study, high PA reduced insulin-induced Akt and eNOS phosphorylation (Fig. 3). Interestingly, C3G not only restored PI3K/Akt axis signaling altered by PA, but also increased Akt and eNOS phosphorylation upon insulin stimulation. These data provide a molecular support to the hypothesis of an endothelium-dependent vasorelaxation activity of anthocyanins. In fact, it has been reported that many plant polyphenols have a vasodilating effects, both in vivo and in vitro [38, 39].

Several data support the hypothesis that adaptive and pharmacologically induced expression of Nrf2/EpRE-regulated cytoprotective proteins contributes to the atheroprotective and anti-inflammatory phenotype in endothelial cells [40]. We have previously demonstrated that C3G significantly inhibits endothelial NF-κB proinflammatory pathway induced by PA in HUVECs, and these effects have been attributed to the activation of Nrf2/EpRE pathway [20]. Our previous study has in fact demonstrated that C3G induces Nrf2 nuclear localization and transcription of antioxidant and cytoprotective proteins both in the absence of a specific stimulus and after high PA exposure in endothelial cells [20]. Although the exact mechanisms are not fully understood, Nrf2 is associated with insulin signaling pathway and insulin resistance [41, 42]. In this study we tried to dissect the role of Nrf2 in the protective effects of C3G against insulin resistance induced by high PA. We observed that the activation of Akt and eNOS induced by C3G was completely quenched by a transient silencing of Nrf2 (Fig 5). Although the beneficial properties of anthocyanins have always been attributed to an unspecific antioxidant activity, these data for the first time demonstrate that C3G exerts protective effects through an indirect mechanism involving Nrf2.

It has been demonstrated that IKKβ and JNK, two serine/threonine kinases, can directly reduce insulin signal transduction by directly phosphorylating serine (S307) of IRS-1 [43]. In our experimental conditions, IKKβ and JNK (Fig. 3) were activated by PA in endothelial cells and this effect should be responsible for the negative regulation of IRS-1 in response to insulin. Interestingly, C3G pretreatment restored IKKβ and JNK activation to the levels observed in absence of high PA. These results suggest that C3G inhibition of PA-induced activation of serine/threonine kinases JNK and IKKβ is one important mechanism responsible for the modulation of serine specific IRS-1 phosphorylation (Fig.2) and reduced insulin resistance. Also in this case, these effects were linked mostly to the activation of Nrf2 pathway by C3G. In fact, knockdown of the endogenous Nrf2 by siRNA, inhibited C3G effects and enhanced the PA-mediated activation of IKKβ and JNK (Fig. 4).

Although this mechanism has been previously proposed in other endothelial experimental models [18, 44], data obtained by Nrf2 silencing originally demonstrate that, similarly to other molecules belonging to the "broad" class of polyphenols [45], C3G protective effects on high PA-induced endothelial dysfunction are directly related to the "hormetic" activation of Nrf2. In view of the data reported in this work, we can now assume an indirect mechanism involving the Nrf2 pathway activity and not mitigating ROS levels.

4.1 Conclusions

In conclusion, the presence of C3G counters the dysregulation of insulin-modulated Akt/eNOS axis and insulin-increased NO availability in endothelial cells induced by high FFAs. This effect is associated to the inhibition of IRS-1 serine phosphorylation exerted by IKKβ and JNK after PA exposure. In the present study, we firstly demonstrated that this modulation is directly related to the hormetic activation of Nrf2/EpRE pathway since Nrf2 siRNA transfection totally abolished C3G protective effects.

Finally, our data suggest that C3G is potentially able to ameliorate insulin resistance-related endothelial dysfunction caused by lipotoxicity, leading to a "rebalancing" of a proper vasocontriction and vasodilator actions of insulin. These observations contribute to provide a molecular background to the beneficial effects of molecules of nutritional interest in the prevention and treatment of cardiovascular disease related to insulin resistance, diabetes, and metabolic diseases.

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Figure legends

Figure 1. Changes in NO release (A), ET-1 (B) and PAI-1 (C) mRNA expression in HUVECs pretreated with C3G for 24 h and then incubated with 100 μM PA for 3 h, followed by addition of 100 nM insulin for 15 min. Cells treated with vehicles were used as control. NO levels were expressed as concentration (μM) into cell medium; ET-1 and PAI-1 gene expression were expressed as $2^{-\Delta\Delta Ct}$ normalized to control. Data are reported as mean ± SD of at least three separate experiments. *P < 0.05 vs control; °P < 0.05 vs PA + Insulin; $^{\#}$P< 0.05 vs Insulin 100 nM.

Figure 2. Tyrosine (Tyr895) (A) and serine (Ser307) (B) phosphorylation of IRS-1 in HUVECs pretreated with 20 μM C3G for 24h, and then incubated with 100 μM PA for 3h followed by addition of 100 nM insulin for 15 min. Results by densitometry are reported as fold of change against control and expressed as mean ± SD of at least three separate experiments. Intensity values were normalized to the corresponding β-actin value. (C) Serine (Ser307) phosphorylation of IRS-1 in HUVECs pretreated with 20 μM C3G for 24h, and then incubated with 100 μM PA for 3h, but not exposed to insulin. In all experiments cells treated with vehicles were used as control. *P< 0.05 vs control; $^{\#}$P< 0.05 vs insulin; °P< 0.05 vs PA.

Figure 3. Phosphorylation Akt (A, B) and eNOS (A, C) in HUVECs pretreated with 20 μM C3G for 24 h and then incubated with 100 μM PA for 3 h, followed by addition of 100 nM insulin for 15 min. Cells treated with vehicles were used as control. Results by densitometry are reported as fold change against control and expressed as mean ± SD of three experiments. Intensity values were normalized to the corresponding β-actin value. (D) Phosphorylation of Akt and eNOS in HUVECs transfected with 10 nM negative control siRNA or Nfr2 siRNA for 24 h. After transfection, cells were treated as above described. Negative control siRNA transfected cells treated with vehicles followed by addition of insulin (100 nM) for 3h were used as control. *P< 0.05 versus control; $^{\#}$P< 0.05 versus insulin; °P< 0.05 versus PA + insulin.

Figure 4. Phosphorylation of IKK (A) and JNK (B) in HUVECs pretreated with 20 μM C3G for 24h and then incubated with 100 μM PA for 3h. Cells treated with vehicles were used as control. Results by densitometry are expressed as fold change against control and expressed as mean ± SD of at least three separate experiments. Intensity values were normalized to the corresponding β-actin value.

(C) Phosphorylation of IKK and JNK in HUVECs transfected with 10 nM negative control siRNA or Nfr2 siRNA for 24 h. After transfection, cells were treated as above. Negative control siRNA transfected cells treated with vehicles alone were used as control. *P< 0.05 versus control; °P< 0.05 versus PA.

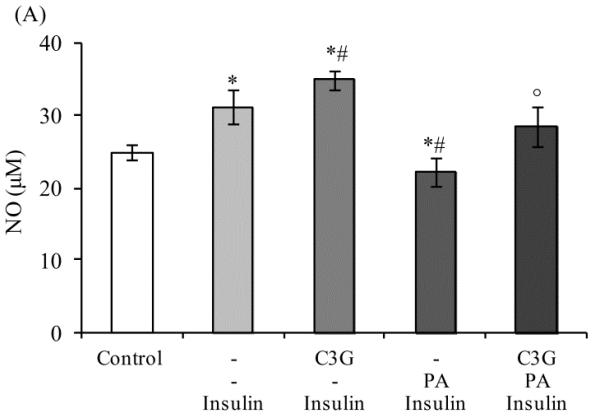

Figure 1A

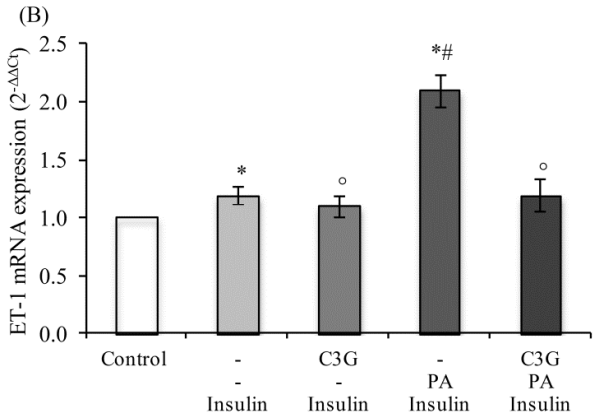

Figure 1B

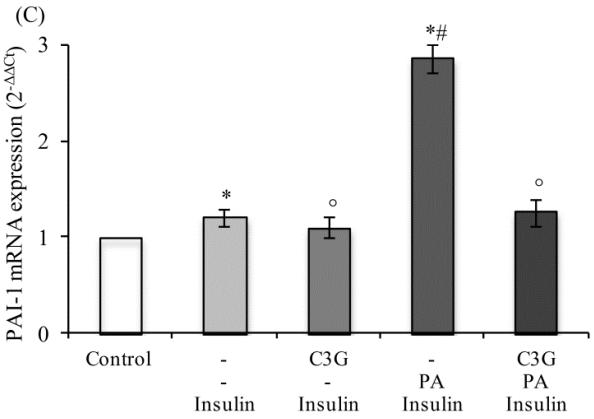

Figure 1C

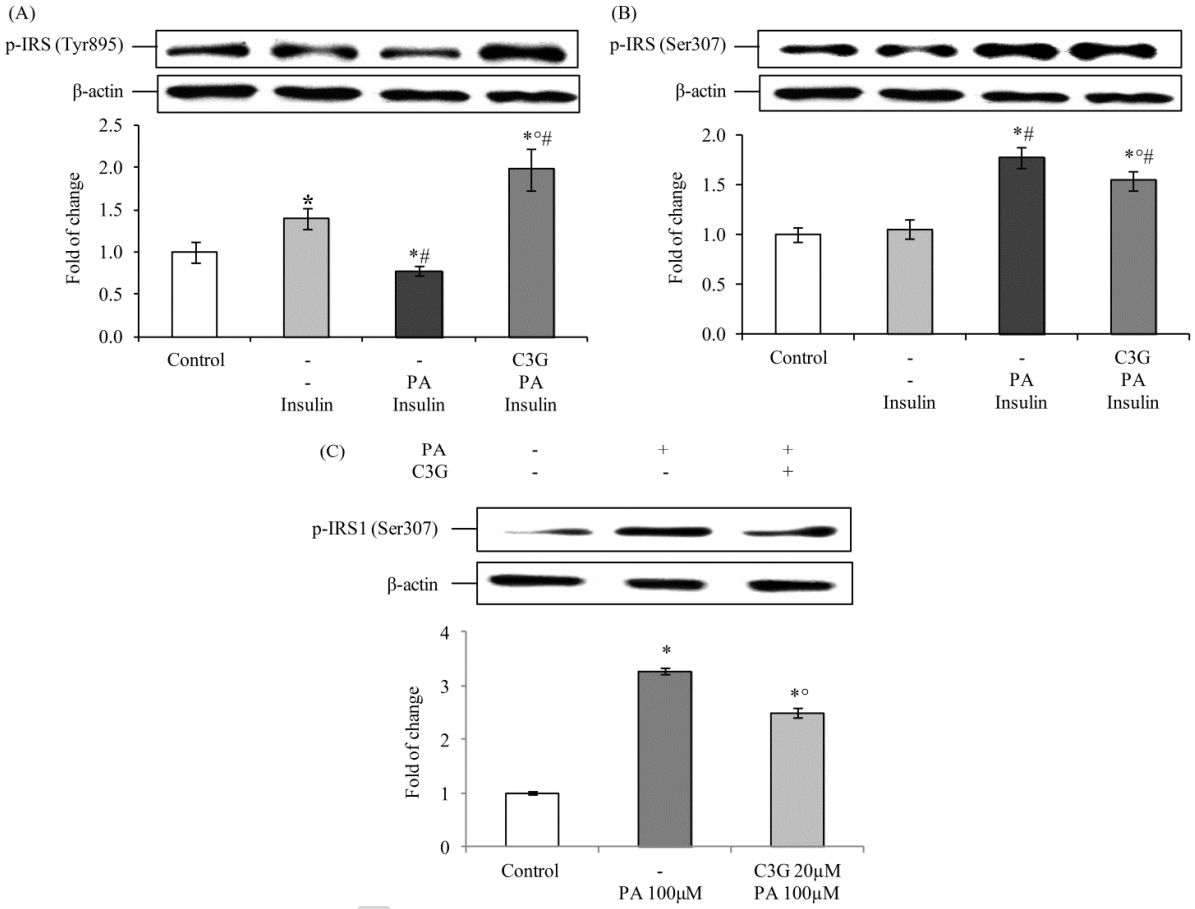

Figure 2

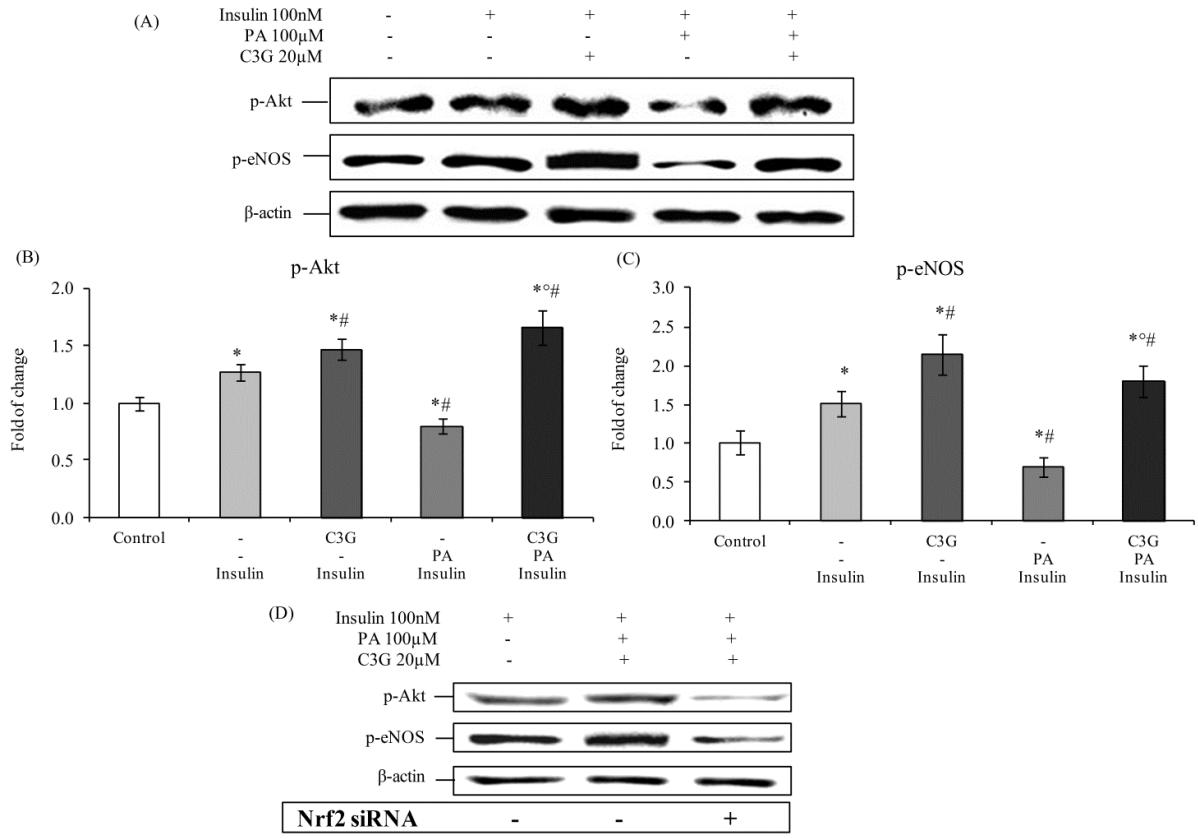

Figure 3

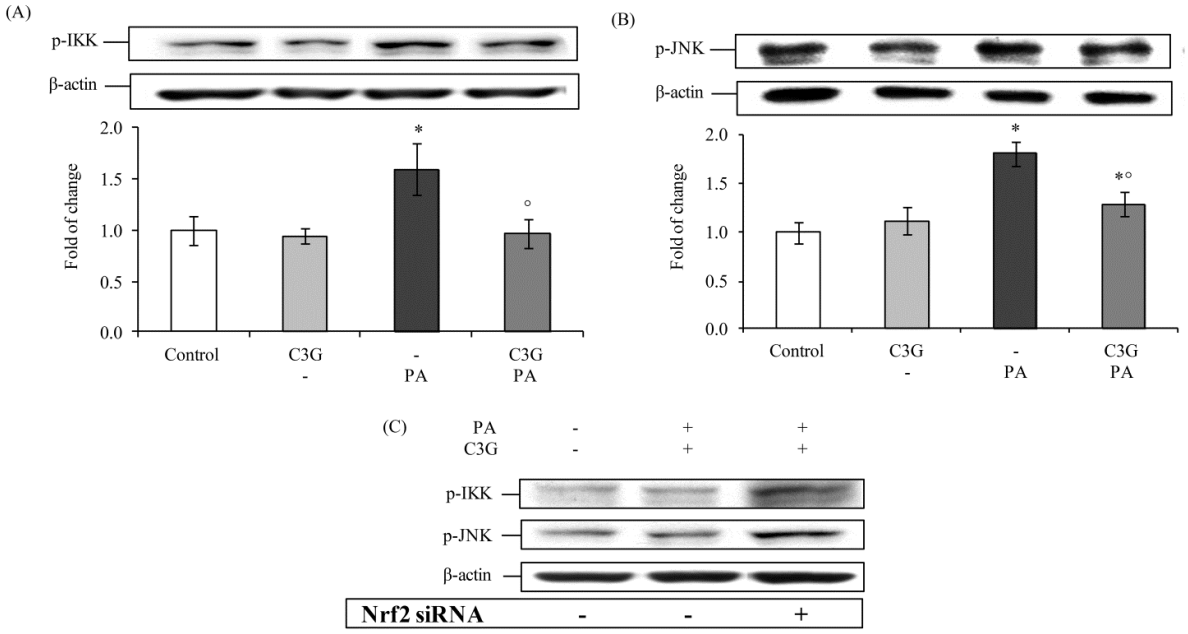

Figure 4

Highlights

- C3G counters FFAs-induced dysregulation of insulin-modulated Akt/eNOS axis
- C3G restores NO availability altered by FFA
- C3G ameliorates insulin resistance, induced by FFA, through Nrf2 activation