Colonic metabolites from flavanols stimulate nitric oxide production in human endothelial cells and protect against oxidative stress-induced toxicity and endothelial dysfunction.

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Abbreviations:

AKT/PKB, protein kinase B; AMPK, adenosine monophosphate-activated protein kinase; DAF-FM-DA, 4-amino-5-methylamino-2,7-difluorofluorescein diacetate; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DHBA, 2,3-dihydroxybenzoic acid; DHPAA, 3,4-dihydroxyphenylacetic acid; eNOS, endothelial nitric oxide synthase; ERK, extra cellular regulated kinase; GPx, glutathione peroxidase; GSH, glutathione; HPPA, 3-hydroxyphenylpropionic acid; JNKs, Jun N-terminal kinases; NO, endothelial-derived nitric oxide; PI3K, phosphatidylinositol-3-kinase; *t*-BOOH, tert-butyl hydroperoxide;

Abstract

Oxidative stress is involved in endothelial dysfunction, the key player in the development of vascular events. Flavanols, the major antioxidants in cocoa have been related to vascular protection and lower cardiovascular risk. However, the bioavailability of cocoa flavanols is very low and their bioactivity *in vivo* seems to be greatly mediated by the derived phenolic metabolites formed by intestinal microbiota. Hence, we investigated whether microbial-derived flavanol metabolites 3,4-dihydroxyphenylacetic acid (DHPAA), 2,3-dihydroxybenzoic acid (DHBA), 3-hydroxyphenylpropionic acid (HPPA) and a mix of them could influence endothelial function and prevent oxidative stress in human endothelial cells (Ea.hy926). Our results revealed that a mixture of flavanol colonic metabolites significantly increased phosphorylation of endothelial nitric oxide synthase (eNOS) and nitric oxide (NO) production. By using specific inhibitors, we also established the participation of the adenosine monophosphate-activated protein kinase (AMPK) and protein kinase B (AKT) in eNOS activation. Likewise, flavanol metabolite mix protected against oxidative stress-induced endothelial dysfunction and cell death by preventing increased ROS generation and activation of signalling pathways related to oxidative stress. We concluded that flavanol colonic metabolites could exert beneficial effects in endothelial cells and prevent oxidative stress-induced vascular dysfunction.

Keywords: Cocoa flavanols, endothelium, oxidative injury, signalling pathways, cardiovascular diseases.

Highlights

- Flavanol colonic metabolites stimulate NO production in human endothelial cells
- Increased NO production induced by colonic metabolites is related to the activation of eNOS via AKT and AMPK signalling
- Flavanol colonic metabolites prevent oxidative stress-induced endothelial dysfunction, cytotoxicity and cell death

1. Introduction

Endothelial-derived nitric oxide (NO), produced by the endothelial nitric oxide synthase (eNOS), plays a central role in maintaining vascular integrity and endothelial function (Moncada et al., 1991). In the presence of cardiovascular risk factors such as hypertension, hypercholesterolemia or diabetes mellitus, the endothelium undergoes functional and structural alterations resulting in decreased NO bioavailability and endothelial dysfunction, the first step of atherosclerosis (Liao, 2013). Although multiple mechanisms are involved in endothelial cell injury, increased oxidative stress seems to be the common underlying mechanism for the development of endothelial dysfunction (Magenta et al., 2013). Consequently, considerable efforts have been made in the last recent years for the identification of natural antioxidant compounds that may provide valuable strategies to prevent oxidative stress and the development of cardiovascular disease (CVD).

Accordingly, epidemiological and clinical studies have indicated that regular consumption of cocoa or cocoa products rich in flavanols has a beneficial effect in normal endothelium and in consequence reduces the risk of CVD (Lin et al., 2016; Zhang et al., 2013). Furthermore, the results obtained from clinical trials with obese, hypertensive or smokers individuals also support that cocoa flavanols could reestablish endothelial dysfunction in abnormal endothelium (Davison et al., 2008, Grassi et al., 2008, Heiss et al., 2005). For that and other reasons, in 2012 the European Food Safety Authority (EFSA) claimed for the first time that a cause-and-effect relationship may be established between the consumption of cocoa flavanols and maintenance of normal endothelium-dependent vasodilation (European Food Safety Authority, 2012). EFSA also proposed an increase of NO production by eNOS as the most likely mechanism by which cocoa flavanols could induce an acute effect on endothelium-dependent vasodilation.

Flavanols present in cocoa consist predominantly of monomeric catechins (mainly epicatechin) and oligomeric flavanols (procyanidins) ranging from dimers to decamers. Among these, epicatechin has been proposed as the responsible for the acute effects of cocoa flavanols in endothelial function as they can be found in plasma two hours following consumption of cocoa (Actis-Goretta et al., 2012). Nevertheless, blood concentrations of epicatechin after a single dose of cocoa flavanol, returns to baseline 2-4 h after ingestion (Actis-Goretta et al., 2012); therefore, the relative contribution of epicatechin to the long-term effects of cocoa flavanols on fasting endothelium-dependent vasodilation is imprecise. However, procyanidins and the fraction of epicatechin which is not absorbed in the small intestine reach the colon where they are largely metabolised by the microbiota into various phenolic acids, including phenylpropionic, phenylacetic and benzoic acid derivatives (Del Rio et al 2013). These small phenolic acids are then absorbed and could appear in plasma 8-12 hours after ingestion of cocoa products (Urpi-Sardá et al., 2009). In fact, the C_{max} of these colonic metabolites is reached about 6 hours after the flavanol intake and its half-life ($T_{1/2}$) values ranged from 3.1–7.6 hours (Ottaviani et al, 2016). Hence, these phenolic metabolites produced by colonic microbiota could certainly contribute to the long-term effects of cocoa flavanols on fasting endothelium vasodilatation. More importantly, some of them have been shown to possess potential health beneficial effects (Álvarez-Cilleros et al., 2018; Fernández-Millán et al., 2014; Qiu et al., 2010). However, up to date, whether these cocoa flavanol microbial metabolites exert a direct effect on fasting endothelium-dependent vasodilation remains to be elucidated.

In this context, the aim of the present study was to investigate the *in vitro* effects of the microbial-derived flavanol metabolites 3,4-dihydroxyphenylacetic acid (DHPAA), 2,3-dihydroxybenzoic acid (DHBA) and 3-hydroxyphenylpropionic acid (HPPA) and a mixture

of them on endothelial function in human endothelial cells (EA.hy926) and the mechanism involved. Furthermore, the protective effect against oxidative stress-induced cell toxicity and endothelial dysfunction was also evaluated.

2. Material and Methods

2.1. Materials and Chemical

3,4-dihydroxyphenylacetic acid (DHPAA), 2,3-dihydroxybenzoic acid (DHBA), 3-hydroxyphenylpropionic acid (HPPA), o-phthalaldehyde (OPT), gentamicin, penicillin G, streptomycin, bovine serum albumin (fraction V), 6-[4-(2-piperidin-1-ylethoxy)phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine (Comp C), LY294002 (LY) and *tert*-butyl hydroperoxide (*t*-BOOH) were purchased from Sigma Chemical (Madrid, Spain). The fluorescent probes 2',7'-dichlorofluorescein diacetate (DCFH-DA) and 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM-DA) were from Molecular Probes (Eugene, OR). Anti-protein kinase B (AKT), anti-phospho-Ser473-AKT (p-AKT), anti-adenosine monophosphate-activated protein kinase (AMPK), anti-phospho-Thr172-AMPK (p-AMPK), anti-phospho-Ser1177-endothelial nitric oxide synthase (p-eNOS), anti-extra cellular regulated kinases (ERKs), anti-phospho-ERKs (p-ERKs), anti-c-Jun N-terminal kinases (JNKs) and anti- β -actin were obtained from Cell Signaling Technology (Izasa, Madrid, Spain). Anti-eNOS (eNOS), anti-phospho-JNKs (p-JNKs) and anti- α -actinin were purchased from Santa Cruz (sc-376751, sc-6254, sc-17829, respectively, Quimigen, Madrid, Spain). Materials and chemicals for electrophoresis and the Bradford reagent were from BioRad (BioRad Laboratories S.A., Madrid, Spain). Cell culture dishes were from Falcon (Cajal, Madrid, Spain) and cell culture medium and foetal bovine serum from Lonza (Madrid, Spain).

2.2. Cell culture and treatments

Experiments were performed using the human endothelial cell line EA.hy926 (Edgell et al., 1983) derived from fusing human umbilical vein endothelial cells with the

permanent human cell line A549. The cells (a gift from Drs. Patricio Aller and Carmelo Bernabeu, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain) were maintained in a humidified incubator containing 5 % CO₂ and 95 % air at 37 °C. They were grown in DMEM media, supplemented with 10 % foetal bovine serum (FBS) and 1 % of the following antibiotics: gentamicin, penicillin and streptomycin. The different concentrations of DHPAA, DHBA and HPPA (1-10 µM) were diluted in DMEM culture medium (4.5 g/L glucose; 0.9 g/L of glutamine) and added to the cell plates for 18 h. The doses of colonic metabolites employed in this work are considered physiologically relevant since concentrations ranging between 0.1–10 µM have been found in biological fluids after regular consumption of cocoa and cocoa derived products (Urpi-Sarda et al., 2009). A mixture of microbial metabolites (MIX) at 4 µM each one (4 µM of DHPAA + 4 µM of DHBA + 4 µM of HPPA) was also added to the culture medium in order to test the potential additive and synergist effects of the metabolites. In the experiments with the pharmacological inhibitors, cells were pre-incubated with 10 µM of LY294002 (AKT inhibitor), or 5 µM of Compound C (AMPK inhibitor) for 1 h prior to DHPAA, DHBA, HPPA and MIX treatment.

To evaluate the protective effect of microbial phenolic metabolites against an oxidative stress, after DHPAA, DHBA, HPPA and the MIX treatment the medium was discarded and fresh medium containing 25-100 µM of *t*-BOOH and the different treatments was added for 18 hours more.

2.3. Evaluation of cell viability and ROS production

Cell viability was determined by using the crystal violet assay. EA.hy926 cells were seeded at low density (2×10^5 cells per well) in 24-well plates. After the different treatments, cells were incubated with crystal violet (0.2 % in ethanol) for 20 min. Plates were rinsed with distilled water, allowed to dry, and 1 % sodium dodecyl sulfate (SDS) added. The absorbance of each well was measured using a microplate reader at 570 nm (Bio-Tek, Winooski, VT, USA).

Cellular ROS were quantified by the DCFH assay (Wang and Joseph, 1999) using a microplate reader. For the assay, cells were plated in 24-well multiwells and incubated with the different treatments. After that, 10 μ M DCFH was added to the wells for 30 min at 37 °C. After being oxidized by intracellular oxidants, DCFH will become dichlorofluorescein (DCF) and emit fluorescence. ROS generation was evaluated in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Bio-Tek, Winooski, VT, USA).

2.4 Determination of reduced glutathione (GSH) concentration and glutathione peroxidase (GPx) activity

The concentration of GSH was evaluated by a fluorometric assay previously described in (Quéguineur et al., 2012). The method takes advantage of the reaction of GSH with OPT at pH 8.0 and fluorescence was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm. The determination of GPx activity is based on the oxidation of reduced glutathione by GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH by GR (Quéguineur et al., 2012).

2.5. Determination of nitric oxide production

NO production by endothelial cells was measured using the fluorescence probe DAF-FM which is a fluorinated DAF-2 derivative with improved NO sensitivity. The diacetate form of DAF-FM (DAF-2-DA) cross the cell membrane where is cleaved by intracellular esterases to form the negatively charged parent compound DAF-2 which cannot cross the cell membrane and thus accumulates inside the cells. In the presence of NO, DAF-2 is transformed in a highly fluorescent triazole (DAF-2T). NO production was detected by spectrofluorometric following the optimized method for the detection of NO in the low-nM range developed for Leiker et al. (Leiker et al., 2001). Briefly, cells were plated in 24-well multiwells (2×10^5 cells per well) and incubated with the different treatments. After that, 0.1 μ M DAF-2 was added to the wells for 30 min at 37 °C. Then, cells were rinsed three times with PBS. The fluorescence of cells and supernatants (PBS) was evaluated in a fluorescent microplate reader at an excitation wavelength of 495 nm and an emission wavelength of 515 nm (Bio-Tek, Winooski, VT, USA). The auto-fluorescence obtained from PBS without cells was subtracted from each value. Intracellular NO was also visualized by fluorescence microscopy. To this end, cells were seeded on 24-well plates and exposed to the desired treatments. Then, cells were loaded with 2.5 μ M of DAF-2-DA for 30 min at 37 °C, rinsed three times with PBS and observed under an inverted fluorescence Leica AF6000 LX microscope (excitation wavelength: 488 nm; emission wavelength: 515 nm).

2.6. Preparation of cell lysates for Western blotting

To detect eNOS, p-eNOS, AKT, p-AKT, AMPK, p-AMPK, ERKs, p-ERKs, JNKs and p-JNKs, cells were lysed at 4 °C in a buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM 1,4-Dithiothreitol, 0.1% Triton X-100, 200

mM β -glycerolphosphate, 0.1 mM Na_3VO_4 , 2 $\mu\text{g/mL}$ leupeptin, and 1 mM PMSF. Supernatants were collected, assayed for protein concentration by using the Bradford reagents, aliquoted and stored at -80°C until used for Western blot analyses.

2.7. Protein determination by Western Blotting

Equal amounts of protein (100 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters (Protein Sequencing Membrane, BioRad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated antirabbit Ig (GE Healthcare, Madrid, Spain). Blots were developed with the ECL system (GE Healthcare). Normalization of Western blot was ensured by β -actin or α -actinin and bands were quantified using a scanner and accompanying software.

2.8. Determination of carbonyl groups

Protein oxidation of cells was measured as carbonyl groups content according to a published method (Fernández-Gómez et al., 2016). In brief, samples containing 500 μg of protein were treated for duplicate. One of the samples received 2,4-dinitrophenylhydrazine (0.2%) in 2 M HCl and the other one the same volume of 2 M HCl (blanks). Protein was precipitated by addition of trichloroacetic acid (TCA, 20%) followed by centrifugation for 5 min at 11,750g. After that, the pelleted protein was washed 3 times with 1.0 mL ethyl acetate:ethanol (1:1). Pellets were allowed to air dry and resuspended in 6 M guanidine. Absorbance was measured at 360 nm and corrected by the blanks. Carbonyl content was expressed as nmol/mg protein using an extinction coefficient of 22 000 nmol/L/cm. Protein concentration was measured by the Bradford reagent.

2.9. Statistics

Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. The level of significance was $P < 0.05$. A SPSS version 23.0 program has been used.

3. Results

3.1. Effects of microbial phenolic metabolites on redox status and viability in endothelial cells.

Firstly, in order to discard a potential cell injury by microbial phenolic metabolites, EA.hy926 cells were treated with realistic doses of DHPAA, DHBA and HPPA (1-10 μ M) and the MIX (12 μ M) during 18 hours and several parameters related to the cellular redox status and antioxidant response were evaluated. As shown in table 1, none of the tested concentrations of phenolic metabolites increased ROS generation and crystal violet staining after 18 h, indicating no cellular stress or damage. Similarly, treatment with metabolites evoked no changes in the cellular store of GSH and the activity of GPx. These results ensure that EA.hy926 cells treated with microbial phenolic metabolites (low μ M doses) are absolutely functional and in competent conditions to face a stressful challenge.

3.2. Effects of microbial phenolic metabolites in NO production in endothelial cells.

Endothelial cells play a crucial role in the maintenance of vascular homeostasis by releasing several vasoactive factors being the most important the endothelium-derived nitric oxide (NO). Therefore, to investigate the role of colonic metabolites in endothelial function we first examined whether physiological concentrations of these metabolites could induce the production of NO in EA.hy927 cells. Hence, the MIX (12 μ M) and the colonic metabolites individually at 4 and 10 μ M were added to the cells during 1 or 2 hours (short-term) or during 18 hours (long-term) and the production of NO was evaluated. As shown in figure 1A, treatment of cells with 4 μ M of each metabolite individually failed to increase the production of NO. However, the mix of these metabolites at the same μ M dose (4 μ M of DHPAA + 4 μ M of DHBA + 4 μ M of HPPA) significantly augmented the generation of

NO in endothelial cells at 1 and 2 h while had no significant effect at 18 h. Similarly, concentrations of 10 μ M of DHPAA, DHBA and HPPA individually were also able to increase the production of NO in EA.hy927 cells at the same short-times. Insulin (100 nM for 15 min) was used as positive control.

According to these results, the MIX (12 μ M) and the dose of 10 μ M of DHPAA (the one that individually provokes the greatest increase of NO production) were those selected for subsequent experiments.

3.3. DHPAA and the colonic metabolite MIX induce the phosphorylation of eNOS and NO production in human endothelial cells.

Since endothelial NO synthase (eNOS) is the key enzyme involved in NO synthesis in endothelial cells, we next investigated the effect of colonic metabolites on eNOS activation. To this end, endothelial cells were cultured for 2 h with 10 μ M of DHPAA and 12 μ M of the MIX and then immunoblots were performed using phospho- Ser1177 and non-phospho-antibodies against eNOS. As shown in figure 1B, the levels of p-eNOS were significantly increased in the presence of DHPAA and the MIX. Moreover, there was no difference in the total levels of eNOS in any treatment, suggesting that metabolites are able to phosphorylate/activate eNOS without increasing its protein expression. According to that, the stimulatory effect of colonic metabolites was accompanied by an increase in intracellular NO production (as shown before) that was additionally confirmed by fluorescence microscopy (Figure 1C).

3.4. Colonic flavanol metabolites increased endothelial function via AKT and AMPK.

Next, to elucidate the mechanisms underlying the increase in eNOS phosphorylation in response to colonic metabolites, AMPK and AKT, upstream transducers of eNOS phosphorylation, were also evaluated. To this end, endothelial cells were treated with 10 μ M of DHPAA and 12 μ M of the MIX during 2 h and then immunoblots were performed using the phosphorylated and total antibodies against AKT and AMPK. As shown in figure 2, both DHPAA and the MIX increased the phosphorylated levels of AKT and AMPK; however, the activation/phosphorylation of AKT induced by DHPAA was larger than that induced by the MIX. Both compounds had no effect on total AMPK and AKT.

To further determine the potential role of these pathways in eNOS activation induced by colonic metabolites, we next investigated the effects of specific inhibitors of AKT and AMPK on eNOS phosphorylation and NO production induced by DHPAA and the MIX. Accordingly, cells were pre-incubated with 10 μ M LY (AKT inhibitor) or 5 μ M of Comp C (AMPK inhibitor) one hour before and during the 2 hours of DHPAA and MIX treatment. As shown in figure 3A, DHPAA- and MIX-induced phosphorylation of eNOS was partially blocked with LY and Comp C pre-incubation. Accordingly, the production of NO induced by DHPAA and the MIX was partially blocked with the pharmacological inhibition of AKT and AMPK (Figure 3B). Altogether, these results suggest that both AKT and AMPK are involved in the upregulation of NO production induced by colonic metabolites in EA.hy927 cells.

3.5. Colonic flavanol metabolites protect endothelial cells against t-BOOH induced oxidative stress and cell death.

Since oxidative stress has been related to endothelial cell death and dysfunction, in the following experiments we investigated the potential of microbial phenolic metabolites

to protect endothelial cells against chemically-induced oxidative stress. To this end, we used a chemical compound commonly used to induce oxidative insults in biological systems (*t*-BOOH). The first goal was to determine the conditions of *t*-BOOH culture leading to increased oxidative stress and cell death in EA.hy972 cells. To this purpose, cells were treated for 18 h with different concentrations of *t*-BOOH, and ROS generation and cell viability were determined. Figure 4A reveals that increasing concentrations of the pro-oxidant induced a dose-dependent increase in the production of ROS and in cell death. Since the dose of 100 μ M *t*-BOOH caused 50-60% increase in ROS generation and cell death, that was the concentration chosen for the following experiments.

Then, endothelial cells were treated with 10 μ M DHPAA and 12 μ M of the MIX during 18 hours and further submitted to 100 μ M *t*-BOOH for 18 hours more. As shown in figure 4, treatment of cells with *t*-BOOH enhanced ROS generation (Figure 4B) and protein cell damage (measured as carbonyl groups) (Figure 4C) resulting in a remarkable decrease of endothelial cell viability (Figure 4D). Nevertheless, 18 hours of pre-treatment with DHPAA or the MIX significantly prevented the condition of oxidative stress induced by *t*-BOOH including cell viability.

3.6. Colonic flavanol metabolites protect endothelial cells against t-BOOH induced endothelial dysfunction.

A reduced in endothelial-derived NO production is considered the major mechanism of endothelial dysfunction; therefore, we next investigated the effect of oxidative stress in eNOS activation. As expected, *t*-BOOH treatment for 18 h significantly decreased the activation/phosphorylation of eNOS whereas the pre-incubation with 10 μ M DHPAA and 12 μ M of MIX partially restored the eNOS phosphorylation (Figure 5A). To further explore

the potential signalling pathway contributing to the protective effect of DHPAA and the MIX, we examined the phosphorylation of ERK and JNK kinases. As shown in figure 5B, *t*-BOOH remarkably activated the phosphorylation of both kinases at 4 hours; on the contrary, DHPAA and the MIX pre-treatment effectively mitigated the effects of *t*-BOOH on ERK and JNK phosphorylation.

Altogether, these results clearly indicate that the chemically-induced oxidative stress and endothelial dysfunction in EA.hy972 cells were significantly recovered with realistic concentrations of DHPAA and the MIX.

4. Discussion

More than 80% of flavanols ingested in the diet are metabolized by colonic microbiota generating several phenolic metabolites, which may contribute in a great extend to their reported biological activities (Monagas et al., 2010). In fact, these derived colonic metabolites are currently considered as the most plausible candidates responsible for the health properties derived from flavanol consumption. According to this, studies aimed to investigate the potential beneficial effects of circulating metabolites at physiological concentrations and the characterization of their mechanisms of action are at present receiving much interest. In the present study, we demonstrated for the first time that phenolic acids derived from flavanol microbial metabolism can induce *in vitro* NO production in human endothelial cells. Likewise, we showed that this effect was largely mediated via AMPK and PI3K/AKT pathways and the subsequent eNOS phosphorylation/activation. More importantly, under conditions of oxidative stress, derived flavanol colonic metabolites were also able to protect against endothelial dysfunction and cell death by reducing ROS generation and ERK and JNK activation.

After regular consumption of flavanols-rich foods (mainly cocoa and cocoa derived products) mono- and di-hydroxylated phenylpropionic, phenylacetic and hydroxybenzoic acids and their conjugated form (sulphates and glucuronidates) are the most abundant metabolites in both urine and fasting plasma (Urpi-Sardá et al., 2009). However, it has been proved that conjugation could be a reversible process *in vivo*; therefore, the observed effects *in vivo* may to be due to the deconjugated forms (Menéndez et al., 2011; Pérez-Vizcaino et al., 2012). Consequently, in this work, we investigated the potential beneficial effect of some of these phenolic acids (3,4-DHPAA, 2,3-DHBA and 3-HPPA) on endothelial function. The use of these flavanol-derived colonic metabolites *in vitro* reflects

physiological conditions more accurately than the use of pure dietary compounds such as monomeric catechins and procyanidins (Monagas et al., 2010). Besides, we used realistic doses (1-10 μM) since phenolic acids and its conjugated forms could be detected in plasma at low μM concentrations after the intake of foodstuff containing flavanols (Rodriguez-Mateos et al., 2016; Urpi-Sarda et al., 2009).

Interestingly, we found that individual metabolites only were able to induce the production of NO in human aortic endothelial cells at the highest concentration tested (10 μM). However, a mix of the lowest dose of microbial-derived flavanol metabolites (4 μM of DHPAA + 4 μM of DHBA + 4 μM of HPPA) was equally effective to increase NO production in endothelial cells. These results are in agreement with a recent *in vitro* study showing that a mixture of ellagitannin-derived metabolites increased NO bioavailability to a greater extent than the single metabolites alone (Spigoni et al., 2016). More significantly, our data strongly support the hypothesis that beneficial effects of flavanol-rich diets will be mainly due to cumulative or additive activities of several circulating metabolites working together in a synergistic way (Di Gesso et al., 2015).

Cocoa and its flavanols have been described to improve endothelial function, reducing thus the risk of cardiovascular morbidity and mortality (Grassi et al., 2015). This positive effect has been largely attributed to epicatechin, a major flavanol in cocoa, which has been demonstrated to induce endothelium-dependent relaxation both in animals and humans (Smicht and Dirsch, 2009). Particularly, epicatechin phosphorylates and activates eNOS via the PI3K/AKT/PKA and Ca^{2+} -CaM/CaMKII (Ca-calmodulin-dependent protein kinase II) pathways leading to enhanced NO production in human coronary artery endothelial cells (Ramirez-Sanchez et al., 2010). Likewise, under Ca^{2+} -free conditions, epicatechin can also induce eNOS phosphorylation through the formation of an active

complex between eNOS, AKT, and heat shock protein 90 (HSP90) (Ramirez-Sanchez et al., 2012). However, studies currently examining the effect on endothelial function of flavanol-derived colonic metabolites, which are the main circulating metabolites in plasma 6-12 hours after flavanol intake, are scarce. Accordingly, in this study we showed that a mixture of microbial phenolic acids derived from flavanols significantly enhanced eNOS phosphorylation at Ser1177 increasing thus NO production in cultured endothelial cells. Our results are consistent with those from Najmanove et al. (2016) demonstrating that several flavonoid metabolites formed by colon microbiota caused vasodilation of isolated rat aortic rings; more importantly, we provided a potential mechanism of action of the observations reported by them. Of interest, these outcomes extend the recent progress achieved regarding the beneficial effect of colonic phenolic compounds modulating endothelial function. In particular, small phenolic acids derivatives from anthocyanins were able to induce heme oxygenase-1 (HO-1) and modulate eNOS activity, resulting in reduced superoxide production and improved NO bioavailability in human umbilical vein endothelial cells (Edwards et al., 2015). More recently, Lee et al. (Lee et al., 2017) have demonstrated that a major microbial metabolite of proanthocyanidin (5-dihydroxyphenyl-valerolactone) attenuated THP-1 monocyte-endothelial adhesion in human umbilical vein endothelial cells. Taken together, these findings clearly confirmed that 1) microbial phenolic metabolites may independently have bioactivity improving vascular function and 2) this effect may be definitely crucial since they are probably the main active compounds *in vivo*.

In an effort to identify specific signaling pathways involved in flavanol colonic metabolites-induced effects, we examined the participation of PI3-kinase/AKT and AMPK, which have been described as importantly implicated in the physiological modulation of

eNOS phosphorylation (Zheng et al., 2011). AKT, the major downstream target of PI3K, is known to phosphorylate/activate eNOS at Ser1177, playing a crucial role in regulating endothelial NO production (Kang et al., 2013). Likewise, previous studies have shown that AMPK also induces phosphorylation of eNOS at serine-1177 and activates NO generation in endothelial cells (Morrow et al., 2003). According to that, we found that the mixture of flavanol colonic metabolites were able to increase AKT and AMPK phosphorylation in endothelial cells. Besides, the presence of specific inhibitors of these pathways significantly attenuated eNOS phosphorylation and NO production in response to colonic metabolites, suggesting that the effect of flavanol metabolites in eNOS activity is partly mediated by the PI3-kinase/AKT and AMPK signaling pathways. Similar mechanisms of eNOS phosphorylation have also been observed in response to several polyphenolic compounds, including genistein (Liu et al., 2004), epigallocatechin-3-gallate (Lorenz et al., 2004), hesperidin (Rizza et al., 2011), epicatechin (Ramirez-Sanchez et al., 2012), quercetin (Shen et al., 2012) or resveratrol (Liu et al., 2016) and in a number of phytomedicinal preparations (Smicht and Dirsch, 2009). The identification of colonic metabolites of dietary polyphenols that can influence endothelial NO production and the characterization of their mechanism of action further support the cardioprotective effects of these natural compounds.

It is interesting to note that natural antioxidant compounds not only may positively influence endothelial NO production but also alleviate the status of oxidative stress, promoting then endothelial function (Luangaran et al., 2007). According to that, it has been recently demonstrated that some phenolic metabolites produced by colonic microbiota could possess anti-oxidant effects (Fernández-Millán et al., 2014; Mele et al., 2017; Qiu et al., 2013). Therefore, we also investigated the potential protective effect of these flavanol colonic metabolites against an oxidative stress induced by *t*-BOOH, a pro-oxidant that has

been commonly used to study ROS-induced alterations in various cell types (Martín et al., 2014, Martín et al., 2010; Rodríguez-Ramiro et al., 2012). As expected, we found that *t*-BOOH treatment increased ROS generation and cell oxidative damage, resulting in reduced cell viability in endothelial cells. Likewise, *t*-BOOH treatment provoked the activation of signaling pathways related to oxidative stress such as ERK and JNK kinases and the subsequent decrease in eNOS phosphorylation. However, all these effects were attenuated in cultured cells pre-treated with colonic metabolites, suggesting that flavanol colonic metabolites can also prevent ROS generation and the activation of specific free radical-generating pathways to avoid oxidative stress and endothelial dysfunction. Similar results have recently been described by Qian et al. (Qian et al., 2017) showing that the flavanol colonic metabolite 3-HPPA prevented the increase in ROS production evoked by high glucose conditions preserving thus insulin stimulated eNOS phosphorylation and NO production. Nevertheless, we cannot rule out that the ability of these colonic metabolites to activate eNOS via AKT/AMPK pathways could also contribute to protect endothelial cells against oxidant-induced endothelial dysfunction by increasing NO bioavailability. Therefore, the protective effects of these compounds could depend both on their modulation on the oxidative status and their direct activation of AKT/AMPK/eNOS pathway. In any case, these results provide strong evidence that colonic metabolites from flavanols may clearly avoid endothelial dysfunction related to oxidative stress, indicating that their vascular benefits might also result from their antioxidant properties.

In conclusion, in this study we show for the first time that flavanol metabolites formed by colonic microbiota are able to induce NO release via eNOS phosphorylation in human endothelial cells and that this effect seems to be mainly mediated by PI3K/AKT and AMPK pathways. Furthermore, flavanol colonic metabolites can also prevent the increase

in ROS generation and the activation of stress-related pathways induced by oxidative insults avoiding thus cell death and endothelial dysfunction. Taken together, these results provide new evidence that the beneficial effects of cocoa flavanols on endothelial function could result, at least in part, from the bioactivity of their colonic metabolites. Interestingly, there are several colonic metabolites in biologic fluids after flavanol intake and we still do not know the full spectrum of these compounds and even less their biological effects.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Legends to Figures

Figure 1.- Effect of colonic flavanol metabolites on NO production in endothelial cells.

(A) EA.hy926 cells were treated with insulin (100nM) for 15 min or with DHPAA (4 and 10 μ M), DHBA (4 and 10 μ M), HPPA (4 and 10 μ M) or the MIX (12 μ M) for 1, 2 and 18 h and loaded with DAF2-DA as described under “Materials and Methods”. Fluorescence intensity was monitored at 485nm excitation and 515nm emissions using a fluorescence microplate reader. Data represent means $\pm$ SD of 6-8 samples per condition. Different letters denote statistically significant differences, $P < 0.05$. (B) EA.hy926 cells treated with insulin (100nM, 15 min) or DHPAA (10 μ M, 2 h) or the MIX (10 μ M, 2 h) were subjected to Western blot analysis using specific antibodies to p-eNOS and eNOS. Bands are representative of four to six experiments. Normalization of Western blots was ensured by β -actin. Graphic represents the percentage values of p-eNOS/eNOS relative to the control condition (mean $\pm$ SD). Means without a common letter differ, $P < 0.05$. (C) EA.hy926 cells treated with insulin (100nM, 15 min) or DHPAA (10 μ M, 2 h) or the MIX (12 μ M, 2 h) were loaded with DAF2-DA and intracellular NO production was viewed using a fluorescence microscopy as described in “Material and Methods”. Emission of green fluorescence is indicative of NO production. Phase contrast views of cells corresponding to images in the upper panels are also shown. Scale bars: 75 μ m.

Figure 2.- DHPAA and the colonic MIX induce phosphorylation of AKT and AMPK

in human endothelial cells. EA.hy927 cells treated with 10 μ M DHPAA and 12 μ M of the MIX for 2 h were subjected to Western blot analysis using phospho-specific antibodies to AKT and AMPK. The same cell lysates were subjected to Western blot analysis using

corresponding non-phospho-specific antibodies to detect total AKT and AMPK. (A) Bands are representative of four to six experiments. (B) Percentage values of p-AKT/AKT and p-AMPK/AMPK relative to the control condition (mean $\pm$ SD). Normalization of Western blots was ensured by β -actin. Means without a common letter differ, $P < 0.05$.

Figure 3.- DHPAA and the MIX increased endothelial function via AKT and AMPK.

EA.hy927 cells were pre-incubated with 10 μ M LY (selective inhibitor of AKT), and 5 μ M of Comp C (selective inhibitor of AMPK) for 1 h prior to DHPAA (10 μ M) and MIX (12 μ M) treatment for 2 h. (A) Control and treated-cells were subjected to Western blot analysis using antibodies to p-eNOS and eNOS. Bands are representative of four to six experiments. Normalization of Western blots was ensured by β -actin. Graphic represents the percentage values of p-eNOS/eNOS relative to the control condition (mean $\pm$ SD). (B) Control and treated-cells were loaded with DAF2-DA and NO levels were detected as described under “Materials and Methods”. Fluorescence intensity was monitored at 485nm excitation and 515nm emissions using a fluorescence microplate reader. Data represent means $\pm$ SD of 6-8 samples per condition. Different letters denote statistically significant differences, $P < 0.05$.

Figure 4.- DHPAA and the colonic metabolite MIX protect human endothelial cells against oxidative stress and cell death.

EA.hy927 cells were treated with 25-100 μ M of *t*-BOOH and ROS generation and cell viability was determined at 18 h (A). EA.hy927 cells treated with 10 μ M DHPAA and 12 μ M of the MIX for 18 h were further exposed to 100 μ M *t*-BOOH for 18 hours more and intracellular ROS generation (B), carbonyl group levels

(C) and cell viability (D) were measured. Data represent the mean $\pm$ S.D. of 10–12 samples per condition. Different letters denote statistically significant differences, $P < 0.05$.

Figure 5.- DHPAA and the colonic metabolite MIX protect human endothelial cells against endothelial dysfunction induced by oxidative stress. EA.hy927 cells were treated with 10 μ M DHPAA and 12 μ M of the MIX for 18 h. (A) Control and treated-cells were exposed to 100 μ M *t*-BOOH for 18 hours and subjected to Western blot analysis using antibodies to p-eNOS and eNOS. Bands are representative of four to six experiments. Graphic represents the percentage values of p-eNOS/eNOS relative to the control condition (mean $\pm$ SD). Normalization of Western blots was ensured by β -actin. (B) Control and treated-cells were exposed to 100 μ M *t*-BOOH for 4 hours and subjected to Western blot analysis using antibodies to p-ERKs, ERKs, p-JNKs and JNKs. Bands are representative of four to six experiments. Graphic represents the percentage values of p-ERKs/ERKs and pJNKs/JNKs relative to the control condition (mean $\pm$ SD). Normalization of Western blots was ensured by α -actinin. Different letters denote statistically significant differences, $P < 0.05$.

Table 1.- Effect of 18 h treatment with noted concentrations of DHPAA, DHBA, HPPA and the colonic MIX on cell viability, intracellular ROS generation, GSH and GPx activity in human endothelial cells.

		<i>% ROS</i>	<i>% cell viability</i>	<i>% of GSH</i>	<i>% GPx</i>
Control		100 ± 4 ^a	100 ± 7 ^a	100 ± 9 ^a	100 ± 4 ^a
DHPAA	1 µM	100 ± 1 ^a	101 ± 7 ^a	103 ± 10 ^a	100 ± 7 ^a
	4 µM	100 ± 4 ^a	101 ± 2 ^a	105 ± 10 ^a	100 ± 1 ^a
	10 µM	101 ± 5 ^a	101 ± 3 ^a	106 ± 16 ^a	107 ± 7 ^a
DHBA	1 µM	100 ± 2 ^a	101 ± 6 ^a	100 ± 17 ^a	101 ± 2 ^a
	4 µM	100 ± 3 ^a	101 ± 4 ^a	100 ± 9 ^a	101 ± 6 ^a
	10 µM	101 ± 3 ^a	101 ± 5 ^a	103 ± 16 ^a	106 ± 7 ^a
HPPA	1 µM	100 ± 1 ^a	100 ± 10 ^a	100 ± 12 ^a	100 ± 5 ^a
	4 µM	100 ± 3 ^a	100 ± 10 ^a	103 ± 13 ^a	101 ± 6 ^a
	10 µM	101 ± 3 ^a	100 ± 7 ^a	105 ± 4 ^a	110 ± 10 ^a
MIX	12 µM	101 ± 5 ^a	102 ± 10 ^a	100 ± 20 ^a	102 ± 8 ^a

Data represent means ± SD of 8-10 samples per condition. Different letters denote statistically significant differences, P < 0.05.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5